

**REGULATION OF CYCLIN D1 EXPRESSION BY PKC SIGNALING IN
INTESTINAL EPITHELIAL CELLS**

by

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ABSTRACT

Members of the protein kinase C (PKC) family of serine/threonine kinases are recognized as major players in cell cycle regulation in numerous systems. Previous studies in this laboratory have demonstrated that PKC signaling, and PKC α in particular, negatively regulates cell cycle progression in intestinal epithelial cells. Activation of the phorbol ester responsive PKC isozymes, PKC α , PKC δ , and PKC ϵ , or of PKC α alone, in IEC-18 nontransformed intestinal crypt cells induces hallmark molecular events of G0 exit, including downregulation of cyclin D1. Conversely, depletion of PKC α , δ and ϵ is associated with hyperinduction of cyclin D1 protein in these cells. Cyclin D1 is limiting for cell cycle progression in intestinal epithelial cells and plays a critical role in tumor progression; thus, understanding of its regulation by PKC signaling, which is markedly reduced in intestinal tumors, is of key importance. Elucidation of the molecular pathways underlying the bimodal regulation of cyclin D1 expression determined by the activation status of PKC isozyme(s) was the focus of the current study. In the first part of this work, we (a) characterized the pathways involved in PKC agonist-induced downregulation of cyclin D1 and (b) determined the specific PKC isozyme involved. The data point to PKC α as a major regulator of cyclin D1 translation. PKC/PKC α activation does not alter cyclin D1 transcription, mRNA stability, or mRNA transport to reduce cyclin D1 protein levels. Furthermore, pulse-chase analysis and exogenous expression of cyclin D1 proteolysis mutants excluded a role for changes in cyclin D1 protein stability. Instead, PKC/PKC α activity was shown to inhibit cyclin D1 synthesis, in association with blockade of key regulators of both translation initiation and elongation. Upon

PKC/PKC α activation, the function of eukaryotic initiation factor 4E (eIF4E) was rapidly blocked via activation of its inhibitory binding factor, eIF4E binding protein 1 (4E-BP1), indicating PKC-induced shut-down of cap-dependent translation initiation. Furthermore, PKC/PKC α activation promoted rapid inhibition of eukaryotic elongation factor 2 (eEF2), a key regulator of the elongation step of translation, via increased phosphorylation of Thr56.

These negative modulatory effects of PKC/PKC α signaling on regulators of translation initiation and elongation were reversed coincident with downregulation of PKC isozymes, paralleling the recovery of cyclin D1 levels and re-entry of cells into the cell cycle. Interestingly, continued absence of PKC signaling resulted in aberrant hyperinduction of cyclin D1 protein and increased proliferation of IEC-18 cells. Understanding of this effect and its implications for intestinal tumor development was the focus of the second phase of this study. Levels of cyclin D1 in PKC-depleted cells were found to be comparable with those seen in neoplastic intestinal cells, offering a perspective on the frequent reduction/absence of PKC/PKC α signaling in intestinal malignancies. Findings from biosynthetic labeling experiments revealed enhanced synthesis of cyclin D1 in PKC-depleted cells, in the absence of changes in cyclin D1 message levels, suggesting the involvement of post-transcriptional mechanisms. Several lines of evidence obtained in the current study point to a role of PKC α signaling, in particular, in repressive control of cyclin D1 accumulation. First, selective inhibition of PKC α activity was found not only to block PKC agonist-induced downregulation of cyclin D1, but also to markedly elevate cyclin D1 steady state levels in cycling cells.

Second, targeted silencing of PKC α mRNA, but not of PKC δ or PKC ϵ mRNA, resulted in aberrant elevation of cyclin D1 protein in IEC-18 cells. Third, abrogated PKC α expression in association with aberrantly high levels of cyclin D1 was detected in colonic adenocarcinoma cell lines derived from human tumors and *Apc^{min}* mouse adenomas. Based on these findings, PKC α signaling is proposed to be a key regulator of cyclin D1 expression in intestinal epithelial cells. Reduced PKC α signaling may provide a proliferative advantage to intestinal cells through relief of its preventive role on cyclin D1 accumulation, and thus contribute to malignant transformation. This study also highlights the potential importance of translational control in maintenance of intestinal epithelial homeostasis.

TABLE OF CONTENTS

Section	Page
Title page	i
Abstract	ii
Table of Contents	v
List of Figures	xiv
List of Tables	xxi
List of Abbreviations	xxii
Acknowledgements	xxiv
Dedication	xxvii
I. INTRODUCTION	1
I.A The PKC family of Ser/Thr-specific protein kinases	2
I.A.1 Structure of PKC isozymes	4
I.A.2 Model for activation of PKCs	8
I.A.3 PKC agonists and inhibitors	15
I.A.4 Role of PKC in the cell cycle	18
I.A.5 PKC and cancer	23
I.A.5.1 PKC α and intestinal neoplasia	26

I.B D-Type cyclins and their role in cell cycle progression	28
I.B.1 The mammalian somatic cell division cycle	28
I.B.2 Summary of CDK regulation	31
I.B.3 Mechanisms of regulation of cyclin D1	35
I.B.3.1 Transcriptional Regulation of cyclin D1	38
I.B.3.2 Regulation of cyclin D1 via alteration in mRNA stability	40
I.B.3.3 Regulation of cyclin D1 mRNA transport	41
I.B.3.4 Regulation of cyclin D1 via alterations in protein stability	42
I.B.3.4a Glycogen Synthase Kinase-3 β (GSK-3β) and its regulation of cyclin D1 protein stability	43
I.B.3.4b Regulation of cyclin D1 protein stability via the N-terminal “destruction-box” motif	51
I.B.3.4c Regulation of cyclin D1 protein stability by the calpain protease	52
I.B.3.5 Regulation of cyclin D1 protein synthesis	52
 I.C Regulation of gene expression at the level of translation	 53
I.C.1 Translation Initiation	53
I.C.2 Translation Elongation	57
I.C.3 Regulation of global translation machinery components can impact expression of specific genes	59
I.C.3a Regulation of eIF4E in mammalian cells	64
I.C.3b Regulation of eIF2 α in mammalian cells	68
I.C.3c Regulation of eEF2 in mammalian cells	70

I.C.4 Regulation of key translation factors by the cell cycle	72
I.D Objectives	74
II. Materials and Methods	79
II.A Antibodies	79
1. Antibodies to PKC isozymes	79
2. Antibodies for cyclin D1	79
3. Antibodies for translational control molecules	79
4. Conjugated secondary antibodies	80
II.B Cell Culture Procedures	80
1. IEC-18 cell cultures	80
2. PKC agonists and activation protocols	81
3. PKC inhibition	82
4. Proteasome inhibition	82
5. Inhibition of translation and preservation of polyribosomal complexes	82
II.C Cloning and transfection procedures	83
1. Cloning of cyclin D1 mutants	83
2. Transfection of IEC-18 cells	84
II.D Polyribosome Analysis	84

II.E In vitro Cap-affinity Assay	87
II.F Immunoprecipitation-reverse transcriptase polymerase chain reaction (IP-RT-PCR) assay	88
II.G [S^{35}]-Met/Cyst pulse-chase analysis	90
II.H Biosynthetic labeling experiments	91
II.I Cell Lysate Preparations	92
1. Protein isolation	92
2. Subcellular Fractionation	93
3. Total RNA isolation	94
4. RNA fractionation	94
5. Immunoprecipitation (IP)	95
II.J Western Blot Analysis	97
II.K Northern Blot Analysis	98
II.L Immunofluorescence Staining of <i>Apc^{min}</i> mouse adenomas for cyclin D1	100
II.M Immunohistochemical analysis of <i>Apc^{min}</i> mouse adenomas for PKCα	101

II.N II.N Silencing of PKCα, PKCδ and PKCϵ using <u>RNA interference</u> (RNAi) technology	102
III. RESULTS	103
III.A PKC-induced cell cycle arrest in cultured intestinal epithelial cells involves early loss of cyclin D1 protein.	103
III.A.1. PMA induces an early loss of cyclin D1 protein	103
III.A.2. Other PKC agonists also induce early loss of cyclin D1 protein	104
III.A.3. Depletion of PKC isozyme protein or inhibition of PKC activity blocks PMA-induced cyclin D1 downregulation in IEC-18 cells	105
III.A.4. Inhibition of PKC α signaling blocks PMA-induced loss of cyclin D1	107
III.B Analyses of cyclin D1 regulation in response to PKC/PKCα signaling	109
III.B.1 PKC/PKC α -mediated downregulation of cyclin D1 protein does not involve changes in cyclin D1 gene expression or stability of its mRNA	109
III.B.2. PKC/PKC α activation does not alter the transport of cyclin D1 message	111
III.B.3 PKC/PKC α signaling does not alter the stability of cyclin D1 protein	113
III.B.4a. Addressing the possible involvement of GSK-3 β in PKC/PKC α -induced downregulation of cyclin D1	117

III.B.4a.I Inhibition of GSK-3 β activity does not block PKC α -mediated loss of cyclin D1	120
III.B.4a.II. PKC/PKC α activation does not lead to changes in GSK-3 β phosphorylation on Ser9	121
III.B.4a.III. PMA downregulates a GSK-3 β non-phosphorylatable cyclin D1 mutant	123
III.B.4b. PKC/PKC α -mediated loss of cyclin D1 does not involve engagement of “destruction-box” motif-mediated degradation pathways	125
III.C Analyses of regulation of cyclin D1 translation in response to PKC/PKCα signaling	128
III.C.1 PKC/PKC α activation leads to inhibition of cyclin D1 synthesis	128
III.C.2 PKC/PKC α -activation leads to major changes in the translation machinery	130
III.C.2.1 PKC/PKC α signaling leads to inhibition of cap-dependent translation initiation	130
III.C.2.2 Impact of PKC/PKC α -activation on levels of eIF4E	131
III.C.2.3 PKC/PKC α activation leads to rapid, strong and sustained phosphorylation of eIF4E	132
III.C.2.4 PKC/PKC α activation leads to rapid and sustained activation 4E-BP1	135
III.C.2.5 Activation of 4E-BP1 is PKC α -dependent	136

III.C.2.6 Increased association of 4E-BP1 with eIF4E upon PKC/PKC α activation	140
III.C.2.7 PKC α -activation leads to acute sequestration of cyclin D1 mRNA in 4E-BP1-associated complexes	142
III.C.3 PKC activation does not lead to changes in the activation status of eIF2 α	144
III.C.4 PKC activation leads to rapid inhibition of eEF2	146
III.C.4.a PMA-induced inhibition of eEF2 is PKC α -dependent	148
III.C.4.b PKC signaling does not alter the activity of eEF2K	150
III.C.5. PMA does not alter the polyribosome profile of cyclin D1 mRNA	151
III.C.6 PKC/PKC α -induced alterations in 4E-BP1 and eEF2 could account for the observed decrease in levels of cyclin D1 degradation-mutants	154
III.D Analysis of the relationship between loss of PKC/PKCα signaling and regulation of cyclin D1 expression in intestinal epithelial cells	156
III.D.1 Loss of PKC/PKC α signaling is associated with hyperinduction of cyclin D1 protein and accelerated IEC-18 cell growth	156
III.D.2 Depletion of PKC/PKC α is associated with recovery of cap-dependent translation initiation and translation elongation, increased cyclin D1 mRNA levels as well as enhanced synthesis of cyclin D1 protein	164
III.D.3 Loss of PKC signaling and increased cyclin D1 expression are	168

associated with accelerated growth of intestinal epithelial cells	III.D.4 Intestinal
neoplasms are characterized by decreased/abrogated	170
PKC α expression and elevated levels of cyclin D1	
IV. DISCUSSION	173
IV.A PKCα signaling inhibits translation of cyclin D1 in intestinal epithelial cells undergoing G0 withdrawal	175
IV.A.1 PKC/PKC α activation inhibits cap-dependent translation initiation of cyclin D1 in IEC-18 cells	179
IV.A.2 PKC signaling inhibits translation elongation in IEC-18 cells	186
IV.A.3 Evidence for the role of inhibition of cap-dependent translation initiation and translation elongation in PKC-induced downregulation of cyclin D1	187
IV.A.4 Tight regulation of cyclin D1 expression and of the translation machinery is crucial for maintenance of cell growth control	188
IV.B Keeping cyclin D1 expression in check by the presence of PKCα	190
IV.C Maintenance of high levels of cyclin D1 may provide the basis for frequent loss of PKCα expression in colon tumorigenesis	192
IV.D Future Directions	194
IV.D.1 Addressing the role of loss of Akt signaling in mediating PKC-induced inhibitory effects on translation and cell cycle progression of intestinal epithelial cells	194
IV.D.2 Addressing other consequences of loss of PKC α signaling that may contribute to cellular transformation	196

IV.D.3 Addressing the anti-tumorigenic effects of PKC α overexpression in intestinal tissue using animal models	196
Appendix A PI3K and mTOR signaling pathways modulate components of the translation machinery	198
Appendix B Optimization of cyclin D1 antibodies raised in rat for Western blot and IP protocols	204
Appendix C PMA does not change the inhibitory phosphorylation on GSK-3 β	207
Appendix D Control CIP-reaction to confirm the phospho-specificity of phospho-Thr56-eEF2 and phospho-Ser366-eEF2K rabbit polyclonal antibodies	210
Appendix E Proteasome is involved both in regular turnover of cyclin D1 and PKC-induced downregulation of cyclin D1 in intestinal epithelial cells	212
V. REFERENCES	214-278

LIST OF FIGURES

Figure Title	Page
Figure 1 Molecular architecture of the PKC isozymes	6-7
Figure 2 The addition of phosphate to the <i>Turn Motif</i> and <i>Hydrophobic Motif</i> sites in cPKCs is reflected in a shift in electrophoretic mobility	9
Figure 3 Current Model for activation of cPKCs	11
Figure 4 Molecular events involved in the progression of the mammalian somatic cell cycle	29
Figure 5 Principles of CDK-regulation	32
Figure 6 Inhibitory regulation of GSK-3 β by mitogenic stimuli	47
Figure 7 Diagrammatic representation of insulin-governed signaling pathways activating the cell cycle machinery, glycogen storage, and protein synthesis by converging on inactivation of GSK3 β	48
Figure 8 Regulation of GSK-3 β activity by WNT-signaling	49
Figure 9 Translation initiation in eukaryotes	55-6

Figure 10	Translation elongation in eukaryotes	58
Figure 11	Model for mRNA hierarchy for cap-dependent translation initiation	62
Figure 12	Predicted secondary structure of cyclin D1 leader sequence	63
Figure 13	Cap-structure	64
Figure 14	Regulation of cap-dependent translation through the modulation of eIF4E activity via binding of 4E-BP1	67
Figure 15	Models of PKC-mediated cell cycle withdrawal in IEC-18 cells.	76
Figure 16	Effect of PMA treatment on the expression of cyclin D1, cyclin E, cdk4 and cdk2 in IEC-18 cells.	77
Figure 17	Comparison of PKC-isozyme expression patterns of IEC-18 cells and rat small intestinal tissue	78
Figure 18	Changes in cyclin D1 expression in response to PKC-activation with PMA	103
Figure 19	Changes in cyclin D1 expression in response to PKC-activation by various PKC agonists	104

Figure 20	PMA-induced changes in cyclin D1 expression is PKC-dependent	106
Figure 21	The PKC α -selective inhibitor Gö6976 blocks PMA-induced loss of cyclin D1	108
Figure 22	PKC-induced downregulation of cyclin D1 protein is not due to changes in regulation of cyclin D1 message	110
Figure 23	PKC signaling does not alter nucleocytoplasmic transport of cyclin D1 message	112
Figure 24	PKC-activation does not alter stability of cyclin D1 protein	114
Figure 25	Adverse effects of prolonged methionine/cysteine starvation on cyclin D1 steady state levels in IEC-18 cells	116
Figure 26	Rapid and transient loss of Akt signaling upon PKC activation in IEC-18 cells	118
Figure 27	Crosstalk between PKC and PI3K/Akt/GSK-3 β signaling pathways	119
Figure 28	Inhibition of GSK-3 β by Li ⁺ does not prevent PKC-induced loss of cyclin D1	120

Figure 29	PKC activation does not decrease the levels of inhibitory phosphorylation of GSK-3 β	122
Figure 30	PMA downregulates a Thr286A cyclin D1 mutant in IEC-18 cells	124
Figure 31	Generation of the cyclin D1 “destruction-box”-mutants	126
Figure 32	PMA downregulates the “destruction-box” cyclin D1 mutants	127
Figure 33	PKC signaling suppresses cyclin D1 synthesis in IEC-18 cells	129
Figure 34	Effect of PKC signaling on levels of eIF4E	131
Figure 35	Impact of PKC activation on eIF4E phosphorylation at Ser209	133
Figure 36	PKC-induced phosphorylation of Ser209 on eIF4E is Erk/MAPK-dependent	134
Figure 37	PKC activation promotes hypophosphorylation of 4E-BP1	135
Figure 38	PMA-induced activation of 4E-BP1 is PKC signaling-dependent	136
Figure 39	Other PKC-agonists can induce activation of 4E-BP1	137

Figure 40	PMA can dephosphorylate 4E-BP1 in PKC δ/ϵ -depleted cells	138
Figure 41	The PKC α selective inhibitor Gö6976 blocks PMA-induced dephosphorylation of 4E-BP1	139
Figure 42	PKC activation results in increased association of 4E-BP1 with eIF4E	141
Figure 43	Activation of PKC leads to sequestration of cyclin D1 mRNA in 4E-BP1-associated complexes	143
Figure 44	PKC activation does not lead to changes in phospho-Ser51-eIF2 α levels	145
Figure 45	PKC activation promotes rapid inhibition of eEF2	147
Figure 46	PMA-induced inhibition of eEF2 is dependent on PKC signaling	148
Figure 47	PKC α alone can promote modest inhibition of eEF2	149
Figure 48	PMA does not alter Ser366 phosphorylation of eEF2K	150
Figure 49	No change is detected in the polysome profile of cyclin D1 message 1 h after PKC activation	
Figure 50	Hypophosphorylation of 4E-BP1 and increased	155

phospho-Thr56-eEF2 levels are observed in samples expressing
cyclin D1 degradation mutants following 2 h-PMA-treatment

Figure 51	Restoration of cyclin D1 levels following PMA-induced downregulation coincides more closely with the disappearance of PKC α	157
Figure 52	PKC/PKC α depletion is associated with a marked restoration /hyperinduction of cyclin D1 protein	158
Figure 53	Regulation of cyclin D1 levels by various PKC agonists in IEC-18 cells correlates with their effects on PKC α expression/activity	160
Figure 54	Both a general PKC-inhibitor and a PKC α -selective inhibitor results in hyperinduction of cyclin D1 in IEC-18 cells	161
Figure 55	Silencing of PKC α , but not PKC δ/ϵ results in hyperinduction of cyclin D1	163
Figure 56	Enhanced cyclin D1 protein synthesis is associated with reversal of the inhibition by 4E-BP1 hypophosphorylation and loss of inhibitory phosphorylation of eEF2 on Thr56	166
Figure 57	The hyperinduction of cyclin D1 that accompanies loss of PKC signaling in IEC-18 is associated with increased mRNA expression and enhanced synthesis of the protein	167

Figure 58	Loss of PKC/PKC α signaling in intestinal crypt cells is associated with prolonged hyperinduction of cyclin D1 protein and accelerated growth	169
Figure 59	Intestinal neoplasms are characterized by decreased/abrogated PKC α expression and elevated levels of cyclin D1	171-2
Figure 60	Model of the role of PKC α signaling in regulation of cyclin D1 levels in intestinal epithelial cells	174
Figure A.1	PI3K and mTOR signaling pathways modulate components of the translation machinery	199
Figure B.1	Optimization of cyclin D1 antibodies raised in rat for Western blot and IP protocols	206
Figure C.1	PMA does not change the inhibitory phosphorylation on GSK-3 β	209
Figure D.1	Control CIP-reaction to confirm the phospho-specificity of phospho-Thr56-eEF2 and phospho-Ser366-eEF2K rabbit polyclonal antibodies	211
Figure E.1	Proteasome Inhibitors block PKC-induced downregulation of cyclin D1 protein	213

LIST OF TABLES

Table	Page
Table 1 PKC isozyme subfamilies	3
Table 2 Cell cycle effects of PKC isozyme overexpression	22



LIST OF ABBREVIATIONS

Bis-I	bisindolylmaleimide I
Bryo	Bryostatin-1
CAK	cdk-activating kinase
cdk	cyclin-dependent kinase
CKI	cdk inhibitory protein
DAG	diacylglycerol
DiC ₈	1,2-dioctanoyl- <i>sn</i> -glycerol
DME	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
Erk	extracellular signal-regulated kinase
FBS	fetal bovine serum
HRP	horseradish peroxidase
RT-PCR	Reverse transcriptase polymerase chain reaction
MAPK	mitogen-activated protein kinase
PBS	phosphate-buffered saline
PdBu	phorbol 12,13-dibutyrate
PKC	protein kinase C
PS	phosphatidylserine
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

TBS	Tris-buffered saline
eIF4E	eukaryotic initiation factor 4E
4E-BP1	eIF4E binding protein
eIF4G	eukaryotic initiation factor 4G
eIF4F	eukaryotic initiation factor 4F
eIF4A	eukaryotic initiation factor 4A
eEF2	eukaryotic elongation factor 2
eEF2K	eukaryotic elongation factor 2 kinase
PtdIns-4,5-P ₂	phosphatidylinositol- 4,5 -bisphosphate
PtdIns-3,4,5-P ₃	phosphatidylinositol-3,4,5,-trisphosphate
PI3K	phosphatidylinositol 3-kinase
PDK-1	3-phosphoinositide-dependent protein kinase-1
PTEN	phosphatase and tensin homologue deleted from chromosome 10
mTOR	mammalian target of rapamycin
Met-tRNA _i	Methionyl-tRNA
p70S6K	p70 S6 (ribosomal protein) kinase
MAPKAP-K1	<u>MAPK</u> - <u>a</u> ctivated p <u>r</u> otein <u>k</u> inase <u>1</u> (also known as RSK)
GSK-3 β	glycogen synthase kinase-3 β
<i>Apc</i> ^{<i>min</i>}	adenomatous polyposis coli gene with <i>min</i> mutation
APC	anaphase promoting complex
GAP	GTPase Activating Protein
5'/3' UTR	5'/3' Untranslated Region

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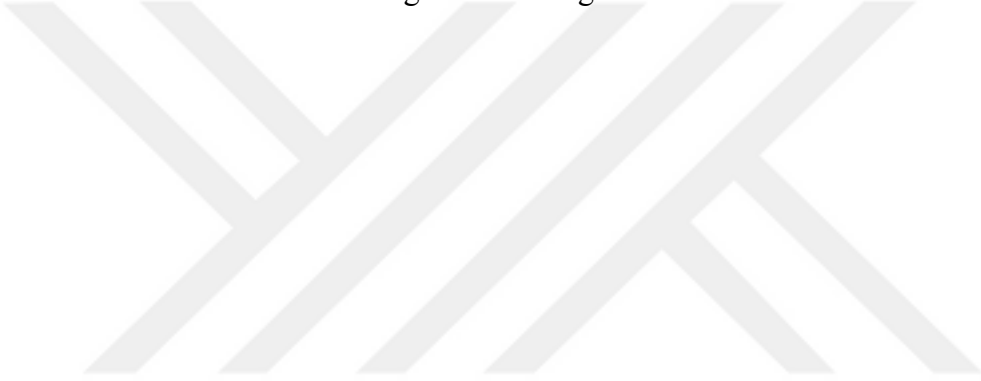
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DEDICATION

This work is dedicated to my mother, Mehlika Hizli, who will always be uniquely special in my life, and to the memories of an always-to-be-remembered uncle and my father's great friend Mr. Yekta Okur, my aunt Macide Islen and my dear teacher, Ms. Iffet Esen, whom I lost really prematurely and with whom I had originally discovered the taste of learning and knowing.



I. INTRODUCTION

In recent years, several studies have highlighted the key participation of the PKC family of Ser/Thr kinases in signal transduction pathways related to cell growth, cell cycle progression, differentiation, apoptosis, cytoskeletal remodeling, modulation of ion channels and secretion in various cell systems including the intestinal epithelium (Black 2000, references therein). Subsequent findings reporting frequent deregulation of several members of the family including PKC α , β I, β II, δ , and ζ in both rodent and human colonic malignancies underscored the importance of retaining normal expression levels and activity of these kinases to maintain homeostasis in this continually self-renewing tissue (Black 2001, references therein).

In previous studies involving pharmacological manipulation of PKC isozyme activity in IEC-18 intestinal crypt cells and *in situ* imaging techniques in rat intestine, we demonstrated that activation of PKC α signaling is tightly associated with cessation of cell cycle progression and commitment to differentiation (Frey et al. 2000a). A set of hallmark molecular events indicative of G1 arrest and G0 exit, including rapid loss of cyclin D1 protein, was shown to take place upon agonist-induced activation of PKC α . Conversely, agonist-induced depletion of PKCs was shown to result in sustained hyperinduction of cyclin D1.

In this study, we have attempted to characterize the molecular mechanisms underlying the loss of cyclin D1 promoted by PKC α and the hyperinduction of the

protein associated with abrogated PKC signaling in IEC-18 cells. Our results indicate that (a) PKC α activation modulates key factors of the translation machinery in order to inhibit cyclin D1 synthesis in cells undergoing G0 exit, and (b) the hyperinduction of cyclin D1 associated with loss of PKC α signaling is achieved by enhanced cyclin D1 protein synthesis involving reversal of inhibition of key translation regulators and post-transcriptional alterations. Below, we first present a brief summary of current understanding of A) PKC signal transduction, B) Regulation of cyclin D1 expression, and C) Control of gene expression at the level of translation. We also discuss D) Objectives of this study, as well as IEC-18 cells as an *in vitro* model to study PKC α -mediated regulation of cyclin D1 in the intestinal epithelium.

1.A The PKC family of Ser/Thr-specific protein kinases

To date, 10 mammalian isozymes have been identified as members of the protein kinase C (PKC) family of signal transduction molecules. These enzymes belong to the protein kinase **A**, protein kinase **B** (also known as Akt), and Protein Kinase **C** (**ABC**) superfamily of Ser/Thr protein kinases (Toker and Newton, 2000, Newton 2004, and Hofmann 2004). The members of the PKC family are related through a conserved kinase domain but differ in their regulatory moieties (Newton 2004). These isozymes are therefore subject to differential biochemical regulation, intracellular localization, and tissue distribution (Kazanietz 2000). As summarized in Table 1, PKC isozymes are classified into three main groups on the basis of the domain composition in the regulatory moiety: (a) Conventional PKCs (**cPKCs**; α , β I, β II, and γ). PKC β II is an alternatively spliced variant of PKC β I, which differs only in the last 43 residues. cPKCs require both

Ca²⁺ and phosphatidylserine (PS), phorbol esters or diacylglycerol (DAG) for optimal activation. DAG is an endogenous agonist which is a breakdown product of phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol- 4,5 -bisphosphate (PtdIns-4,5-P₂). (b) novel PKCs (nPKCs; δ , ϵ , θ , and η) require PS, DAG or phorbol ester, but not calcium, for activation. (c) atypical PKCs (aPKCs; λ in mouse / ι in human, and ζ) require neither Ca²⁺ nor DAG for activation. PKC ζ has a splice variant which only encodes the catalytic domain (PKM ζ). aPKC are known to require only PS or cholesterol sulfate for activation (Newton 2003 and Hofmann 2004).

Table 1 PKC Isozyme subfamilies. This table, which is originally adapted from Nishizuka (1992), is taken from Frey (2000b)

Subfamily	Isozymes	Activator/cofactor sensitivity	Tissue distribution
Conventional	α	Responds to Ca ²⁺ , PS, DAG, phorbol esters	universal
	β I	Responds to Ca ²⁺ , PS, DAG, phorbol esters	limited tissues
	β II	Responds to Ca ²⁺ , PS, DAG, phorbol esters	widely expressed
	γ	Responds to Ca ²⁺ , PS, DAG, phorbol esters	brain, spiral cord, some other tissues
Novel	δ	Responds to PS, DAG, phorbol esters, but not Ca ²⁺	universal
	ϵ	Responds to PS, DAG, phorbol esters, but not Ca ²⁺	some tissues, incl. brain, skin, epithelial tissues
	η	Responds to PS, DAG, phorbol esters, but not Ca ²⁺	some tissues, incl. lung, skin, muscle, heart, intestine
	θ	Responds to PS, DAG, phorbol esters, but not Ca ²⁺	many tissues
Atypical	ζ	Insensitive to DAG, Ca ²⁺ , phorbol esters; may respond to PS	universal
	ι	Insensitive to DAG, Ca ²⁺ , phorbol esters; may respond to PS	Some tissues, incl. brain, ovary, testis, epithelial tissues

1.A.1 Structure of PKC isozymes

As mentioned above, PKC isozymes have a conserved kinase core region, which is also shared by other members of the AGC superfamily (Newton 2003). The regulatory region not only controls the kinase activity by acting as an autoinhibitory module, but also plays a role in targeting the enzyme to the appropriate subcellular localization (Newton 2003). Autoinhibition is mediated through steric blockade of the active site by the so-called “pseudosubstrate” sequence (depicted in green rectangles in Figure 1). Binding of cofactors like DAG induces conformational changes to relieve the active site from autoinhibition. The membrane-targeting modules in the regulatory moiety as well as specific protein-protein interactions determine the precise localization of the active enzyme (Newton 2003) (Figure 1).

The regulatory moiety of PKC isozymes consists of two domains: the **C1 domain**, which confers sensitivity to DAG, and the **C2 domain**, which is a Ca^{2+} sensor. These modules can be found in ligand-binding or ligand non-binding forms. In accordance with their activation requirements (Table 1), (a) cPKCs have functional C1 and C2 domains, and therefore, respond to both DAG and Ca^{2+} ; (b) nPKCs have a functional C1 domain but a non-functional C2 domain, and therefore they respond only to DAG, but not to Ca^{2+} ; and (c) aPKCs have a non-functional C1 domain and no C2 domain, and respond neither to DAG nor to Ca^{2+} .

The C1 domain acts as a DAG sensor through the affinity of its cysteine-rich tandem repeats for this agonist. Phorbol esters and nonhydrolyzable analogues of the

endogenous ligand also bind to this site. Both cPKCs and nPKCs have two C1 domains, **C1A** and **C1B**, only one of which seems to be engaged to the ligand at a time. In aPKCs, the ligand-binding region of the C1A domain is impaired; therefore, these isozymes do not respond to DAG or phorbol esters. The C1 domain also binds to PS. Other non-kinase molecules also have C1 domains, which can be bound by DAG and phorbol esters. Therefore, PKC-dependence of a given effect must be addressed by various approaches as we will discuss further in the Results Section.

The C2 domain senses Ca^{2+} levels. In conventional PKCs, the C2 domain serves as a membrane-targeting module binding anionic phospholipids in a Ca^{2+} -dependent manner. Novel PKCs lack the Ca^{2+} -binding sequences in their so-called C2-like domain; therefore, they neither bind Ca^{2+} nor phospholipids. Other non-PKC molecules have also been reported to have C2 domains. *In vivo* PKC, is largely maintained in an inactive conformation through binding of the pseudosubstrate sequence to the substrate-binding cavity, which masks the active site. C1 and C2 domains also act as membrane-docking sites when the enzyme encounters its activation stimuli, DAG and Ca^{2+} . Membrane-binding is a key event that allows PKC substrate-binding and catalysis.

PKCs are also found in lower eukaryotes. *Caenorhabditis elegans* has four PKCs while *Saccharomyces cerevisiae* has only one PKC, PKC1, which contains apparently non-ligand-binding C1 and C2 domains, and responds to neither diacylglycerol nor Ca^{2+} .

Figure 1

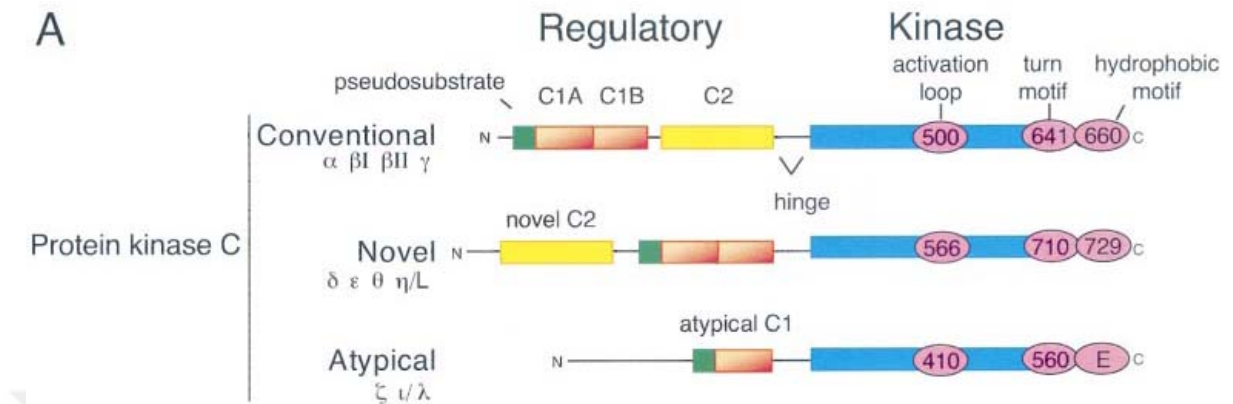


Figure 1 (a) Molecular architecture of the PKC isozymes The C1 domain, which is almost 8 kDa in size, is present in all PKCs and mediates binding to DAG (red rectangles). The C2 domain is 12 kDa and is present only in conventional and novel PKCs (yellow rectangle). The hinge region serves as the cleavage site for calpain and trypsin. The three critical phosphorylation sites, which are conserved in all members of the ABC superfamily, are located in the kinase domain (depicted in pink balls). Without these priming phosphorylations, these kinases remain catalytically inactive and incompetent for activation. The phosphorylation of the *Activation Loop Site*, located near the entrance to the active site, is an important determinant of substrate phosphorylation. Mutation of this crucial Thr residue to nonphosphorylatable residues abolishes activity while converse mutations generate catalytically competent enzyme. The *Turn Motif* is the next conserved phosphorylation site, C-terminal to activation loop site. The phosphate at the turn motif has a role in stabilization of the kinase core. Finally, in the extreme C-terminus lies the *Hydrophobic motif* site which, like the turn motif residue, can be either a Ser or a Thr, but flanked by hydrophobic residues. Phosphorylation of this site also increases the stability of the kinase core (Figure is taken from Newton, 2003).

B

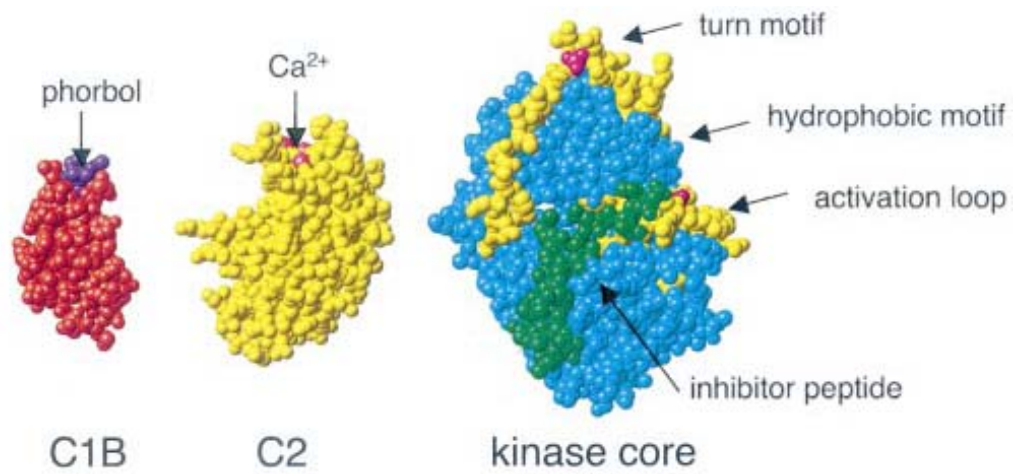


Figure 1 (b) Visualization of the C1B, C2, and the kinase core regions based on information obtained from molecular modeling techniques. The *Kinase core* is thought to have a bilobal structure separated by a cleft region that is involved in binding ATP and the substrate. The N-terminal lobe is primarily composed of β sheet, while the C-terminal lobe is primarily α helix.

I.A.2 Model for activation of PKCs

The molecular mechanisms governing the life-cycle of a nascent PKC have been the focus of many studies. There are discrepancies among these reports regarding the details of each stage of the PKC life-cycle. For the sake of brevity, this section will only include a summary of the most widely accepted paradigm for cPKC activation.

Therefore, molecular events related to activation which may be different for some of the isozymes, or still subject to debate in the field, will not be discussed in detail.

The newly synthesized PKC species has an “open-structure” in which the autoinhibitory pseudosubstrate region is *not* folded into the substrate cavity. In this open conformation PKC seems to make multiple weak interactions with anionic lipids in the membrane through its C1, C2, and the basic pseudosubstrate domains. These three interactions are thought to provide sufficient tethering of PKC in an open conformation to the membrane to allow the PDK-1-mediated crucial phosphorylation at the *Activation Loop* to take place. PDK-1 docks on the unphosphorylated hydrophobic site, catalyzes the phosphate transfer onto the Thr residue of the Activation Loop and is released from the C-terminal docking-site leaving the two other critical phosphorylation sites required for activation exposed. Next, at least in the case of conventional PKCs, the hierarchical phosphorylation of the *Turn Motif* and the *Hydrophobic Motif* are achieved via intramolecular autophosphorylation mechanisms. The addition of the phosphates to the *Turn Motif* and *Hydrophobic Motif* sites, but not the *Activation Loop* site, is reflected in

electrophoretic mobility shifts each of which results in retardation of migration equivalent of 2 kDa (Newton 2003) (Figure 2 and 3).

Figure 2



Figure 2 The addition of phosphate to the *Turn Motif* and *Hydrophobic Motif* sites in cPKCs is reflected in a shift in electrophoretic mobility IEC-18 cells were treated with vehicle or 100 nM Bryostatin-1 (**Bryo-1**) for 2 h. Protein samples were resolved on 10% SDS-PAGE gels and immunoblotted for PKC α . The mature form of PKC α (M), phosphorylated at the *Activation Loop*, *Turn Motif*, and *Hydrophobic Motif* sites, migrates at approximately 80 kDa. Treatment with Bryo-1 results in removal of phosphate from all three sites (Leontieva and Black, 2004). Deposphorylation of the *Turn Motif*, and *Hydrophobic Motif* sites is reflected in a 4 kDa shift, to produce a faster migrating form of 76 kDa (arrow). Loss of phosphate at the *Activation Loop* does not alter the migration of the protein on SDS-PAGE gels (Keranen et al., 1995).

Phosphorylation of these three sites in the C-terminus results in critical conformational changes, which lock PKC into a more thermally stable, protease- and phosphatase-resistant state. Phosphorylation of the extreme C-terminus (*Hydrophobic Motif*), in particular, has been suggested to lock this segment on the upper lobe of the kinase domain, clearing the substrate cavity for the binding of the pseudosubstrate region. Therefore, relocation of the pseudosubstrate region from the point of membrane-attachment to the substrate cavity (active-site) not only shifts the “open” PKC conformation to a “closed” one, but also releases the enzyme into the cytosol. This species of cytosolic PKC in a “closed” conformation is called the “mature” enzyme, and the process in which this conformation is achieved “maturation”. Mature PKC is inactive as a result of autoinhibition, but is competent to become activated. Upon binding of agonists to the C1 and C2 domains, which removes the autoinhibiting pseudosubstrate sequence, the mature fully-phosphorylated PKC associates with the membrane where the catalytically active enzyme binds its substrates and promotes downstream signaling (Newton 2003) (Figure 3).

Figure 3

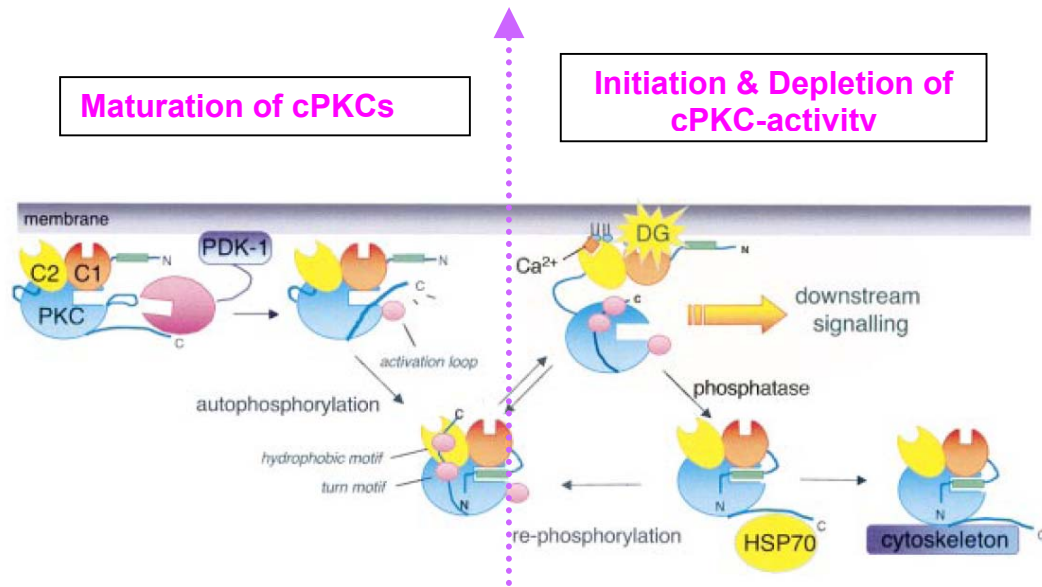


Figure 3 Current Model for activation of cPKCs The biphasic activation of cPKC-signaling is composed of two stages, maturation and agonist-mediated activation. A detailed description of the molecular events is provided in the text. Note that the activated PKC can be rapidly dephosphorylated, and bound by the chaperone Hsp70 at its unphosphorylated *Turn Motif*. Binding of dephosphorylated PKC by Hsp70 stabilizes PKC and allows re-phosphorylation and, thereby, re-entry into the pool of signaling-competent PKC. In the absence of Hsp70, or as a result of chronic activation, PKC is degraded by the proteasome (Figure is taken from Newton 2003).

Since inhibitors of PI3K do not affect the rate of PKC maturation, PDK-1-mediated rate-limiting phosphorylation of the *Activation Loop* is considered independent of phosphatidylinositol 3-kinase (PI3K) activity (Newton 2003). For cPKCs, most of the mature enzyme is localized in the cytosol where it diffuses freely and probably makes occasional collisions with the membrane. However, conventional isozymes are unable to make permanent contacts with the membrane until they encounter a flux of Ca^{2+} and DAG. Binding of Ca^{2+} to the C2 domain immediately promotes the first and rather weak engagement of PKC to the membrane. Subsequent diffusion through the plane of the membrane and encounter with membrane-embedded DAG molecules allows PKC to make stronger membrane contacts through its DAG-binding C1 domain. By virtue of this second engagement, the substrate-cavity is relieved from the autoinhibitory pseudosubstrate region yielding the catalytically active enzyme. Lacking the functional C2 domain, novel PKCs have a reduced chance of being found in the membrane and regulatory mechanisms governing activation of atypical PKCs, which lack the ability of binding Ca^{2+} and DAG, are far less well-understood (Newton 2003) (Figure 3).

Active PKC has increased sensitivity for dephosphorylation. Several studies have demonstrated that prolonged exposure to phorbol esters results in dephosphorylation and subsequent proteasomal degradation of the enzyme, although there is evidence that, at least for the α and δ isozymes, dephosphorylation is not be a pre-requisite for ubiquitin-mediated degradation (Leontieva and Black, 2004, and Srivastava et al. 2002). Experimental evidence suggests that agonist-mediated activation and membrane association of PKCs is closely coupled to their rapid inactivation/downregulation

(Blumberg et al. 1994, Kraft and Anderson 1983, and Young et al. 1987). In fact, one study showed that phorbol ester-stimulated growth of mouse melanocytes correlated with PKC downregulation rather than its activation (Brooks et al., 1991). Therefore, data obtained with the use of PKC activators must be supplemented with data obtained through different approaches before making conclusions about PKC function in a given context.

Lipid cofactor-mediated activation of PKC isozymes is further fine-tuned for substrate specificity by differential subcellular localization which is thought to be mediated by PKC-interacting proteins (Hofmann 2004). Among them are receptor for inactive C kinases (**RICKs**), scaffolding proteins, substrates that interact with C kinases (**STICKs**), PKC activators, receptor for activated C kinases (**RACKs**), and proteins interacting with C kinases (**PICKs**). These adaptor proteins may either isolate PKC from its substrates or bring PKC in proximity of its substrate (Hofmann 2004). Adaptor proteins do not have catalytic activity but are considered to act as a bridging factor connecting an isozyme to a specific signaling pathway. This type of mechanism may account for the observation that different isozymes can play divergent roles in cellular processes as in the case of the cell cycle.

Identification of *in vivo* substrates of PKCs still remains an unconquered challenge of the PKC field, although some candidate proteins, which seem to have PKC-phosphorylation consensus sites, have been shown to be phosphorylated by PKC *in vitro*. Like some other protein kinases (cAMP- or cGMP-dependent Protein Kinases), PKC also

requires presence of basic amino acid residues near the phosphoacceptor group of its substrates (Kennelly and Krebs, 1991). Arginine seems to be preferred over lysine in most cases. Although PKC substrates may have either an N- or C- terminal basic group in close proximity to the phosphoacceptor site, optimal phosphorylation is observed in substrates that have basic groups on both sites (Kennelly and Krebs, 1991). For example, Kennelly and Krebs report that a survey of eight proteins phosphorylated by PKC *in vivo* shows examples of sites that are doublesided (**6/11**), that have only C-terminal basic residues (**2/11**), or that have N-terminal basics only (**3/11**), a pattern resembling that displayed *in vitro*. Determination of a definitive consensus sequence for PKC is complicated by potential substrate specificity differences among different PKC isozymes. However, these authors summarize current understanding of PKC consensus phosphorylation sequences as follows; [N-(R/K₁₋₃, X₂₋₀)-**S*/T***-(X₂₋₀, R/K₁₋₃)-C] is the more frequently preferred than [N-**S*/T***-(X₂₋₀, R/K₁₋₃)-C] which is more or equally preferred with [N-(R/K₁₋₃, X₂₋₀)-**S*/T***-C] (Kennelly and Krebs, 1991).

p53 (Baudier et al., 1992 and Takenaka et al., 1995), annexins I/II (Mochly-Rosen et al. 1991), lamin B (Fields et al., 1988 and 1990), myristoylated alanine-rich C kinase substrates (**MARCKS**) (Albert et al. 1987 and Stumpo et al. 1989), adducins (Chapline et al., 1993 and Fowler et al. 1998), and ribosomal protein S6 kinase betaII (Valovka et al., 2003) are a few examples of candidate PKC-substrates awaiting *in vivo* confirmation. Some of these substrates such as p53 are not well-accepted substrates of PKC.

I.A.3 PKC agonists and inhibitors

Several compounds that can activate PKCs have been either synthesized or isolated from natural sources, and have become widely used tools for studying the cellular functions of PKCs. Compounds such as phorbol esters [e.g., phorbol 12-myrsitate 13-acetate (**PMA**) and phorbol 12, 13-dibutyrate (**PdBu**)] or membrane permeant DAG-analogues [e.g., 1,2-dioctanoyl-*sn*-glycerol (**DiC₈**)] bypass the requirement for upstream receptor-mediated signaling which normally controls PKC activation. These reagents bind the same region as the physiological agonist DAG (Ono et al., 1989 and Sharkey et al., 1984). Binding of phorbol esters to the C1 domain has been shown not to induce a conformational change, but rather to cover a hydrophilic site on the protein exterior forming a contiguous hydrophobic region which can readily insert into the bilayer (Ron and Kazanietz 1999, Zangh et al., 1995). Although phorbol esters and DAG-analogues can all activate PKC, these compounds differ in their potency toward individual PKC family members. Furthermore, DAG analogues (eg., DiC₈) are known to be less potent agonists due to their rapid metabolism in cells, thus providing a much more transient activation of PKC isozymes.

Among the naturally-existing agonists of PKC, Bryo-1 is a widely-studied compound. Bryostatin-1 is isolated from the marine organism *Bugula neritina* (Pettit et al., 1991). Like phorbol esters, Bryo also targets the same DAG-binding region in the regulatory domain of PKC. In intact cells, similar to the case with phorbol esters, binding of Bryo results in translocation of PKC from the cytosol to the plasma membrane and

activation of the enzyme. Administration of Bryo-1 in some systems is reported to be growth inhibitory, while it can stimulate growth in others. However, unlike PMA, Bryo-1 is not known to be a tumor-promoting agent (Hofmann 2004). Treatment with Bryo is known to produce a subset of PMA-induced effects in many systems and to antagonize effects of PMA that it cannot produce by itself. For example, in monocytic leukemia cells (U937) and IEC-18 intestinal epithelial cells, Bryo-induced G0/G1 arrest is weaker compared with that produced by PMA (Vrana et al. 1998 and Clark and Black et al., 2004). Bryo is currently being evaluated as a single anti-tumor agent or in combination with other anti-neoplastic drugs in clinical trials (Hofmann 2004).

Compared with its activators, most of the earlier synthesized inhibitors of PKC were rather weak in inhibition and more promiscuous in terms of targeting other key kinases (Martiny-Baron et al. 1993). **Sphingosine** and **Calphostin C**, which interact with the regulatory moiety, peptide inhibitors related to the pseudosubstrate sequence that compete with the substrate binding cavity, and **Staurosporine** and **H7**, which are competitive inhibitors of ATP-binding, are from this older generation of PKC inhibitors (Martiny-Baron et al. 1993). For example, staurosporine, which was known as the most potent inhibitor of PKC, was also shown to inhibit p70 S6 kinase, phosphorylase kinase, c-AMP-dependent protein kinase, and src kinase with similar efficiencies (Martiny-Baron et al. 1993). Compounds generated more recently such as bisindolylmaleimide I (**BIM I**) (Toullec et al. 1991) and the indolocarbazole **Gö6976** (Martiny-Baron et al. 1993) are recognized as more potent and selective inhibitors of PKC activity; thus, they have been used as pharmacological tools in numerous studies.

BIM I, which is a general inhibitor of all PKC isozymes, was developed as a staurosporine-related compound (Toullec et al. 1991). Since it does not inhibit binding of PdBu to PKC, its inhibitory activity is not a result of its interaction with the regulatory moiety. BIM I is thought to compete with ATP for binding to the ATP-binding site which is conserved in several other protein kinases. Therefore, the selectivity of BIM I for PKC family members is probably due to high affinity of the BIM I molecule for the tertiary structure of the ATP-binding site of PKCs (Toullec et al., 1991). Gö6976 is a BIM I derivative that has high selectivity for conventional isozymes, PKC α , PKC β I and II, and γ . This compound has no recorded inhibition of novel PKCs (Martiny-Baron et al. 1993). Nonetheless, being competitors for the ATP-binding site in PKCs, there is always the risk that these compounds may inhibit other yet-to-be discovered kinases, which may have similar tertiary ATP-binding domains to that of PKCs (Toullec et al., 1991).

I.A.4 Role of PKC in the cell cycle

The role of PKC isozymes in control of cell cycle progression is now well recognized (reviewed in Black 2000). An avalanche of data has been obtained (over 1500 papers in PubMed) characterizing the function of PKC isozymes in cell cycle progression. Early observations made with PKC1 (the single PKC-homologue in budding yeast) underscore the crucial role of PKC-signaling in cell cycle regulation (Levin et al., 1990). In this study, depletion of PKC1 resulted in arrest of cell division at a point subsequent to DNA replication, but prior to mitosis. Interestingly, in contrast to the phenotype of cell division cycle (**cde**) mutants, which continued to grow in the absence of cell division, PKC1-depleted cells arrested growth with small buds.

Subsequent studies showed that mammalian PKCs also play major roles in control of cell cycle progression at the G0/G1, G1/S, and G2/M transitions (reviewed in Black 2000). Most of these studies obtained information on how PKC family members impact cell cycle progression through overexpression or depletion of individual isozymes, or through modulation of endogenous PKC activity using relatively isozyme-specific agonists or inhibitors (reviewed in Black 2000). In the majority of the systems examined, the effects of PKC signaling on cell cycle progression were found to be inhibitory, although there are reports on the involvement of PKC in positive control of both the G1/S and G2/M transitions (reviewed in Black 2000). Overall, it is possible to conclude that, while PKC α , δ , and η generally appear to have a cell cycle-inhibitory and differentiation-inducing role, PKC ϵ and β II are implicated in promoting growth and cell cycle

progression. This conclusion is supported by studies in which different isozymes were overexpressed in the same cell line. For example, overexpression of PKC ϵ in rat R6 fibroblasts results in cell transformation (Cacace et al. 1993), while introduction of PKC α into the same cell line leads to growth inhibition (Borner et al., 1995). Similarly, PKC δ inhibits the growth of colonic epithelial cells, while overexpression of PKC ϵ leads to increased growth and transformation (Perletti et al. 1999, Perletti et al. 1998, Perletti et al. 1996).

It must be pointed out, however, that the role of a given isozyme can be dependent on cellular background. Therefore, some of the information obtained regarding the cell cycle regulatory functions of certain PKC isozymes can be controversial. For example, although PKC α activity has been reported to be growth-suppressive in several cell lines including leukemic, normal and transformed intestinal, and prostate cancer cells, there is evidence that it may act as a growth-promoting molecule in certain breast cancer cells and glioblastomas (summarized in Table 2). Similarly, while overexpression of PKC β I in rat fibroblasts enhances growth (Housey et al., 1988), overexpression of the same isozyme in colon adenocarcinoma cell lines is associated with inhibition of growth and reduced tumorigenicity in nude mice (Choi et al., 1990). The impact of PKC β II activity on cell cycle progression is another example of such controversy. In erythroleukemia cells, PKC β II is involved in cell proliferation (Murray et al., 1993), while in HL60 myelocytic leukemia cells the same isozyme has been implicated in differentiation (Gamard et al., 1994). PKC β II activity is thought to play a role in promotion of the G2/M transition by mediating mitotic nuclear envelope breakdown

(Goss et al., 1994). These opposing results may reflect differences in the molecular background of these cell systems. The molecular mechanisms accounting for such discrepancies are still not well understood, although differences in the profile of PKC isozymes expressed and the expression and localization of PKC-interacting proteins may be involved.

Studies addressing the consequences of PKC activation on cell cycle progression using PKC-agonists showed that treatment of vascular endothelial cells (Zhou et al., 1993), vascular smooth muscle cells (Sasaguri et al., 1993), melanoma cells (Coppock et al., 1992), intestinal epithelial cells (Frey et al., 1997), fibroblasts (Hamada et al., 1996), and mammary epithelial cells (Slosberg et al., 1999) results in accumulation of these cells at the G1/S transition. Administration of phorbol esters in Chinese Hamster Ovary (CHO) cells overexpressing PKC δ (Watanabe et al., 1992), HeLa cells (Barth and Kinzel, 1994), melanoma cells (Coppock et al., 1992), and fibroblasts (Barboule et al., 1999) also resulted in G2/M arrest. The cell cycle regulatory effects of PKC were mediated through modulation of various components of the cell cycle machinery. Regulation of the G1/S or G2/M transitions by PKC-signaling appears to involve alterations in pRb, p107, and p130 phosphorylation state, modulation of E2F family members and cyclin/cdk kinase activities, regulation of cyclin-dependent kinase inhibitor (CKI) expression levels (e.g., p21^{CIP1/WAF1} and p27^{KIP1}), and control of the DNA licencing factor cdc6 (reviewed in Black 2000). Major molecular targets of PKC signaling in negative control of the G1/S transition appear to be the activities of cyclin D/cdk4/6 and/or cyclin E/cdk2 complexes. The PKC-induced decrease in the activity of these key G1 kinases does not appear to be

due to altered expression levels of the cdk subunit, although, in some systems, it can be due to reduced phosphorylation of the Thr160 site of cdk2, achieved either by increased dephosphorylation or decreased phosphorylation by cdk activating kinase (CAK). Increased binding of p21^{CIP1/WAF1} or p27^{KIP1}, appears to be a major mechanism of inhibition of cdk2-associated activity in the majority of the systems examined (Black 2000).

Negative regulation of the cyclin D/cdk4/6 complex by PKC is achieved through changes in the levels of the cyclin D1 subunit in some systems. For example, overexpression of PKC δ in vascular smooth muscle cells (Fukumoto et al., 1997) and overexpression of PKC ϵ in R6 fibroblasts (Han et al., 1995) have been shown to result in reduction of cyclin D1 levels. Delayed nuclear translocation of cyclin D1 was seen in PKC δ -overexpressing rat microvascular endothelial cells (Ashton et al., 1999). Phorbol ester treatment of melanoma cells (Coppock et al., 1995), pancreatic cancer cells (Detjen et al., 2000), and intestinal cells (Frey et al., 2000a) resulted in transient suppression followed by hyperinduction of cyclin D1, an event which can promote cellular transformation if it occurs in the context of other gained mutations (Wang et al., 1994, Bodrug et al., 1994, Lovec et al., 1994, and Quelle et al., 1993). As will be discussed further in the following section, deregulation of PKC levels and activity can profoundly impact cell cycle control, and adverse effects resulting from these alterations are likely to contribute to the process of carcinogenesis in several epithelial systems.

<i>Isozyme</i>	<i>Cell type</i>	<i>Effect</i>	<i>Reference</i>
α	intestinal epithelial cells	extends phorbol ester-induced cell cycle arrest	Frey, 2000
	mammary epithelial cells	sensitizes to PMA-induced G1 delay	Slosberg, 1999
	3Y1 fibroblasts	decreases basal E2F activity & sensitizes to PMA induced E2F inhibition	Nakaigawa, 1996
	prostate cancer cells	Increased p21 ^{CIP1/WAF1} expression, hypophosphorylation of pRb	Zhao, 1997
	bovine aortic endothelial cells	decreased proliferation, G2M delay	Rosales, 1998
	R6 fibroblasts	decreased growth	Bonner, 1995 #58
	B16 melanoma cells	Decreased tumorigenicity & partially differentiated phenotype	Gruber, 1992
	colon carcinoma cells	Increased p21 ^{CIP1/WAF1} expression & growth inhibition	Scaglione-Sewell, 1998
	Glioma cell lines	Upregulation of p21 ^{CIP1/WAF1} & positive impact on cell cycle progression	Besson and Yong, 2000
	MCF7 breast cancer cells	Increased tumorigenicity	Ways, 1995
	MCF7 breast cancer cells	Suppressed tumorigenicity	Manni, 1996
	bovine aortic endothelial cells (BAEC)	decreased proliferation and accumulation at G2/M	Rosales, 1998
	BAEC	Shortened doubling-time	Rosales, 1998
	R6 fibroblasts	enhanced growth/transformation	Bonner, 1995
	colon carcinoma cells	growth arrest/tumor suppression	Choi, 1990
βI			
βII	colonic epithelium (transgenic mice)	hyperproliferation	Murray, 1999
γ	human glioma cells	Increased growth	Mishima, 1994
	vascular endothelial cells	p27 ^{KIP1} induction, cell inhibition	Ashton, 1999
δ	3Y1 fibroblasts	Sensitizes to PMA-induced E2F inhibition	Nakaigawa, 1996
	vascular smooth muscle cells	G1 arrest p27 ^{KIP1} induction, decreased cyclin D1 expression	Fukumoto, 1997
	CHO cells	arrest in telophase	Watanabe, 1992
	human glioma cells	decreased growth	Mishima, 1994
	NIH 3T3 fibroblasts	decreased growth & saturation density	Mischak, 1993
	3Y1 fibroblasts	sensitizes to PMA-induced E2F inhibition	Nakaigawa, 1996
ε	NIH 3T3 fibroblasts	increased growth & tumorigenicity	Cacace, 1993
	Rat 6 fibroblasts	increased growth & tumorigenicity	Perletti, 1996
	colonic epithelial cells	increased saturation density & tumorigenicity	Perletti, 1996
η	NIH 3T3 fibroblasts	Cell cycle inhibition, increased CIP/KIP & cyclin E expression, decreased cdk2 activity	Livneh, 1996
θ	vascular endothelial cells	Dominant-negative inhibits G2/M progression	Tang, 1997

Table 2 Cell growth/cell cycle effects of PKC isozyme overexpression or activation. This table is taken and adapted from Frey (2000b).

1.A.5 PKC and cancer

The role of PKC signaling in human cancer has been extensively studied since the discovery that PKCs are the major receptors for tumor-promoting phorbol esters (Castagna et al., 1982). As summarized in a comprehensive recent review by Hofmann (2004), alterations in PKC isozyme expression/function have been detected in a variety of human cancers including tumors of the central nervous system (CNS), breast, pituitary and thyroid, leukemias, skin, colon, and prostate (see references therein). PKCs have been implicated in control of various processes known to be dysregulated during tumorigenesis, including cell proliferation, differentiation, antitumor drug-resistance, and apoptosis in a highly context-dependent manner. Thus, despite a vast amount of information, it is still not possible to assign a specific tumor-promoting or tumor-suppressive role to a given isozyme that applies to all types of cancer. However, trends are beginning to emerge concerning the function of certain PKC in regulating cell transformation.

Increasing evidence from studies in different tumor tissues points to a role for PKC α , δ , β I, and η in negative regulation of cell proliferation and/or survival (Black 2001 and Hofmann 2004). Other reports, however, support the involvement of PKC α , β II, ϵ , ζ , and λ in positive regulation of cell proliferation, tumor vascularization, and survival processes in various malignancies (Beson and Yong, 2000, Murray et al., 1999, and

Hofmann 2004). For example, *in vivo* studies involving targeted expression of PKC α , PKC δ , or PKC ϵ to mouse skin (Jensan et al., 2001) showed that, while PKC α expression had no effect on skin tumor promotion by PMA, exogenous expression of PKC δ resulted in a marked reduction in papilloma burden. Interestingly, while PKC ϵ expression also dramatically reduced papilloma formation, it promoted the development of squamous cell carcinoma in this system (Jensan et al., 2001). Activation or overexpression of PKC δ has been linked to pro-apoptotic signaling in a broad spectrum of tumor types, including U937 and HL60 cells, CaCo2 colon carcinoma cells, and glioblastoma cells (Emoto et al. 1995, Ghayur et al., 1996, Griffiths et al. 1996, Denning et al. 1998, Cerda et al., 2001, and Hofmann 2004). Furthermore, activation or increased expression of PKC α or PKC δ in colon, prostate, or pancreatic cancer cells, or melanoma cells has been associated with inhibition of tumorigenicity (Cerda et al., 2001, Hofmann 2004, Kazanietz 2004, and Detjen et al., 2000). Since an in-depth discussion of the extensive literature on the role of each PKC isozyme in human cancer is beyond the scope of this study, we will limit our focus to findings regarding the involvement of alterations in PKC α signaling in colon carcinogenesis.

Activation of several PKC isozymes has been associated with cell cycle exit and cell differentiation in the normal intestinal epithelium (Saxon et al., 1994, Verstovsek et al. 1998, Jiang et al., 1995, Kahl-Rainer et al., 1996, and Assert et al., 1999). PKC α , β I, β II, δ , ϵ , η , θ , ζ and ι are mostly cytosolic in proliferating intestinal and colonic crypt cells (Saxon et al., 1994, Verstovsek et al. 1998, Jiang et al., 1995, Davidson et al., 1994, Kahl-Rainer et al., 1996, and Assert et al., 1999), and an increase in total levels of

expression and membrane/cytoskeletal association (indicative of enzyme activation) of individual isozymes occurs coincident with enterocyte and colonocyte growth arrest/differentiation (Saxon et al., 1994, Verstovsek et al. 1998, Jiang et al., 1995, Kahl-Rainer et al., 1996). Notably, PKC α , which has been linked to regulation of cell cycle exit in non-transformed intestinal epithelial cells (Frey et al., 1997, 2000a, and Clark and Black et al., 2004), maintains high expression and membrane association throughout the post-mitotic compartment (Figure 15 and data presented in Saxon et al. 1994 and Frey et al. 1997).

In contrast to the expression pattern seen in normal intestinal or colonic cells, total PKC activity and expression of individual PKC isozymes have been reported to be reduced in colonic neoplasms and colon adenocarcinoma cells compared with normal colonic tissue (Kahl-Rainer et al., 1994 and 1996, Kusunoki et al., 1992, Doi et al., 1994, Kuranami et al., 1995, Guillem et al., 1987, Levy et al., 1993, Kopp et al., 1991, Pongracz et al. 1995, Verstovsek et al. 1998, Davidson et al., 1994, Craven and DeRubertis, 1994, and Assert et al., 1999). Decreased PKC activity has been observed in preneoplastic colonic mucosa and in colonic adenomas, indicating that dysregulation of PKC isozyme levels occurs early in the process of multistage colon carcinogenesis (Baum et al., 1990 and Black 2001). Parallel to these observations, loss of PKC activity associated with reduction in isozyme expression levels has been reported in 1,2-dimethylhydrazine-induced rat colonic tumors and in adenomas of the *Apc^{min}* mouse model of Familial Adenomatous Polyposis (**FAP**) (Wali et al., 1991, Baum et al., 1990, and Klein et al., 2000). Current evidence supports the notion that loss of PKC α , β I, δ , and

ζ provides a growth/survival advantage to intestinal cells, thus promoting intestinal carcinogenesis. Conversely, restoration of PKC α , β I, δ , or ζ expression promotes suppression of growth or reduced survival (Black 2001). It should be noted that, while the majority of PKC family members seem to be downregulated during intestinal tumor progression, elevated levels of PKC β II and PKC ϵ have been detected in experimental models of colon cancer and in human colon carcinoma cell lines and tissues (Wali et al., 1995, Gokmen-Polar et al., 2001, Sauma et al., 1996, Murray et al., 1999, 2002, and 2004). Thus, individual PKC isozymes may contribute in opposing ways to the development of intestinal neoplasms.

1.A.5.1 PKC α and intestinal neoplasia

Marked reduction or abrogation of PKC α activity/expression (at both mRNA and protein levels) has been detected in premalignant and malignant colonic biopsies (Verstovsek et al., 1998, Kahl-Rainer et al., 1994, Levy et al., 1993, Pongracz et al. 1995, Assert et al., 1999, and Suga et al., 1998), and in colon adenocarcinoma cell lines (Leontieva, O.V., Bateman, N., Marinaro, J., Black A.R., and Black J.D., unpublished data). Consistent with a growth-suppressive function, overexpression of PKC α sense-constructs in CaCo2 human colonic adenocarcinoma cells decreased proliferation, enhanced differentiation, and resulted in an attenuated tumor phenotype compared with empty vector-transfected control cells (Scaglione-Sewell et al., 1998). Conversely, downregulation of PKC α activity in CaCo2 cells by overexpression of anti-sense PKC α constructs resulted in enhanced proliferation and decreased differentiation, as well as in a

more aggressive transformed phenotype compared with control cells (Scaglione-Sewell et al., 1998). Together, these findings indicate that PKC α signaling has tumor suppressive properties in the intestine and that loss of PKC α activity confers a growth/survival advantage to intestinal cells.

A major objective of the work presented in this thesis was to gain a better understanding of the cell cycle regulatory effects of PKC α signaling in normal intestinal epithelial cells and to determine the molecular consequences of loss of PKC α -mediated control in neoplastic intestinal cells. Based on (a) findings from our laboratory linking PKC/PKC α signaling to control of cyclin D1 expression levels in non-transformed IEC-18 intestinal crypt cells (Frey et al., 2000a and Clark and Black et al., 2004), (b) increasing evidence that loss of PKC/PKC α expression is an early event in intestinal carcinogenesis (Black, 2001), (c) evidence from the literature highlighting cyclin D1 overexpression as a common abnormality in colorectal cancers (Weinstein 2000) and demonstrating that increased expression of cyclin D1 can occur in colonic adenomatous polyps (Sutter et al., 1997, Arber et al., 1996, Bartkova et al., 1994, and Zhang et al., 1997), and (d) a recent report underscoring the significance of aberrant cyclin D1/cdk4 activity in colonic tumors through the demonstration that SW480, HT-29, SW48, and HCT116 colon adenocarcinoma cells are more dependent on cyclin D1/cdk4 activity than cyclin E/cdk2 activity for proliferation (Tetsu and McCormick, 2003), we have focused this study on understanding the mechanisms underlying regulation of cyclin D1 expression levels by PKC/PKC α signaling in the intestinal epithelium.

1.B D-Type cyclins and their role in cell cycle progression

1.B.1 The mammalian somatic cell division cycle

Unlike embryonic or germ cells, the cell cycle of somatic cells in mammals is composed of four phases: S phase, the stage of DNA synthesis, and M phase (mitosis), the stage of cell division, which are separated by two so-called Gap phases, **G1** and **G2** (Figure 4). Progression of cells through these phases is controlled by temporally and spatially regulated activity of complexes consisting of cyclins and cyclin dependent kinases (**cyclin/cdk**). Initiation of distinct cyclin/cdk activities and concomitant termination of others are found to mark the temporal borders of the four phases.

Typically, cdk activity rises from low levels during G1 to allow formation of preinitiation complexes at origins of DNA replication (Sherr and Roberts 2004). As cells advance through the cell cycle, cdk activity peaks, and then rapidly falls back to minimal levels as they exit M phase (Sherr and Roberts 2004). External mitogenic signals stimulate entry of the cells into the cell cycle from quiescence (**G0**) by sequentially inducing expression of D-type cyclins and cyclin E during G1 phase (Sherr and Roberts 1995). Assembly of D-type cyclins with cdk4 and cdk6 catalytic subunits is followed by additional activating post-translational modifications (described in detail in the next section, Figure 5) to achieve active cyclin D/cdk4 and cdk6 complexes in mid-G1. In this respect, D-type cyclins are considered sensors of proliferation and growth-inducing signals. Their activity is required for progression of cells through G1 to reach the restriction point (R), before which withdrawal of mitogens results in prompt inactivation

of cyclin D/cdk4/6 kinase activity and cell cycle arrest in G₁ phase (Sherr and Roberts, 2004).

Figure 4

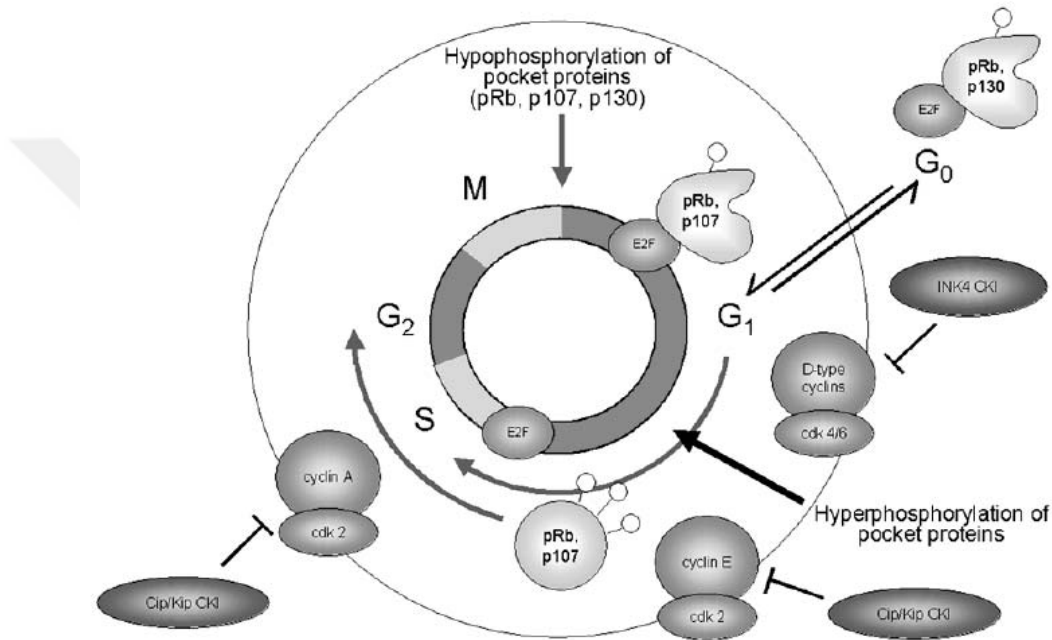


Figure 4 Molecular events involved in progression of the mammalian somatic cell cycle Key molecular events governing progression through the four phases of the cell cycle (G₁, S, G₂, and M phases) are depicted. Detailed description of each phase in terms of the molecular events taking place is provided in the text.

The growth suppressive members of the pocket protein family, retinoblastoma protein (pRb), p107, and p130, are the major targets of the kinases dependent on G1 cyclins. Members of this family negatively regulate the cell cycle by directly binding and repressing the function of the E2F family of transcription factors, which mediate expression of genes required for progression through G1 and transition into S phase. Cells in G0 primarily express p130 and pRb while expression of p107 is restricted to cycling cells (Mayol et al. 1998). Thus, the induction of cyclin D-dependent kinases in early-to-mid G₁ is thought to mediate the phosphorylation of pRb, which results in activation of E2F-dependent transcription, and phosphorylation/destabilization of p130.

Cyclin E is an E2F-target gene. Induction of cyclin E/cdk2 activity allows cells to pass the restriction point whereby their progression into S phase becomes independent of mitogenic signaling. Targets of cyclin E/cdk2 that have been identified include other sites in pRb, p107, and proteins involved in centromere duplication (Tetsu and McCormick 2003). Once cells reach the beginning of S phase, cdk2 is thought to phosphorylate cyclin E at Thr380 allowing the recognition of cyclin E by the ubiquitin proteasome pathway and its subsequent degradation. Concomitant induction of cyclin A and its association with cdk2 allows progression through S phase by maintaining pocket proteins in a hyperphosphorylated state for successful completion of DNA synthesis. Reminiscent of cyclin E's fate at the G1/S border, cyclin A destruction is triggered at the G2/M border coincident with the induction of cyclin B, which complexes with cdk1 to complete progression through M phase. At the end of M phase, cyclin B degrades upon entry of the daughter cells into the next G1 phase.

Within each phase of the cell cycle, a molecular checkpoint circuitry prevents cells from progressing into the next phase prematurely, with the ultimate goal of propagating flawless genetic information and a normal amount of cytoplasmic material to daughter cells. Thus, the aim of each checkpoint response is to provide prompt cessation of cyclin/cdk activity in response to acute suboptimal or defective growth conditions. In the next section, we will give a brief overview of the current model of regulation of cyclin/cdk activity before a summary of the literature on cyclin D1 control.

1.B.2 Summary of CDK regulation

In higher eukaryotes, multiple layers of control are thought to have evolved to control the activities of cdks to prevent cells from progressing through the cell cycle under suboptimal growth conditions, such as extensive genotoxic or cytotoxic stress or incomplete preparation for the next cell cycle (Morgan 1995).

So far, four major molecular switches which provide tight regulation of cdk-associated activity have been characterized; (a) cdk activation by cyclin binding, (b) cdk activation by phosphorylation, (c) cdk inhibition by phosphorylation, and (d) cdk inhibition by binding of inhibitory subunits (cyclin-dependent kinase inhibitors, **CKI**'s) (Morgan 1995) (Figure 5).

Figure 5

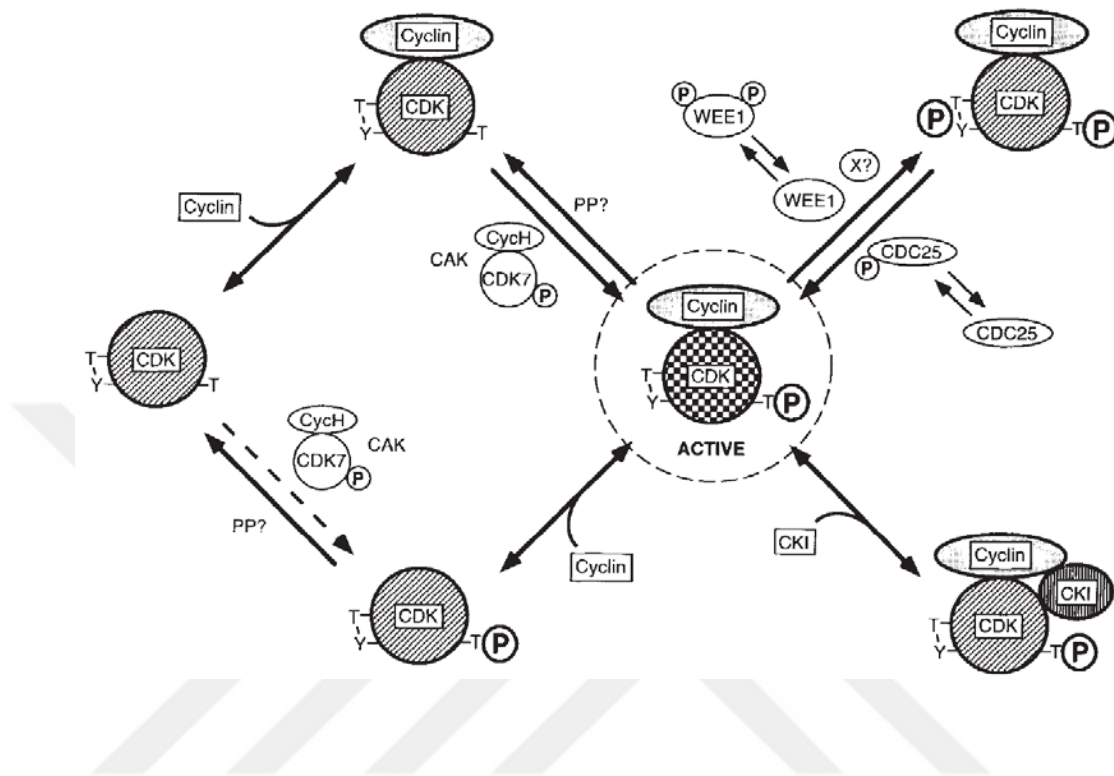


Figure 5 Principles of CDK-regulation At the center is the active cyclin/cdk complex (phosphorylated at the Thr160/161 position). Each arrow points to one of the four characterized major molecular switches which determine the ON/OFF state of cyclin/cdk-associated activity. Arrows pointing away from the central active cyclin/cdk represent “inactivating pathways”, and arrows pointing in represent “activating” pathways (Figure is taken from Morgan 1995).

(a) Cyclin binding is recognized as a major regulator of cdk activity. Members of the cyclin family, which are structurally related, bind and activate cdk catalytic subunits. A so-called “cyclin-box”, which is composed of a 100 amino acid-sequence conserved across cyclin family members, mediates binding to the cdk subunit and activation. The primary mode of regulation of cyclin function involves changes in cyclin levels. So far, studies done in different organisms have shown that modulation of cyclin expression can occur at one or more levels including transcription, mRNA stability, mRNA transport, translation, or protein stability (Morgan 1995). While changes in cyclin B levels (mitotic cyclin) in the *Xenopus* embryo are governed by changes in protein stability, in somatic cells of vertebrates and yeast, cyclin levels are determined by transcriptional mechanisms. In mammalian cells, the orderly induction of the cyclin specific for a given phase of the cell cycle is due to transcriptional changes, while the rapid depletion of that cyclin upon completion of the phase is due to an acute decrease in protein stability. More recently studies have shown that co-translational and mRNA transport mechanisms also impact cyclin gene expression, at least for mammalian cyclin D1. However, whether all of these mechanisms are responsible for the orderly oscillations of cyclin D1 levels during consecutive phases of the cell cycle remains to be determined.

(b) The next level of control for full cdk activation involves phosphorylation at a conserved threonine residue (Thr161 in human cdk1 and Thr160 in cdk2) by a multi-subunit enzyme named cdk activating kinase (CAK). The catalytic subunit of CAK is a cdk-related protein kinase, CDK7, while the second major subunit is a relatively new cyclin family member named cyclin H. *In vitro* demonstration of CAK activity upon

combination of purified cdk7 and cyclin H confirmed that CAK, like its substrates, is a cyclin/cdk complex (Fisher and Morgan, 1994). The kinase activity of CAK appears to be constant throughout the cell cycle, although phosphorylation at the Thr 161/160 residues of its protein substrates appears to oscillate parallel to the binding of cyclins, suggesting that cyclin binding may stimulate this phosphorylation (Morgan 1995). Therefore, it is accepted that modulation of CAK activity does not play a role in normal cell cycle progression; however, different growth inhibitory stimuli, including TGF- β or PKC signaling, have been implicated in targeting CAK activity to provide decreased cyclin/cdk activity and cell cycle arrest (Nagahara et al., 1999 and Black 2000).

(c) Cyclin/cdk complexes can be inhibited through phosphorylation of Thr14 and Tyr15 in cdk1 and cdk2. For example, phosphorylation of Thr14 and Tyr15 in cdk1 is regulated by cyclin binding, and like the phosphorylation of Thr160/161, roughly parallels the increase in cyclin B levels that occurs as cells approach M phase. Thus cyclin B/cdk1 complexes are maintained in an inactive state until the end of G2, when dephosphorylation of Thr14 and Tyr15 activates cyclin B/cdk1 (Morgan 1995). The kinase called Wee1 has been identified as a candidate Tyr15 kinase (originally in *S. pombe*). Dephosphorylation at Thr14 and Tyr15 is mediated by the dual-specificity phosphatase CDC25, whose activity is known to be elevated in mitosis due to a positive kinase activity acting at its N-terminal domain (Morgan 1995). Further studies have shown that rapid destruction of the CDC25A isotype in response to UV or ionizing radiation (IR) constitutes a *bona fide* checkpoint by resulting in termination of CDK2

kinase activity and prevention of cells from entering S phase without repairing the DNA damage (Mailand et al., 2000).

(d) Binding of a diverse family of proteins known as cyclin-dependent kinase inhibitors (**CKIs**) is recognized as the fourth major mechanism controlling CDK activity. There are two major classes of CKIs: the CIP/KIP family including p21 (cip1, Waf1, Sdi1, Cap20), p27 (Kip1), and p57 (Kip2), and the INK4 family consisting of: p16^{INK4a} (p16), p15^{INK4b} (p15), p18^{INK4c} (p18), and p19^{INK4d} (p19). Although members of the CIP/KIP family of CKIs were initially shown to inhibit both cyclin D/Cdk4/6 and cyclin E/cdk2 kinase activity at high concentrations *in vitro*, observations made in later studies indicated that, at lower stoichiometries, **p21^{CIP1/WAF1}** and **p27^{KIP1}** have no inhibitory effect on cyclin D/cdk4/6 kinase activity (Sherr and Roberts, 1999). Instead, they act as assembly factors for the association of cyclin D and cdk4/6. However, their association with cyclin E/cdk2 complexes inhibits cdk2 activity. Members of the INK4 family of CKIs exclusively inhibit cyclin D/cdk4/6 kinase activity.

I.B.3 Mechanisms of regulation of cyclin D1

With 1732 entries in PubMed, cyclin D1 is a gene whose regulation has become an attractive area of research for many investigators since its cloning in 1991 and 1992 by two groups in two independent systems (Matsushime et al., 1991 and Seto et al., 1992).

Dr. Sherr's lab reported the identification of a novel G1 cyclin, which was a genetic target of colony stimulating factor-1 (**CSF-1**) in macrophages (Matsushime et al., 1991). The expression of this new member of the cyclin family, which the Sherr group named cyclin D1, was shown to be dependent on CSF-1. In other words, it was expressed throughout the cell cycle as long as CSF-1 was present; conversely, its levels declined precipitously upon withdrawal of CSF-1. The authors also reported two other members of the cyclin D family, cyclin D2 and cyclin D3. Unlike cyclin D1, cyclin D2 was shown to be induced in late G1 and its expression is periodic, and that of cyclin D3 is tissue-specific. In the same study, cyclin D1 was found to associate with a polypeptide that is antigenically related to p34cdc2, and to rescue a cdk activity from growth factor-deprived macrophages. Later, this catalytic activity, regulated through association of cyclin D, would be termed cdk4 and cdk6.

The second group cloned cyclin D1 as the proto-oncogene PRAD1 (parathyroid adenoma 1) on chromosome 11q13. Overexpression of the PRAD1 gene was linked to a t(11;14)(q13;q32) translocation identified in all of the five B-cell lines tested in the study (Seto et al., 1992). A plethora of subsequent studies comparing levels of cyclin D1 in various carcinomas with their normal epithelial counterpart concluded that similar striking increases in cyclin D1 levels were evident in wide variety of human cancers. For example, cyclin D1 overexpression occurs in approximately 60% of breast cancers, 40% of colorectal cancers, 40% of squamous carcinomas of the head and neck, and 20% of prostate cancers lending support to the notion that cyclin D1 has an oncogenic capacity (Weinstein 2000).

The major catalytic function of cyclin D1/cdk4/6 is to promote progression through G1 phase upon mitogenic stimuli by inactivating pRb. Its other known noncatalytic function, which also indirectly contributes to G1 progression, is its ability to sequester p21^{CIP1/WAF1} and p27^{KIP1} CKIs away from cyclin E/cdk2 complexes, facilitating an increase in the activities of both of these kinases (*“titration model”* Sherr and Roberts, 2004, 1999, and 1995). Overexpression of cyclin D1 has been shown to result in shortening of G1, enhancement of proliferation rates, mitogen-independent growth and transformation in many cell lines (Quelle et al., 1993, Jiang et al., 1993, Rosenwald et al., 1993). Animal models addressing the impact of cyclin D1 deficiency have shown that, in addition to its role in proliferation, this gene plays a critical role in maintenance of cell, organ, and organism size. Sicinski et al. (1995) showed that cyclin D1 knock-out mice had smaller body size compared with litter-mates. Hypoplastic retinopathy, defective breast lobuloalveolar development during pregnancy, and uncharacterized neuropathy were other characterized phenotypes of cyclin D1 *null* animals (Sherr and Roberts 2004). In another study, overexpression of cyclin D1 not only overcame hepatocyte growth arrest in mice treated with rapamycin [inhibitor compound for (mammalian target of rapamycin) **mTOR** kinase activity] following 70% partial hepatectomy, but also induced increased hepatocyte cell size, and promoted growth of the liver (Nelsen et al., 2003). Other noncatalytic (cdk-independent) and novel functions of cyclin D1 include its ability to associate with and regulate the activity of several transcription factors, coactivators, and corepressors that govern histone acetylation and chromatin remodeling, to regulate cellular metabolism, fat cell differentiation, and cellular migration (Fu et al., 2004).

There are two major properties of D-cyclins that distinguish them from cyclin E. First, expression of D-cyclins is dependent on external growth signals while expression of cyclin E is periodic. Second, differences exist in their substrate specificity. D-type cyclins have a preference for pRb (with which they are known to associate), p107, and p130. Moreover, phosphorylation of cyclin D-specific sites in pRb leads to relief of active repression by pRb, which involves recruitment of histone deacetylases to E2F-responsive promoters. Cyclin D/cdk4 was more recently shown to phosphorylate Smad3, whereby it negatively regulates the functions of transcriptional complexes that mediate the cell growth inhibitory effects of TGF- β family members (Matsuura et al., 2004). In contrast, phosphorylation of cyclin E-specific sites terminates pRb-mediated passive repression of E2F-mediated transcription, which is blocked due to steric interference imposed by pRb-binding to E2Fs (Sherr and Roberts 2004, 1999, and 1995, Harbour and Dean, 2000). As mentioned before, in addition to specific sites in pRb, cyclin E-associated kinase activity can also phosphorylate histone H1, p107, and certain proteins involved in centromere duplication.

Another unique property of cyclin D1 compared with other cyclins is that its expression is regulated at multiple stages including 1) **transcription**, 2) **mRNA stability**, 3) **mRNA transport**, 4) **protein stability**, and 5) **translation**.

I.B.3.1 Transcriptional Regulation of cyclin D1

Early studies showed that activation of receptor tyrosine kinase (RTK) signaling led to an increase in cyclin D1 transcription as an early delayed response to growth factors upon cell cycle entry (Matsushime et al., 1991). Subsequent cyclin D1 promoter activity studies in fibroblasts and breast cancer cells confirmed that induction of cyclin D1 at the G0/G1 boundary occurs at the transcriptional level (Lavoie et al., 1996 and Altucci et al., 1996). Transcriptional induction of cyclin D1 is mediated through the activation of the Ras/Raf/MEK/MAP kinase pathway [Mitogen Activated Protein Kinase (MAPK)] in JEG-3 human trophoblast cells and in CV-1 fibroblasts transfected with Simian Virus 40 (SV40) (Watanabe et al., 1996), in Mv1.Lu lung epithelial cells (Albanese et al., 1995), in Chinese Hamster Embryonic fibroblasts (ILC9 cells) stimulated with Platelet-derived Growth factor (PDGF) (Weber et al. 1997a and b), and in cells derived from colon cancers (McCormick and Tetsu 2003). Transcription factors that have been implicated in expression of the *cyclin D1* gene by acting on *cis*-elements identified in the cyclin D1 promoter include signal transducers and activators of transcription (STAT), cAMP-responsive binding (CREB) protein/activating transcription factor 2 (ATF2) (Watanabe et al., 1996, Lee et al., 1999, and Sabbah et al., 1999), nuclear factor- κ B (NF- κ B) (Herber et al., 1994, Joyce et al., 1999, Guttridge et al., 1999, and Hinz et al., 1999), activator protein-1 (AP-1) (Watanabe et al., 1996), Sp1 (Lee et al., 2000), Ets (Albanese et al., 1995), TCF/LEF-1/ β -catenin (Albanese et al., 2003 and Tetsu and McCormick 1999), **Oct-1** (Eto 2000, Boulon et al., 2002, Magne et al., 2003, and **Egr-1** (Yan et al., 1997, Guillemot et al., 2001, and Shin et al., 2004).

Overexpression of cyclin D1 mRNA was soon recognized as a common lesion seen in many cancers. As Russel and colleagues summarized (1999), aberrant increases in cyclin D1 mRNA can result from amplification of chromosome 11q13 (e.g., breast and other cancers, Lammie and Peters, 1993, Motokura and Arnold, 1993), chromosomal translocation (B cell lymphoma, Motokura and Arnold, 1993, Rosenberg et al., 1991, Withers et al., 1991), and intrachromosomal inversion (parathyroid adenomas, Motokura and Arnold, 1993). In addition, targeted overexpression of cyclin D1 in mouse mammary cells leads to carcinoma formation or acceleration of c-myc-induced lymphomagenesis. Together these results suggest that loss of mechanisms that control cyclin D1 mRNA levels *in vivo* can lead to oncogenesis.

I.B.3.2 Regulation of cyclin D1 via alterations in mRNA stability

Changes in RNA stability have been associated with both loss and increased levels of cyclin D1 protein. Rapid disappearance of cyclin D1 RNA as a result of targeted degradation of message is thought to account for the major modulation of cyclin D1 expression via p53-independent mechanisms in UV-irradiated murine macrophages (Miyakawa and Matsushime, 2001). Similarly, loss of cyclin D1 expression upon serum withdrawal in NIH-3T3 cells synchronized in G2 was shown to be a result of message destabilization (Guo et al., 2005). Prostaglandin A2 treatment has been reported to result in destabilization of cyclin D1 mRNA in human non-small cell lung carcinoma cells, possibly via the action of a *trans*-acting factor (AUF1) on a segment of the

3' untranslated Region (**UTR**) (Lin et al., 2000). Treatment of NIH-3T3 cells with rapamycin was also shown to decrease cyclin D1 mRNA stability (Hashemolhosseini et al., 1998).

In continually proliferating cells, an induction of cyclin D1 protein at the S/G2 border in the absence of *de novo* transcription is known to be required for progression of the cells through the next G1 phase (Guo et al., 2002). Although post-transcriptional mechanisms have been proposed to mediate the Ras-dependent induction of cyclin D1 as cells transit from S to G2, whether changes in mRNA stability are involved in cyclin D1 induction at this stage is unclear (Guo et al., 2002). A truncation of the cyclin D1 gene in MDA MB-453 human breast cancer cells was associated with overexpression of short, 3' truncated (1.1-1.3 kb, in contrast to the normally expressed 4.2 kb transcript) and more stable cyclin D1 transcripts, demonstrating that mRNA stabilization mechanisms can contribute to pathological elevation of cyclin D1 expression in breast cancer (Lebwohl et al., 1994).

I.B.3.3 Regulation of cyclin D1 mRNA transport

Control of cyclin D1 gene expression can also occur at the level of mRNA transport (Rosenwald et al. 1993 and 1995, Rousseau et al. 1996). Recognition of this mechanism came from studies demonstrating that overexpression of the key translation initiation factor, eIF4E, led to a dramatic increase in cyclin D1 protein levels in the absence of transcriptional up-regulation of the cyclin D1 gene. Subcellular fractionation

experiments revealed that increased eIF4E levels lead to an increase in the cytoplasmic pool of cyclin D1 mRNA, which correlates with the induction of protein (Rosenwald et al., 1995).

In addition to its crucial cap-structure binding function, which is discussed in detail in the following sections, a second function of eIF4E has recently been unveiled that can account for its ability to enhance cyclin D1 mRNA nucleocytoplasmic transport. eIF4E can translocate to the nucleus as a result of its association with a chaperone called eIF4E Transporter (4E-T) (Dostie et al., 2000, Proud 2002, Topisirovic et al., 2003). Nuclear eIF4E, which represents a significant fraction of the cellular eIF4E pool, is thought to play an important role in transporting cyclin D1 mRNA to the cytoplasm (Rousseau et al., 1996a and Scheper and Proud 2002).

1.B.3.4 Regulation of cyclin D1 via alterations in protein stability

It is known from studies in rodent fibroblasts and macrophages that cdk4 is the dominant catalytic subunit to associate with cyclin D and that levels of cyclin D are far lower than those of cdk4 in cells (Diehl et al., 1997). The half-life of cyclin D1 in fibroblasts is 20-30 min, and both free and cdk4-bound D cyclins are unstable compared with cdk4 ($t_{1/2}$ = 4 hrs) (Diehl et al., 1997). Cyclin D1-cdk4 holoenzymes exist in a dynamic equilibrium, with the bound regulatory subunits being replaced continually by newly-synthesized cyclins (Matsushime et al., 1992). A decrease in cyclin D1 synthesis would, therefore, be accompanied by a decrease in cyclin D1-CDK4 activity, ultimately

leading to arrest in G1 phase of the cell cycle. In order to balance the continual synthesis of cyclin D1, a molecular turnover-machinery promoting continual degradation of cyclin D1 is needed. Below, we will summarize known proteolysis pathways for cyclin D1.

The observation in certain cases of breast cancers that up-regulation of cyclin D1 protein can occur in the absence of gene amplification led to the notion that defects in cyclin D1 proteolysis may also account for the accumulation of this protein in tumors (Buckley et al., 1993, Gillet et al., 1994, and Russell et al., 1999). Russell and colleagues (1999) demonstrated that cyclin D1 and cyclin D3 are ubiquitinated in U2-OS cells, and that a Cul-1 (E3 ubiquitin-ligase)- containing SCF complex associates with both of these D-type cyclins. These investigators also observed that, in some breast and ovarian cancer cell lines, the rate of cyclin D1 turnover was four times slower than that in other breast cancer and osteosarcoma cell lines, suggesting that cancer cells can be differentiated based on their ability to proteolyze cyclin D1 (Russell et al. 1999). Several other groups have reported defects in cyclin D1 turnover as the basis for its accumulation in different cancer cell lines, but the precise mechanism(s) governing cyclin D1 proteolysis was first mapped out in NIH-3T3 cells (Diehl et al., 1998).

I.B.3.4a Glycogen synthase kinase-3 β (GSK-3 β) and its regulation of cyclin D1 protein stability

Diehl and colleagues (1997 and 1998) demonstrated that Thr286 in the carboxy-terminus of cyclin D1 is phosphorylated by GSK-3 β to target the protein for degradation via the ubiquitin proteasome pathway. Phosphorylation at the Thr286 residue was

subsequently shown to be required for binding of cyclin D1 to a nuclear exportin CMR1, which mediates the translocation of cyclin D1 protein from the nucleus to the cytosol for degradation during S phase, when cyclin D1 is known to have a shorter half-life (Alt et al., 2000 and Guo et al., 2005). This finding was further supported by data showing that a T286A mutant remains nuclear throughout the cell cycle, and that overexpression of this mutant in murine fibroblasts leads to cellular transformation and tumor formation in nude mice (Alt et al., 2000).

GSK-3 β was cloned in 1980 (Embi et al.) as the third identified kinase activity that phosphorylates and inhibits glycogen synthase (GS) in rabbit skeletal muscle (Woodgett 1994). Numerous subsequent studies showed that GSK3 β has a key role in several cellular processes including signalling by insulin, growth factors and nutrients, and specification of cell-fate during embryonic development. GSK-3 β is also thought to play a role in cell division, apoptosis, and microtubule function (Cohen and Frame, 2001). Although phosphorylation of Axin and the adenomatous polyposis coli (APC) gene product by GSK3 β does not appear to destabilize these substrates, activation of free GSK-3 β is generally associated with antagonizing proliferative cell processes by priming its substrates for degradation or inactivation.

Free GSK3 β has a unique kinase activity that requires priming phosphorylation of its substrates by another protein kinase at a Ser/Thr residue (called the *priming phosphate*) four residues C-terminal to the site of GSK-3 β phosphorylation (Cohen and Frame, 2001). The kinases that catalyze the addition of the priming phosphates to GSK-

3 β substrates are often not regulated by extracellular signals. ATP citrate lyase, tau protein, myc family proteins (c-myc, L-myc and N-myc), and β -catenin are among substrates of GSK-3 β identified to date, and all are subject to a common negative mode of regulation by GSK-3 β action. When GSK3 β -activity is inhibited, these substrates lose phosphates at the GSK3 β site through the action of protein phosphatases; the priming phosphates, however, remain unaffected. Upon activation of GSK-3 β under growth-limiting conditions, these substrates are thus ready for processing by GSK-3 β activity.

Although there is *in vivo* evidence showing that GSK-3 β requires a tyrosine phosphorylation for full activation (Tyr 216) (Sutherland et al., 1993, Saito et al. 1994; Stambolic and Woodgett 1994; Murai et al. 1996), it is generally thought of as a constitutively active Ser/Thr protein kinase under the control of inhibitory phosphorylation at Ser 9. Upon activation of distinct mitogen signaling pathways by specific growth factors, GSK-3 β is inhibited by phosphorylation at this site, which transforms the amino terminus into a 'pseudo-substrate' inhibitor, blocking access of the priming phosphate on the substrate to GSK3- β 's active site (Figure 6). Insulin, insulin-like growth factor-1 (IGF-1) via direct kinase activity of Akt, other growth factors, amino acids, cyclic-AMP-dependent protein kinase/protein kinase A (PKA), and WNT-signaling all promote inactivation of GSK-3 β through phosphorylation of Ser9 (Figure 6, 7, and 8, all are taken from Cohen and Frame 2001). Conversely, GSK-3 β is activated in serum-starved or non-cycling cells (Stambolic et al. 1996).

The mechanism of GSK-3 β -mediated inactivation of Wnt-signaling operates very differently from the pathways mentioned above (Figure 8). In contrast to the pool of GSK-3 β inactivated by other signaling pathways, the fraction of GSK-3 β that participates in WNT-signaling exists in a multi-protein complex that includes axin, β -catenin, and APC protein. WNT-signaling leads to sequestration of GSK3 β away from this complex into another one composed of Dishevelled (**DVL**) and frequently rearranged in T-cell lymphomas (**FRAT**). β -catenin thus becomes stabilized and liberated to accumulate in the cytoplasm, before migrating to the nucleus to complex with transcription factors of the T-cell factor (**TCF**) family [also known as the lymphoid-enhancer factor (**LEF**)] to activate transcription of a specific array of genes, including cyclin D1. WNT-signaling mediated inactivation of GSK-3 β impacts cyclin D1 regulation in two major ways: alteration in cyclin D1 protein stability and indirect control of cyclin D1 transcription (Rimerman et al., 2000).

McCormick and Tetsu (1999) showed that, in colon tumor cells lacking functional APC, β -catenin that cannot be targeted for turnover by GSK-3 β mediates transcriptional induction of cyclin D1. Subsequent to this study, another group demonstrated that NIH-3T3 cells stably transfected with HFc-epitope-tagged Wnt1 (**Wnt1-3T3**) displayed a four-fold increase in steady state levels of cyclin D1 protein compared with parental cells, an effect largely due to inactivation of GSK-3 β (Rimerman et al., 2000). Wnt1-transfectants also showed a modest increase in levels of cyclin D1 mRNA. These observations confirm the dual role of GSK-3 β function in regulation of cyclin D1 levels. Inhibition of GSK-3 β as a consequence of increased Wnt1 signaling doubles the half-life

of cyclin D1, pointing to a direct role of GSK-3 β in control of cyclin D1 protein stability. The modest increase in cyclin D1 mRNA can be attributed to β -catenin-mediated transcriptional activation of cyclin D1 as a result of decreased turnover of β -catenin under conditions of decreased GSK-3 β activity.

Figure 6

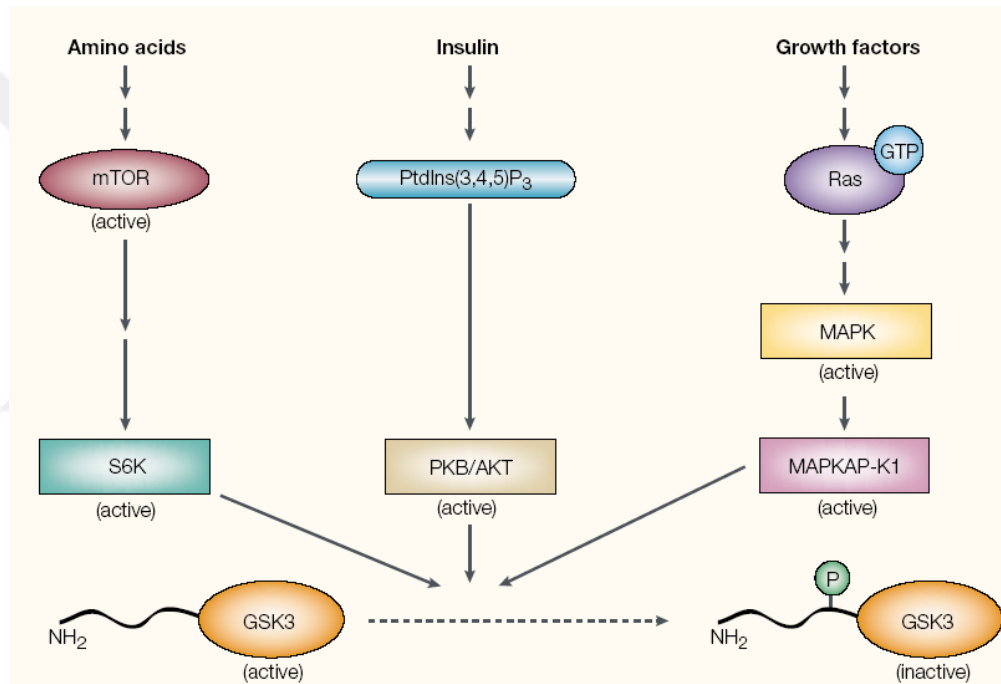


Figure 6 Inhibitory regulation of GSK-3 β by mitogenic stimuli Amino acids activate mTOR, which phosphorylates and activates ribosomal protein p70 S6 kinase (**p70S6K**). p70S6K then targets the same serine residue in GSK-3 β as Akt. Insulin promotes activation of Akt which phosphorylates and inactivates GSK-3 β at Ser9. Growth factors also lead to activation of the MAPK cascade. The ultimate effector kinase of this pathway, MAPK-activated protein kinase 1 (**MAPKAP-K1**, also known as **RSK**) phosphorylates the Akt-targeted site in GSK-3 β .

Figure 7

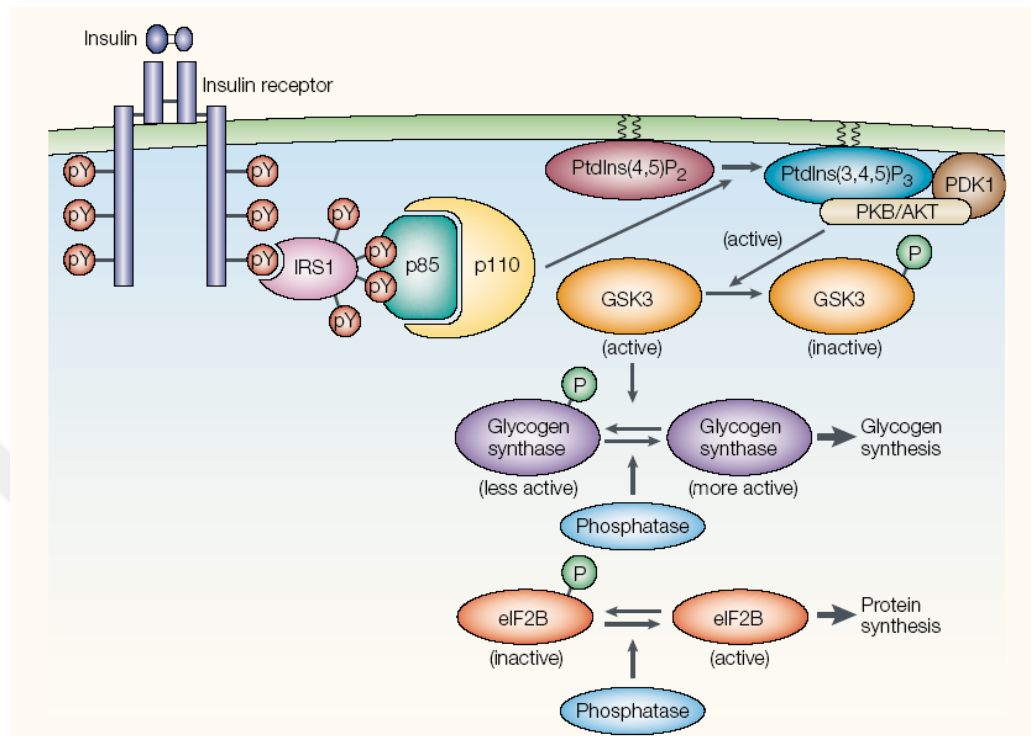


Figure 7 Diagrammatic representation of insulin-governed signaling pathways activating the cell cycle machinery, glycogen storage, and protein synthesis by converging on inactivation of GSK-3 β Binding of insulin to its receptor activates the intrinsic protein tyrosine kinase activity of the receptor resulting in its autophosphorylation at several sites. One phosphotyrosine (pY) residue interacts with the phosphotyrosine-binding (PTB) domain of insulin receptor substrate proteins (IRS1 and IRS2), recruiting them to the plasma membrane, where they are phosphorylated by the insulin receptor. As a result, IRS1 and IRS2 interact with the Src-homology-2 (SH2) domains of the p85 subunit of PI3K. This recruits PI3K to the plasma membrane allowing the (p110) catalytic subunit to catalyze the formation of phosphatidylinositol-3,4,5,-trisphosphate (PtdIns-3,4,5-P₃) from PtdIns-4,5-P₂. PtdIns-3,4,5-P₃ binds to the pleckstrin homology (PH) domain of Akt (Protein Kinase B) and PDK-1, leading to their colocalization at the plasma membrane where PDK1 can catalyze activation of Akt. Ser9 in GSK-3 β becomes a primary target for Akt kinase activity resulting in inhibition of

GSK-3 β and dephosphorylation (activation) of its targets GS and eIF2B (Cross et al., 1995, figure is taken from Cohen and Frame, 2001).

Figure 8

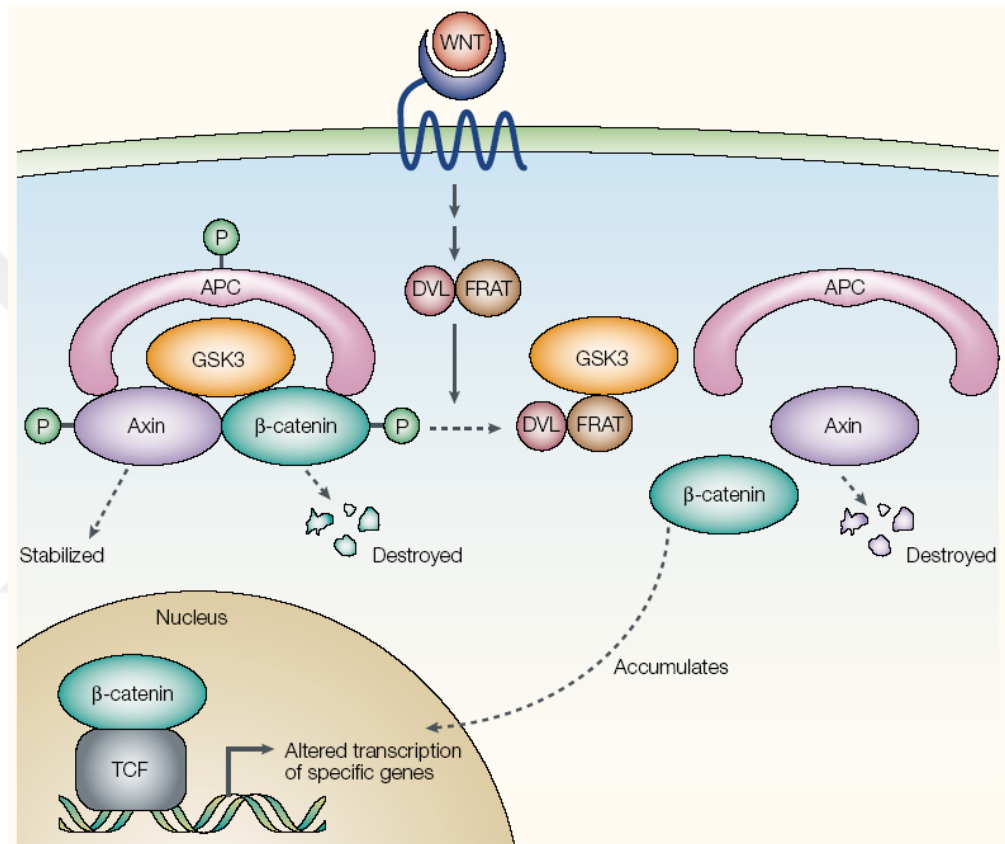


Figure 8 Regulation of GSK-3 β activity by WNT-signaling GSK-3 β complexed with axin, APC, and β -catenin phosphorylates N-terminal residues in β -catenin marking the molecule for ubiquitin-mediated degradation. When Wnt-signaling results in sequestration of GSK-3 β away from the Axin/APC complex into DVL/FRAT complexes, β -catenin becomes stabilized and eventually translocates to the nucleus where it associate with TCF/LEF transcription factors to induce transcription of a specific array of genes including cyclin D1.

Subsequent studies in other epithelial systems, reported increased GSK-3 β -dependent proteolysis of cyclin D1 under conditions of G1 arrest or differentiation. Human embryonal carcinoma cells induced to differentiate with all-*trans*-retinoic acid downregulate cyclin D1 via GSK-3 β -dependent mechanisms (Spinella et al., 1999). Likewise, increased GSK-3 β -mediated turnover of cyclin D1 has been reported in growth-arrested rat fibroblasts which conditionally express Ha-Ras^{Val 12} (Shao et al., 2000).

Notably, ubiquitin proteasome-dependent, but GSK3 β -independent pathways, have also been implicated in modulation of free cyclin D1 (unbound by Cdk4) proteolysis (Germain et al. 2000). A *T286A-T288A* double-mutant of human cyclin D1, transfected alone (i.e. in the absence of co-transfected cdk4) into osteosarcoma cells, could still be degraded, albeit in a delayed fashion (180 min compared with 60 min for the *wild type* cyclin D1). Furthermore, *in vitro*-translated *T286A-T288A* cyclin D1 was ubiquitinated in *in vitro* ubiquitination assays, although to a much lesser extent than the *wild type* protein in the same assay (Germain et al. 2000).

In a more recent study, Thr288 in the C-terminus of cyclin D1 was shown to be phosphorylated by Mirk/dyrk1B leading to destabilization of the protein in Mv1Lu lung epithelial cells (Zou et al., 2004). Mirk, which is an arginine-directed serine/threonine kinase, is activated by the stress-activated mitogen-activated protein kinase kinase, MKK3, but its expression is decreased by the MEK-ERK pathway during G1; thus its function is restricted to G0 and early G1. While stable overexpression of Mirk in colon

carcinoma cells was shown to enhance turnover of cyclin D1, knockdown of its expression by siRNA in differentiating C2C12 myoblasts increased the abundance of cyclin D1 protein (Zou et al., 2004). The Mirk-related kinase Dyrk1A is hypothesized to act as a “priming kinase” for GSK-3 β -mediated phosphorylation of eukaryotic initiation factor 2B ϵ (eIF2B ϵ). The findings that recombinant Mirk and GSK-3 β phosphorylate cyclin D1 in an additive fashion, and that neither LiCl treatment nor mutation at the GSK-3 β site of cyclin D1 prevents Mirk-mediated phosphorylation of Thr288, point to Mirk as a potential priming kinase for GSK-3 β -mediated phosphorylation of cyclin D1 (Zou et al., 2004).

1.B.3.4b Regulation of cyclin D1 protein stability via the N-terminal “destruction-box” motif

Following Diehl’s work on GSK-3 β -mediated proteolysis of cyclin D1 (Diehl et al., 1998), an N-terminal “destruction motif” (RXXL), which is conserved in yeast, mouse, rat, and human cyclin D1, was shown to mediate targeting of cyclin D1 to the ubiquitin proteasome pathway upon DNA damage in MCF7 cells (Agami and Bernards, 2000). Mutation of this motif resulted in an ionizing radiation (IR)-resistant cyclin D1 mutant and abrogation of G1 arrest in the presence of extensive DNA damage (Agami and Bernards, 2000). “Destruction box” motifs are not known to be subject to post-translational modification such as phosphorylation for recognition by ubiquitin ligases. They are conserved motifs found in mitotic cyclins whose proteolytic cleavage is mediated by a multi-component ubiquitin protein ligase, the Anaphase Promoting

Complex (**APC**). In this regard, in non-transfected MCF-7 cells, cyclin D1 and cdk4 could be immunoprecipitated with a component of the APC, cdc27; conversely, cdc27 could be found in cyclin D1 and cdk4 immunocomplexes, but not in complexes containing cdk2 (Agami and Bernards, 2000).

I.B.3.4c Regulation of cyclin D1 protein stability by the calpain protease

Calpain-mediated increased cyclin D1 turnover has been reported to occur in certain systems (Choi et al., 1997). In the study by Choi and colleagues, only inhibitors of calpain protease (calpain Protease I (**LLnL**) and Calpain Inhibitor II (**LLM**)), but not inhibitors of the proteasome (**Lactacystin**) or of other proteases [e.g., phenylmethylsulfonyl fluoride (**PMSF**)], could reverse the loss of cyclin D1 protein in serum-starved NIH-3T3 cells when these compounds were added 12 h after serum withdrawal. Furthermore, cleavage activity of purified calpain on cyclin D1 has been demonstrated *in vitro*, and transient expression of a calpain peptide inhibitor, calpastin, was shown to increase cyclin D1 levels in serum-starved NIH-3T3 cells (Choi et al., 1997).

I.B.3.5 Regulation of cyclin D1 protein synthesis

The paradigm for maintaining cellular levels of a protein involves not only availability of its transcripts in sufficient abundance, but also accessibility of transcripts to the protein synthesis machinery (De Benedetti and Graff, 2004, Proud, 2002).

Regulation of cyclin D1 gene expression at the level of translation has been reported in a number of systems. Key factors involved in regulation of translation initiation and elongation have been implicated in altered cyclin D1 synthesis. Before discussing our current understanding of regulation of cyclin D1 protein synthesis, we will provide a brief summary of current molecular models for translation initiation and elongation. The temporal and spatial order of the molecular events involved in translation initiation is still not well understood. Therefore, the reader should bear in mind that the model presented below is still subject to debate.

1.C. Regulation of gene expression at the level of translation

1.C.1 Translation Initiation

The ultimate goal of translation initiation is to achieve the correct alignment of the start codon in the mRNA with the P-site in the 40S-small ribosomal subunit, to establish the reading frame of the mRNA. Eukaryotic translation initiation requires at least 11 factors, which are known as ekaryotic initiation factors (**eIFs**) (Figure 9) (Dever 2002, Cormier et. al. 2003, and Abbott and Proud 2004). The energy needed for this process is provided by the hydrolysis of ATP and GTP. The highly sophisticated nature of translation initiation requiring the orderly participation of eIF's can be summarized roughly in three main stages:

(1) Priming of the transcript to be translated by its engagement to the eIF4F complex which is composed of three main subunits: (a) **eIF4E** which binds to the cap of the mRNA, (b) the RNA helicase, **eIF4A**, which is responsible for unwinding the

secondary structure in the 5' UTR region of the mRNA, and (c) a large docking factor, **eIF4G**, which maintains direct interactions with eIF4E, eIF4A, and eIF3. eIF3 is another large molecule known to bridge the mRNA:eIF4F complex to the 43S ribosomal complex;

(2) Formation of the 43S preinitiation complex which is composed of the 40S ribosomal subunit, the initiation factors eIF1, eIF1A, eIF3 (which has 5-11 subunits), eIF5, and the ternary complex of eIF2 (which has three subunits, eIF2 α , β , and γ), initiator Methionyl-tRNA (**Met-tRNA_i**), and GTP;

(3) Displacement of the eIF4F complex by the 43S complex upon recognition of the initial methionine codon, and joining of the 60S subunit to form the complete 80S complex.

Figure 9

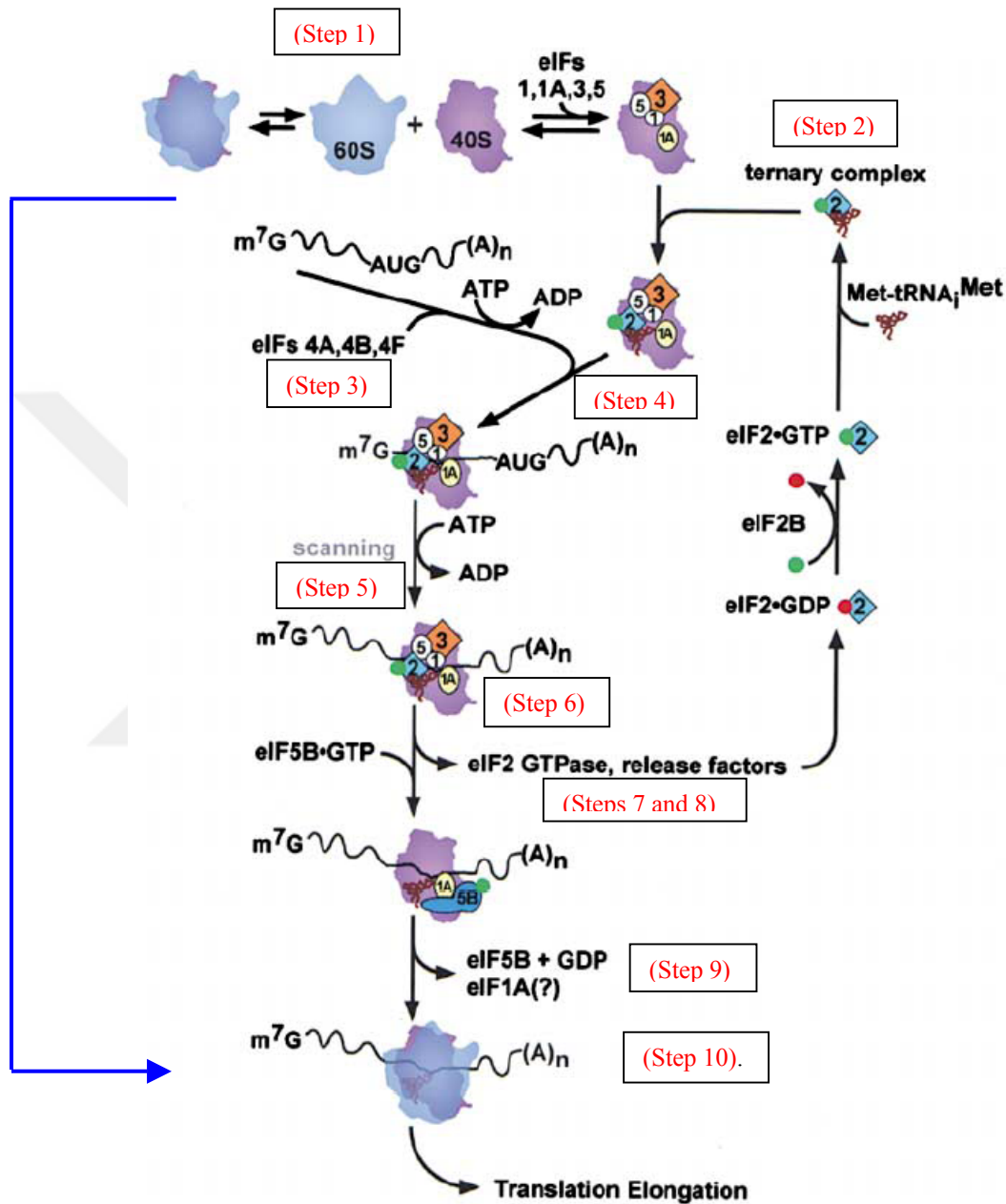


Figure 9 Translation initiation in eukaryotes “(Step 1): The Met-tRNA_i forms a ternary complex with eIF2 bound to GTP, while eIF1, 1A, 3, and 5 associate with the 40S small ribosomal subunit to prepare for the generation of the 43S preinitiation complex. eIF1A promotes Met-tRNA_i binding to the 40S ribosomal subunit by helping to generate a pool of free 40S subunits and by stabilizing ternary complex binding to the 40S subunit.

(Step 2). The ternary complex interacts with the 40S ribosomal subunit and forms the 43S preinitiation complex. Binding of the Met-tRNA_i to the small ribosomal subunit is mediated by eIF2 and is regulated by eIF2B in all eukaryotes. Phosphorylation of eIF2 in the α subunit (i.e., eIF2 α) (note that the α subunit of eIF2 is depicted in this figure) on Serine 51 inhibits translation initiation. Stabilization of the eIF2/GTP/Met-tRNA_i ternary complex is effected by eIF3 which is present in the 43S complex.

(Step 3) eIF4F facilitates the recruitment of ribosomes to the mRNA 5' end. eIF4F is composed of three subunits: (a) eIF4E, which recognizes and binds to the 5' cap structure (m⁷GpppN, where N is any nucleotide); (b) eIF4A, a RNA-dependent ATPase and RNA helicase; (c) eIF4G, a large polypeptide that serves as a scaffolding protein. eIF4F also interacts with the ribosome-associated eIF3 through eIF4G, and thus provides a physical link between the ribosome and the mRNA 5' cap. The RNA helicase activity of eIF4A, in conjunction with eIF4B, is thought to facilitate ribosome binding by unwinding the mRNA secondary structure.

(Step 4) Association of the 43S preinitiation complex with the mRNA bound to eIF4F and eIF4B forms the 48S complex.

(Step 5) The 40S ribosomal subunit is thought to scan from the 5' terminus downstream to the initiation codon. The 48S preinitiation complex pauses upon encountering the initiation codon, which facilitates joining of the 60S ribosomal subunit to form a functional 80S functional ribosome in (Step 10). The Met-tRNA_i becomes delivered directly to the P site of the ribosome in contrast to subsequent aminoacyl tRNAs, which are first delivered to the A site in the elongating ribosomes.

(Step 6) This process requires the hydrolysis of eIF2-bound GTP to GDP in the presence of eIF5.

(Steps 7 and 8) GTP hydrolysis also decreases the affinity between initiation factors and the 40S ribosomal subunit.

(Step 9) The released initiation factors recycle for another round of translation initiation. eIF5B, a recently discovered initiation factor that has ribosome-dependent GTPase activity, plays an important role in the 60S ribosomal subunit joining process" (Cormier et. al. 2003 and Dever 2002).

I.C.2 Translation Elongation

Translation elongation in eukaryotes requires fewer factors than initiation (Abbott and Proud 2004). In this process, amino acids are added to the growing polypeptide chain according to the order specified by the mRNA sequence. The function of eukaryotic elongation factor 1A (**eEF1A**), which delivers the aminoacyl-tRNA to the A-site of the ribosome while hydrolyzing one GTP molecule to GDP, is very reminiscent of the function of initiation factor eIF2. Similar to the mechanism of action and regulation of eIF2, the activity of eEF1A is also dependent on GTP and a guanine nucleotide-exchange factor eEF1B. Like eIF2B, eEF1B recycles the inactive GDP-bound eEF1A to active GTP-bound eEF1A complexes (Figure 10) (Abbott and Proud 2004). eEF1B is composed of three subunits, α , β , and γ , all of which are products of separate genes. Finally, eukaryotic elongation factor 2 (**eEF2**) is required for the translocation of the peptidyl-tRNA from the A-site of the ribosome to the P-site and the movement of the ribosome along the mRNA. This step also requires hydrolysis of a GTP molecule but, interestingly, because eEF2 has high affinity for GTP, there is no additional factor with guanine nucleotide-exchange activity to recycle GDP-bound eEF2s (Browne and Proud 2002) (Figure 10).

Figure 10

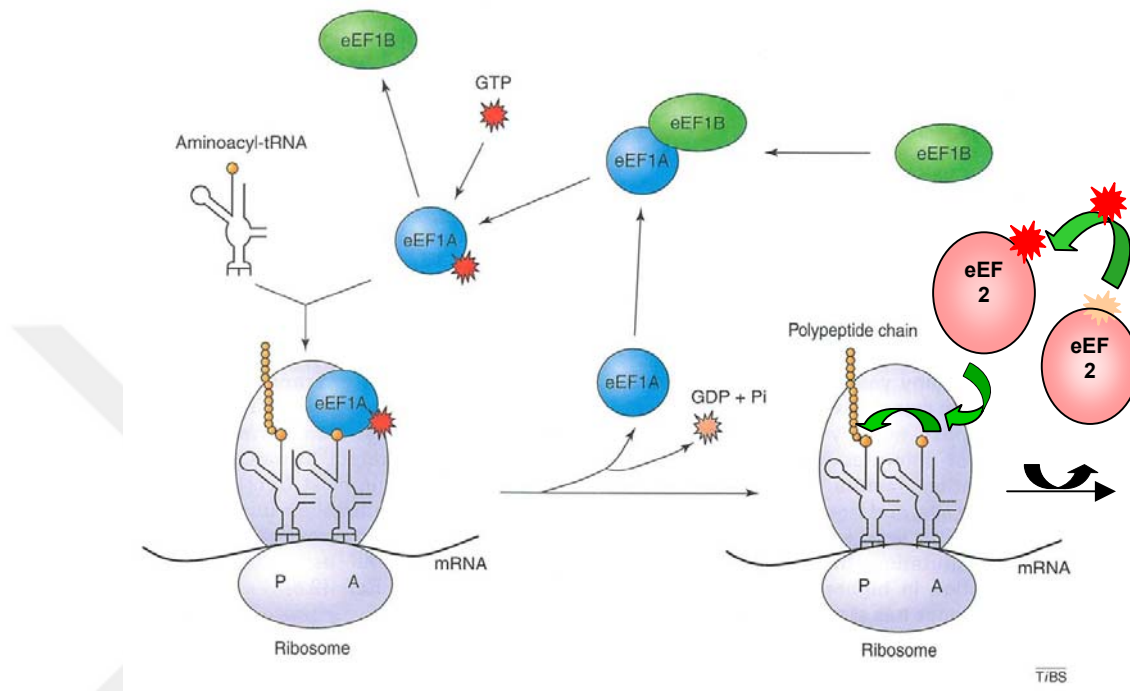


Figure 10 Translation elongation in eukaryotes eEF1A-GTP promotes the delivery of the aminoacylated tRNA to the A-site of the ribosome. Upon codon-anticodon recognition, GTP-bound to eEF1A is hydrolyzed and eEF1A-GDP is released from the ribosome to be recycled back to eEF1A-GTP by the exchange factor eEF1B. Active GTP-bound eEF2 mediates the translocation of the peptidyl tRNA from A site to the P site of the ribosome, and is released as inactive GDP-bound eEF2.

I.C.3 Regulation of global translation machinery components can impact expression of specific genes

The critical role of mRNA translational control in determining changes in cellular growth, proliferation, differentiation and apoptosis is now well established (Mamane et. al., 2004; De Benedetti and Graff, 2004; Bjornsti and Houghton, 2004; Avdulov et. al., 2004). The ability to spare global cellular energy from new rounds of transcription is essential especially for eukaryotes, where the flow of information from genes to proteins is reported to be slow (De Benedetti and Graff, 2004, Proud, 2002). Therefore, modulation of gene expression at the level of translation is believed to allow the cell to respond rapidly to changes in growth conditions. Tight regulation of protein synthesis is required for cellular survival because it is the most expensive process in terms of consumption of metabolic energy. Therefore, in mammals, most of the translational regulation so far elucidated occurs at the level of initiation and elongation (De Benedetti and Graff, 2004, Proud, 2002).

Progressive processing of a given transcript through stages of translation initiation depends heavily on (a) the availability of eIF4E to furnish eIF4F complexes, which are required to scan and locate the initiation codon by unwinding the secondary structure in the 5' UTR region, and (b) the ability to re-cycle eIF2 α -GDP to competent eIF2 α -GTP-Met-tRNAⁱ trimeric complexes, which can deliver the first methionine of a polypeptide chain and form the 43S preinitiation complex. Therefore, eIF4E and eIF2 α constitute the most crucial regulators of global translation. Indeed, the significance of proper regulation

of these two eIFs is underscored by the findings that (a) they are deregulated in a wide variety of human cancers including breast, lung, colorectal, prostate, head and neck, bladder, cervical cancers and lymphomas (Rosenwald 2003 and 2004), and (b) they have potent transforming ability when overexpressed in several cell line models (De Benedetti and Graff, 2004).

Several groups have reported that overexpression of eIF4E in immortalized but nontransformed cell lines of NIH-3T3, CHO, HeLa, or MM3MG results in drastic phenotypic changes including rapid proliferation, inability to undergo growth arrest in response to serum deprivation, failure to undergo apoptosis, induction of vascularization, and ability to survive in soft agar, all of which are indicative of transformation (Lazaris-Karatzas et al., 1990, Kevil et al., 1996, De Benedetti and Rhoads, 1990, and DeFatta and De Benedetti., 2003). Analysis of cell lines overexpressing eIF4E revealed molecular events which can account for the observed phenotypic changes of cellular transformation.

Surprisingly, eIF4E overexpression does not enhance synthesis of all mRNAs, but rather selectively increases translation of a specific group of transcripts whose protein products are potent oncogenic factors and whose translation is repressed under normal cellular conditions (De Benedetti and Graff 2004). cyclin D1, VEGF, c-myc, fos, mdm-2, ornithine decarboxylase (**ODC**), MMP9, Bcl-2, Bcl-xL, and others are among the genes whose expression is induced as a result of eIF4E overexpression (De Benedetti and Graff 2004). Although most of these genes possess Internal Ribosome Entry Site (**IRES**) sequences in their leader mRNA to mediate cap-independent translation, almost all of

them appear to be more heavily dependent on cap-dependent translation under normal growth conditions. Additional support for this observation comes from studies in which eIF4E activity/expression was blocked in transformed cells by overexpression of its inhibitory binding partner, eIF4E-binding protein 1 (**4E-BP1**) (see below) or by antisense eIF4E oligonucleotides. Inhibition of eIF4E led to a reduction in cell growth and tumorigenicity, and increased apoptosis (Rousseau et al., 1996b, Li et al., 2002, De Benedetti et al. 1991, and Graff et al., 1995). Suppression of cyclin D1 and ODC were the major underlying molecular consequences of inactivation of eIF4E function in these studies (Jiang et al 2002, and Graff et al., 1997).

De Benedetti and Graff (2004) explain the inherent sensitivity of oncogenic proteins to fluctuation in eIF4E levels with the paradigm of strong and weak mRNAs. A plethora of studies in various systems have revealed an interesting hierarchy among cellular mRNAs on the basis of their intrinsic ability to be translated. According to this mRNA competition theory, translation of weak mRNAs is outcompeted by strong mRNAs when the rate of translation initiation is reduced (Figure 11 taken from De Benedetti and Graff, 2004). As illustrated by the examples of Vascular Endothelial Growth Factor (**VEGF**) and cyclin D1 in Figures 11 and 12, weak mRNAs share the common property of having lengthy, G+C-rich 5' UTRs with extensive secondary structure. This structural complexity makes them more dependent on the action of RNA helicases in the eIF4F complex. In contrast, the 5' UTR region of strong mRNAs (e.g., those of housekeeping genes like β -actin as shown in Figure 11) are short and unstructured, enabling them to be readily processed by the eIF4F complex. Thus, under

conditions of limiting eIF4F abundance which could be due to 1) decreased eIF4E expression, 2) increased 4E-BP1 expression, or 3) increased association of 4E-BP1 with eIF4E, weak mRNAs will be poorly translated because the efficiently translated strong mRNAs will be outcompeting them for access to eIF4F complexes (De Benedetti and Graff 2004).

Figure 11

eIF-4E in oncogenesis and metastasis
AD Benedetti and JR Graff

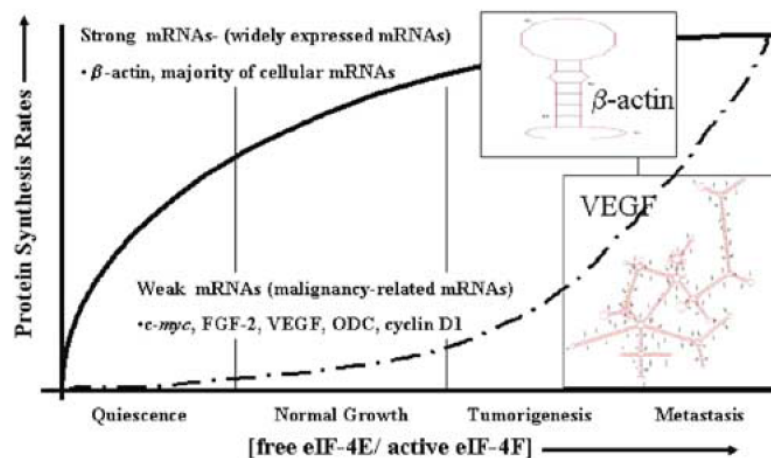


Figure 11 Model for mRNA hierarchy for cap-dependent translation initiation

“mRNA competition for translation initiation. Derived and expanded from the original R^* model (Lodish, 1974), it was developed here from theoretical considerations and experimental observations (De Benedetti and Rhoads, 1990; Rhoads et al., 1993; Graff and Zimmer, 2003). The products of weak mRNAs become selectively increased with higher free eIF-4E, which results in increased active eIF-4F complex. Disproportionately increased translation of these weak mRNAs would increase with malignant progression in relation to increased free eIF-4E. Representative structures for the 5' UTR of β -actin, a strong mRNA and VEGF, a weak mRNA, are depicted. These structures were generated with the Zucker m-fold RNA structure program”

Figure 12

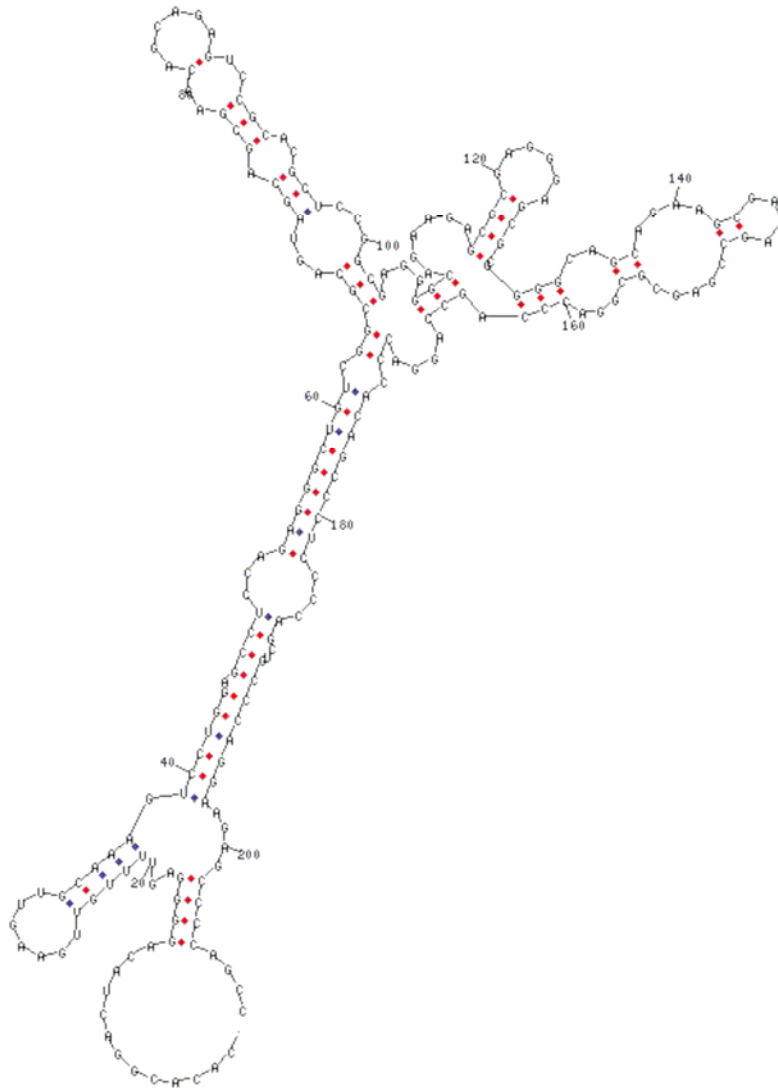


Figure 12 Predicted secondary structure of cyclin D1 leader sequence The most stable structure out of thirteen structures identified in this study which were predicted by the MFOLD algorithm, is depicted ($dG = -88.9$ kcal/mole) (Taken from Shi et al., 2004).

I.C.3a Regulation of eIF4E in mammalian cells

The association of the cap-binding factor, eIF4E, with the cap structure is considered to be the most critical step in cap-dependent translation, because it is the initiating event for the ultimate engagement of a given mRNA to competent ribosomal complexes (De Benedetti and Graff 2004). Most cellular mRNAs in eukaryotes (except those in organelles) are found to have a modified 5' end, referred as the cap structure, $m^7G(5')ppp(5')X$ (where X is any nucleotide) (Mamane et al., 2004) (Figure 13). The mRNA-cap is believed to function by protecting mRNA from the action of phosphatases and nucleases and promoting the initiation of mRNA translation (Shatkin 1976).

Figure 13

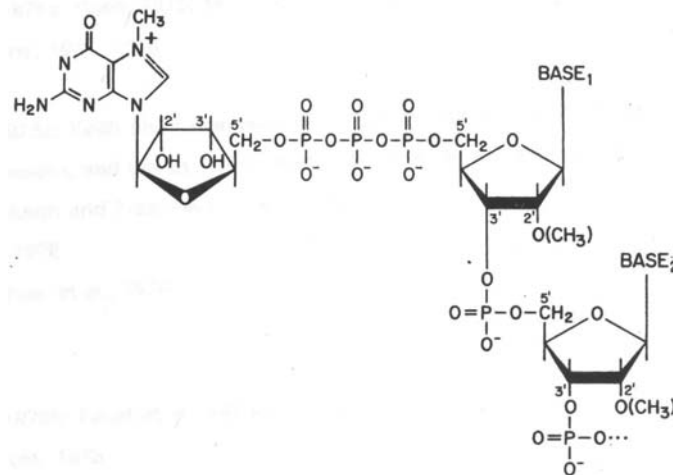


Figure 13 Cap-structure The methylated guanosine is bonded to the first nucleotide at the 5' end by a triphosphate bridge (figure is taken from Shatkin, 1976).

The structure of mammalian eIF4E elucidated by crystallographical studies reveals a baseball-glove shaped molecule in which the cap structure is sandwiched between two conserved tryptophans lying on either side of the cleft. A major site of phosphorylation, Ser209, is at the tip of the cleft neighboring the three phosphates of the mRNA cap (Figure 13 and 14). Consensus binding sites for eIF4G and 4E-BP1 are located in a hydrophobic region on the dorsal surface of eIF4E (Figure 14).

In mammals and insects (at least in *Drosophila*), but not in yeast, eIF4E is subject to an *in vivo* phosphorylation by the MAP-kinase signal-integrating kinases Mnk1 and 2 at the C-terminal Ser209 residue. The influence of eIF4E phosphorylation on the eIF4E/cap interaction and translation initiation has been a controversial issue due to contradictory findings from different studies (Scheper and Proud, 2002). Data from early studies indicated that phosphorylation of Ser209 increases the affinity of eIF4E for the cap, and therefore, must have a positive effect on translation initiation. However, as discussed in detail by Scheper and Proud (2002), the biophysical data showing that phospho-Ser209-eIF4E has increased affinity for the cap could be misleading because the cap analogues used in those studies did not reflect the endogenous substrate (which is a capped oligonucleotide). Thus, more recent data showing that phospho-eIF4E has less affinity for capped oligonucleotides appear to be receiving increasing acceptance (Scheper et al., 2002; Cormier et al 2003). Despite the controversy on how Ser209 phosphorylation influences affinity of eIF4E for the cap, it is well-known that this post-translational modification does not affect binding of eIF4E to either eIF4G or 4E-BP1, which determines the availability of eIF4E competent for initiating translation. In fact,

binding of either of these factors to eIF4E is found to increase affinity of eIF4E for the cap by about 100 fold (i.e. either phosphorylated or unphosphorylated eIF4E bound by eIF4G or 4E-BP1 displayed similar affinity for the cap, which was several fold higher than that of free eIF4E) (Scheper and Proud, 2002).

Although there are studies indicating that stimuli that increase the rate of protein synthesis generally increase the phosphorylation of eIF4E, it has also been reported that insulin, which activates protein synthesis in HEK 293 cells, does not alter the phosphorylation of eIF4E, which remains very low. Studies using an Ala209 eIF4E mutant further indicate that phosphorylation of eIF4E at Ser209 is not required for protein synthesis *in vitro* and *in vivo* (McKendrick et al., 2001).

Figure 14

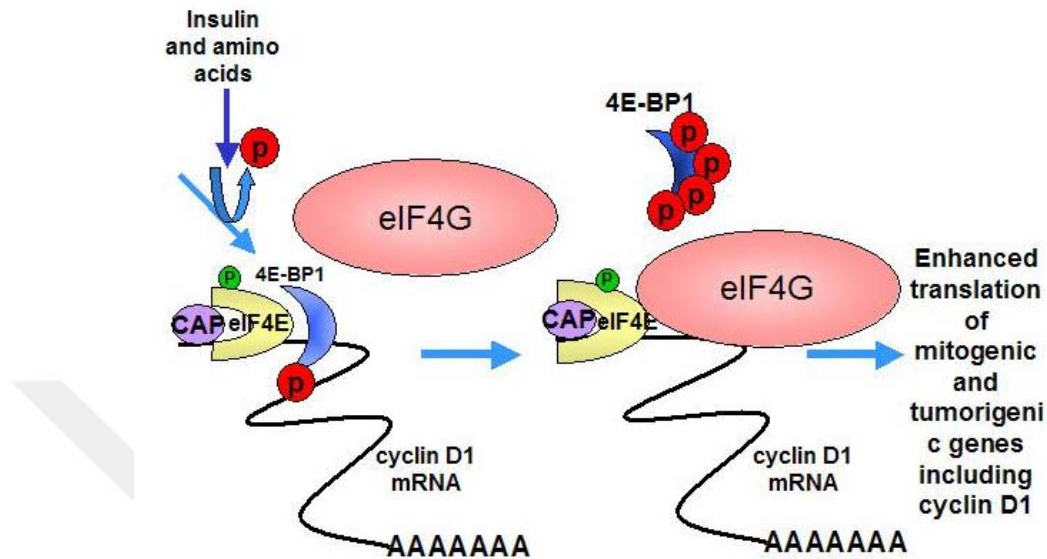


Figure 14 Regulation of cap-dependent translation through the modulation of eIF4E activity via binding of 4E-BP1. Hypophosphorylated 4E-BP1 binds to the same motif in eIF4E as eIF4G and 4E-T. Peptide growth factors and hormones lead to hyperphosphorylation of 4E-BP1, resulting in a marked decrease in its affinity for eIF4E, thereby allowing the association of eIF4G to generate functional eIF4F complexes.

It is well established that levels of active eIF4F complexes are limiting in normal cells because of limited availability of eIF4E resulting from the action of inhibitory factors, 4E-BPs, whose binding to eIF4E abolishes the interaction between eIF4E and eIF4G. There have been three isoforms of 4E-BP identified, 4E-BP1, 4E-BP2, and 4E-BP3, which are expressed in different cell types. Among these, 4E-BP1 has been studied most extensively and is the best understood member of the family in terms of its regulation and function. 4EBP-1 is a 12-15 kDa molecule which shares a common binding motif (YxxxxLΦ) with eIF4G and the chaperone 4E-T on eIF4E. When 4E-BP1 is hypophosphorylated, it outcompetes eIF4G for binding to eIF4E and, as a result of this binding, interaction of eIF4E with eIF4G is abrogated and cap-dependent translation is inhibited. Mitogenic and survival signaling promotes a hierarchical series of phosphorylation events on six serine/threonine residues in 4E-BP1. The hyperphosphorylated form of the molecule acquires reduced affinity for eIF4E (Gingras et al., 1998, 1999, and 2001; Mothe-Satney et al., 2000; Herbert et al., 2002), allowing formation of eIF4F complexes and promoting cap-dependent translation initiation.

I.C.3b Regulation of eIF2 α in mammalian cells

A variety of cellular stress conditions such as nutrient deprivation, ER-stress, viral infections, and oxidative stress elicit a specific response which involves activation of eIF2 α -kinases to inhibit general translation and induction of a special class of genes (Clemens 2004). Phosphorylation of the α -subunit of eIF2 at Ser51 converts the eIF2B-substrate eIF2-GDP to inhibitory phospho-Ser51-eIF2 α -GDP, ultimately resulting in the

accumulation of 'dead end' eIF2B-phospho-Ser51-eIF2 α -GDP complexes (Clemens 2004; Dever 2002). Although relative amounts of eIF2 and eIF2B vary among different tissues and organisms, eIF2 is always present in excess of eIF2B; therefore, phosphorylation of a fraction of the eIF2 in a cell ensures a quantitative inhibition of eIF2B and blockade of protein synthesis (Dever 2002).

PERK is an ER-transmembrane signaling protein. The ER is a calcium-rich and oxidative environment where nascent chains of secretory pathway proteins undergo modifications required for proper folding. Disruption of normal traffic of protein folding in the ER due to perturbations in ER-redox potential, calcium levels, or the glycosylation machinery triggers a complex chain of events termed the Unfolded Protein Response (UPR). During UPR, PERK becomes activated by oligomerization of its cytoplasmic C-terminal portion (eIF2 α -kinase domain) as a result of the dissociation of the chaperone BiP, which is found complexed to the N-terminal and luminal portion of PERK. Activated PERK phosphorylates eIF2 α at Ser51 (Brewer et al., 1999).

PKR [double-stranded RNA (dsRNA)-activated protein kinase] is a major mediator of the antiviral effects of interferons. Thus, it is believed that several virus genomes have evolved to encode proteins which can interfere with the function of PKR. Interferon-mediated activation of PKR involves its association with virus-induced dsRNA and autophosphorylation at several sites, although dsRNA-independent mechanisms are known to activate PKR as well. Activated PKR acts as a eIF2 α -kinase by phosphorylating eIF2 α at Ser51 (Dever 2002 and Clemens 2004).

Both PERK and PKR-mediated phosphorylation of eIF2 α has been implicated in decreasing cyclin D1 translation (Brewer et al., 1999 and Campo et al., 2002). Activation of PERK in response to tunicamycin-induced UPR in NIH-3T3 cells was reported to block translation of cyclin D1 (Brewer et al., 1999). Treatment of MCF7 cells with the cyclopentanone prostaglandin, 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (**15d-PGJ₂**), resulted in an early downmodulation of cyclin D1 protein due to impairment of cyclin D1 synthesis associated with increased eIF2 α phosphorylation at Ser51 by PKR (Campo et al., 2002).

I.C.3c Regulation of eEF2 in mammalian cells

For the addition of each amino acid to the growing polypeptide chain, two ATP molecules are hydrolyzed to AMP during formation of the amino acyl tRNAs and two GTPs are hydrolyzed to GDP by the elongation factors eEF1A and eEF2 (Proud 2004; Abbott and Proud, 2004; Browne and Proud, 2002). Due to the large energy-consumption involved in the process, it has been proposed that eukaryotic cells have evolved mechanisms of tight control over the translational elongation machinery. Upon cellular stress, amino acid starvation or growth arrest, elongation is inhibited with retention of polysomes so that translation can be resumed once conditions revert back to normal (Browne and Proud, 2002). There are two major advantages of having an ON/OFF switch to regulate the translation elongation machinery: (a) cellular energy reserves are spared because the need to expend a large amount of energy to re-initiate the translation of a given mRNA is avoided, and (b) mRNAs are protected from degradation, thus avoiding

the need for another round of transcription when normal growth conditions are re-attained (Browne and Proud, 2002 and Horman et al. 2002).

eEF2, which plays a key role in control of the eukaryotic translation elongation machinery, is subject to tight inhibitory regulation. In response to cellular stress, eEF2 is phosphorylated at Thr56, which lies within the GTP-binding domain, by a kinase called eEF2 Kinase (**eEF2K**). Phosphorylation of eEF2 at Thr56 results in inhibition of its activity in translocation of the peptidyl-tRNA from the A-site of the ribosome to the P-site, as well as in blockade of the movement of the ribosome along the mRNA and of poly(U)-directed polyphenylalanine synthesis by preventing eEF2 from binding to the ribosome (Browne and Proud, 2002, see Figure A.1). Recently, inhibition of eEF2 through increased phosphorylation at the Thr56 residue has been implicated in controlling the levels of a D type cyclin, cyclin D3, in T lymphocytes (Gutkow et al. 2003).

When eEF2 was identified as the only known substrate for the Ca^{2+} /calmodulin-dependent kinase III, the enzyme became known as eEF2 kinase (**eEF2K**) (Browne and Proud, 2002). In response to increases in Ca^{2+} or calmodulin levels, eEF2K undergoes extensive autophosphorylation and the enzyme becomes constitutively active irrespective of calcium levels. eEF2K, which is 93-100 kDa in size, has multiple phosphorylation sites implicated in its regulation by various signaling pathways. cAMP-dependent PKA, which is known to be activated under conditions of increased energy demand, phosphorylates eEF2K at Ser499 resulting in maximal activation of the enzyme (Browne

and Proud, 2002). Recently, AMP kinase (**AMPK**) has been implicated in negative regulation of eEF2 via indirect mechanisms (Horman et al., 2002). It is thought that activation of AMPK upon energy-stress leads to either positive regulation of eEF2K or negative modulation of a phosphatase which can remove the phosphate on Thr56 of eEF2 (Browne and Proud, 2002).

Insulin, on the other hand, has been reported to promote dephosphorylation of eEF2K in a rapamycin-sensitive manner, suggesting that insulin controls eEF2K activity via an mTOR-dependent pathway (see Appendix A for a detailed discussion and diagrammatic representation, Browne and Proud, 2002). p70S6K (see Appendix A and Discussion), an effector of mTOR, has been shown to phosphorylate eEF2K at Ser366 resulting in inactivation of eEF2K (Wang et al. 2001). p90^{RSK}, a member of the classical MAPK pathway lying directly downstream of Erk, has also been reported to catalyze the phosphorylation of eEF2K at Ser366.

I.C.4 Regulation of key translation factors by the cell cycle

In the previous section, we briefly discussed how changes in key translation factors impact the cell cycle. For example, aberrant increases in eIF4E expression/activity lead to upregulation of key regulators of cell cycle progression such as cyclin D1, while inhibition of eIF4E function leads to a decrease in the levels of this protein. Evidence also exists for modulation of certain key translation factors depending on the phases of the cell cycle (Cormier et al., 2003 and De Benedetti and Graff, 2004).

4E-BP1, for example, is found to be hyperphosphorylated during the G1-S transition, when cap-dependent translation is prevalent in order to allow enhanced protein synthesis rates to meet the requirement for increased cell mass prior to cell division. In G2/M, when cap-dependent translation is inhibited and IRES-dependent translation is turned on, 4E-BP1 becomes hypophosphorylated and remains in this phosphorylation state until the daughter cells reach the mid-G1 phase of the next cell cycle (Cormier et al. 2003). During apoptosis, 4E-BP1 is cleaved to generate a shorter fragment, which can bind eIF4E irreversibly because of the loss of regulatory phosphorylation sites, ensuring termination of protein synthesis (Proud 2002). This aspect of 4E-BP1-regulation reconciles well with what we know about expression of cyclin D1 and other molecular events pertaining to the G1/S transition when increased levels of cyclin D1 lead to induction of E2F-dependent genes.

Studies in yeast, where loss of eIF4E- and eIF3-homologues results in arrest at the G1/S boundary due to inefficient synthesis of CLN3, confirm further that translation of G1 cyclins is a critical determinant of cell cycle progression. In mammalian cells, a 50% reduction in protein synthesis has been reported to prevent DNA replication following mitogenic stimulation (Cormier et al., 2003). Likewise, control of cyclin B translation via modulation of different stages of the synthetic process has been shown to play an important role in the G2/M transition in fission yeast, in onset of meiotic cycles during oocyte maturation and early embryonic development in *Xenopus Laevis* and sea urchin (Cormier et al., 2003 and references therein).

1.E. OBJECTIVES

Previous studies in our laboratory showed that activation of PKC in nontransformed intestinal crypt cells induces G0 exit, involving rapid disappearance of cyclin D1, induction of p21^{CIP1/WAF1} and p27^{KIP1}, rapid dephosphorylation of pRb and p130, as well as disappearance of p107 and cdc 6 (Frey et al., 2000a) (Figure 15). Other cell cycle molecules, including cyclin E, cdk4, and cdk2, whose regulation has been implicated in checkpoints in other systems, remain unaffected upon PKC activation (Figure 16). Analysis of the involvement of individual PKC isozymes in these cell cycle-specific effects demonstrated the ability of PKC α , in particular, to induce all of the G0-related events promoted by PKC agonists. Interestingly, except for cyclin D1, all of these molecular changes are reversed upon depletion of PKC isozymes and re-entry of the cells into the cell cycle. Cyclin D1 instead, is induced to unusually high levels which considerably surpass the low amounts seen in control cycling cells (Figure 16). Notably, aberrant elevation of cyclin D1 and loss of PKC α expression are frequent events in intestinal neoplasms in humans (Tetsu and McCormick, 2003, Hulit et al., 2004, and Black 2001).

To investigate PKC signaling-mediated regulation of cyclin D1 expression in the normal intestinal epithelium, we have chosen the IEC-18 cell line which was originally established by Quaroni and his colleagues in 1978 from newborn rat ileum (Quaroni and May, 1980). This cell line, along with a similar line, IEC-6, is recognized as a reliable and phenotypically preserved model system for intestinal crypt cells (Gordon et al.,

2005). Findings from our laboratory demonstrated that the profile of PKC isozyme expression (i.e., PKC α , δ , ϵ , ζ , and ι) in IEC-18 cells parallels that in rat and human small intestinal crypt cells *in situ* (Frey et al., 1997; Saxon et al., 1994) (Figure 17). Thus, analysis of the cell cycle regulatory roles of PKCs in IEC-18 cells may provide clues about the roles of these kinases in the intestinal epithelium *in situ*. Furthermore, studying the consequences of loss of PKCs in IEC-18 cells may reveal the basis for molecular lesions encountered in intestinal malignancies.

Understanding the molecular events underlying the bimodal regulation of cyclin D1 expression in intestinal epithelial cells is the focus of the work described in this thesis. The first aim of this study was to elucidate the molecular pathways involved in downregulation of cyclin D1 expression in response to PKC signaling in normal intestinal epithelial cells and to determine the PKC isozymes involved. The second aim was to characterize the pathways involved in induction/maintenance of elevated levels of cyclin D1 in IEC-18 cells depleted of PKC activity. Results from these two main arms of the study may reveal (a) important aspects of cyclin D1 regulation during cell cycle exit and (b) why PKC α expression is reduced/abrogated in many intestinal neoplasms in rodents and humans. A better understanding of these pathways may point to novel targets for preventive or therapeutic intervention.

Figure 15

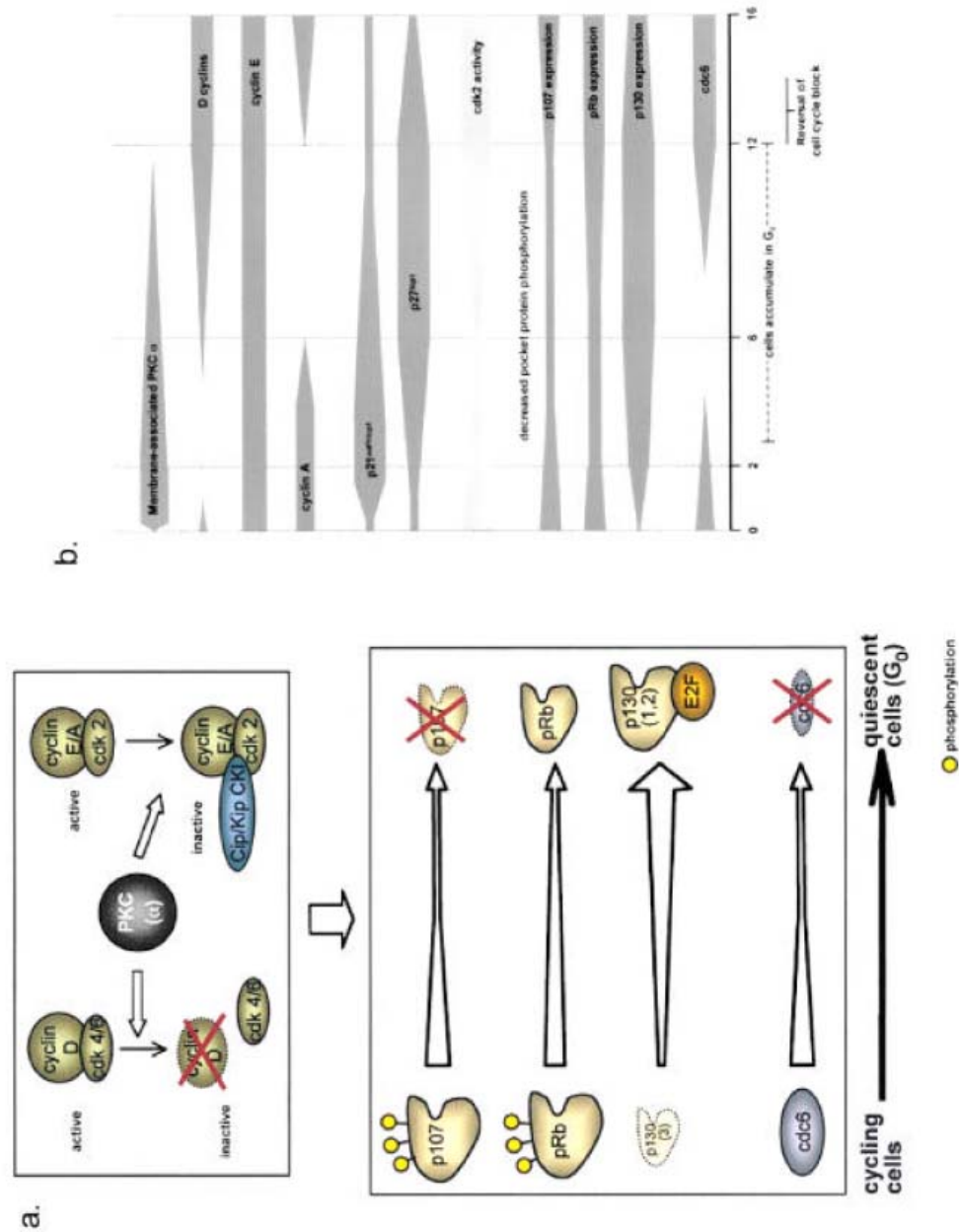


Figure 15 Models of PKC-mediated cell cycle withdrawal in IEC-18 cells. (a)

Proposed PKC-mediated pathways leading to cell cycle withdrawal in intestinal epithelial cells. Activation of PKC (PKC α) leads to induction of p21^{CIP1/WAF1} and p27^{KIP1} and cyclin D1 downregulation. Inhibition of cdk4/6 by cyclin D1 loss and of cdk2 by CKI accumulation results in coordinated hypophosphorylation/ downregulation of p107, hypophosphorylation of pRb, and accumulation of p130 phosphoforms 1/2. These events

indicate a withdrawal of cells from the cell cycle into G0; sustained p27^{kip1} upregulation may also, *in vivo*, be a key event leading to the onset of a differentiation program. (b) Timeline of regulatory changes in cell cycle control molecules following PKC activation in IEC-18 cells (Figure is taken from Frey 2000b).

Figure 16

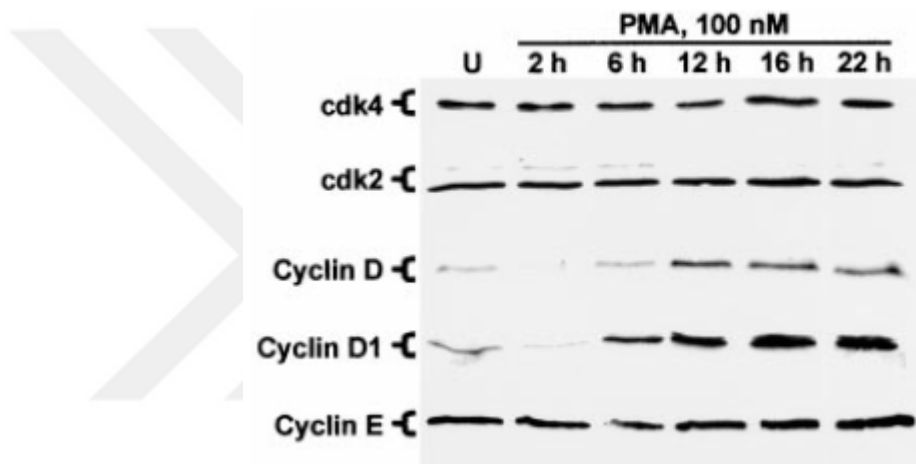


Figure 16 Effect of PMA treatment on the expression of cyclin D1, cyclin E, cdk4 and cdk2 in IEC-18 cells. Cells were treated with 100 nM PMA for the indicated times (U, untreated) and subjected to Western blot analysis using antibodies specific for cdk4, cdk2, and cyclins D (pan), D1, E, and A. Data are representative of three independent experiments (Figure taken from Frey et al., 2000a).

Figure 17

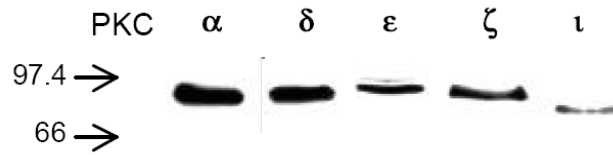


Figure 17 Comparison of PKC-isozyme expression patterns in IEC-18 cells and rat small intestinal tissue To determine the PKC isozyme profile of the IEC-18 intestinal crypt cell line, whole cell lysates (30 mg protein loaded per lane) were prepared from a subconfluent culture of asynchronously growing IEC-18 cells and subjected to Western blot analysis (10% SDS minigels) using antibodies specific for individual isozymes. The figure represents a composite of data from blots performed for the different isozymes, which have been aligned using molecular weight markers as guides. Arrows indicate the migration of 97.4- and 66-kDa molecular mass standards.

II. MATERIALS AND METHODS

II.A Antibodies

1. Antibodies for PKC isozymes

PKC α -, δ -, and ϵ - specific antibodies (sc-8393, sc-213, and sc-214, respectively) were from Santa Cruz Biotechnology (Santa Cruz, CA).

2. Antibodies for cyclin D1

Two of the anti-cyclin D1 antibodies used in this study were purchased from Santa Cruz Biotechnology: (1) sc-753 rabbit polyclonal (H295), raised against full length human cyclin D1 protein, and (2) sc-450 mouse monoclonal (72-13G), raised against recombinant mouse cyclin D1. Three rat monoclonal anti-cyclin D1 antibodies raised against different parts of mouse cyclin D1 were generous gifts from Dr. Martine Roussel (Vallance et al., 1994 and see Appendix B for detailed description of the three reagents).

3. Antibodies for translational control molecules

Rabbit polyclonal antibodies specific for phospho-Ser9-GSK-3 β , phospho-(Ser21/9)-GSK-3 α/β , eIF4E, phospho-Ser209-eIF4E, 4EBP-1, eEF2, phospho-Thr56-eEF2, and phospho-Ser366-eEF2K were obtained from Cell Signaling Technology (Beverly, MA). Antibody specific for phospho-Ser51-eIF2 α was purchased from Stressgen.

4. Conjugated secondary antibodies

Goat anti-rabbit IgG and rat anti-mouse IgG horse radish peroxidase (**HRP**)-conjugated antibodies were obtained from Chemicon International and Jackson ImmunoResearch Labs, respectively. HRP-conjugated goat anti-rat antibody was obtained from Santa Cruz Biotechnology (sc-2006) (Santa Cruz, CA).

II.B Cell culture procedures

1. IEC-18 cell cultures

The IEC-18 cell line (American Type Culture Collection; Rockville, MD; #CRL-1589) is a non-transformed intestinal crypt cell line derived from rat ileal epithelium (Quaroni and May, 1980). Cultures were maintained in complete growth medium (**CGM**), prepared by supplementing Dulbecco's modified Eagle's medium (**DMEM**) (GIBCO BRL) with 10 µg/ml insulin, 4 mM glutamine (GIBCO BRL), and 5% fetal bovine serum (FBS; Intergen; Purchase, NY), in a humidified atmosphere (5% CO₂) at 37 °C; under these conditions, the doubling-time of IEC-18 cells is ~18 h. Medium was replaced every two days and cells were re-plated every 5-6 days before confluence. Serum starvation medium included only 0.5% FBS and 4 mM glutamine (no insulin). To stimulate cells with serum, 1 ml of FBS was added directly to 10 ml medium in each plate.

For preparation of whole cell lysate (**WCL**), cells were generally plated at a density of 3.3×10^5 cells per 100 mm dish and cultured for ~48 h before treatments to carry out experimentation at subconfluent conditions (~75-85%). For cell growth

analysis, cells were plated at 6×10^5 cells per dish and cultured for at least 24 h before treatment. For conditions requiring treatment times of 24 h or longer, cells were plated at $2.75\text{--}3 \times 10^5$ cells per 100 mm dish to ensure equivalent and subconfluent cell density during experimentation. For serum-starvation conditions, cells were plated at a higher cell density ($\sim 4 \times 10^5$ per 100 mm dish) to ensure sufficient protein yield.

2. PKC agonists and activation protocols

PKC isozymes were activated in IEC-18 cells by treatment with 100 nM PMA (Sigma), 100 nM bryostatin (**Bryo**; Biomol or LC Laboratories), 20 $\mu\text{g/ml}$ 1,2-Dioctanoyl-sn-glycerol (**DiC₈**; Sigma), or 1 μM phorbol 12,13-dibutyrate (**PdBu**; Sigma) for various times. PKC agonists were generally added directly to cultures without change of medium; control cells were treated with the appropriate vehicle alone. Phorbol esters and bryostatin were dissolved in ethanol, with a final vehicle concentration in the medium of $<0.1\%$, and DiC₈ was dissolved in dimethyl sulphoxide (**DMSO**).

Depletion of PKC α , δ , and ϵ was accomplished by 24 h exposure to 1 μM PDBu followed by return to CGM (see Results section) before experimentation as we have described (Frey et al., 1997 and 2000a). Selective downregulation of PKC δ and ϵ from IEC-18 cells was accomplished by a 15 min-pulse treatment with 10 nM PMA, followed by two washes in PBS and return to fresh CGM for 24 h before experimentation (see Results section) (Frey et al., 2000a and Clark and Black et al., 2004).

3. PKC inhibition

Inhibition of PKC activity was achieved by a 30 min-pretreatment of cells with 5 μ M bisindolylmaleimide I (**BIM I**) (Alexis; San Diego, CA) and continuous presence of the inhibitor during PKC agonist exposure. **Gö6976** (1 μ M) (Calbiochem; Carpinteria, CA) was used similarly to selectively inhibit PKC α activity in IEC-18 cells. Both inhibitors were dissolved in DMSO.

4. Proteasome inhibition

Proteasome and/or calpain activity was inhibited with 25 μ M lactacystin (Lacta; E. J. Corey, Harvard University), 150 μ M *N*-acetyl-Leu-Leu-Nle-CHO (ALLN; Calbiochem) or 25 μ M Carbobenzoxy-L-leucyl-L-leucyl-L-Leucinal; Z-LLL-CHO (MG132; Calbiochem) with continuous presence of inhibitors during PKC agonist exposure (see Appendix for data). All of the proteasome inhibitors were added 30 min prior to the addition of PKC agonists. ALLN and MG132 were dissolved in DMSO, while lactacystin was resuspended in double-distilled, autoclaved water (**dd H₂O**).

5. Inhibition of translation and preservation of polyribosomal complexes

Polyribosome complexes were blocked with 50 μ M cycloheximide (**CHX**; Sigma) by 5 minute-exposure to the drug immediately before harvest. CHX was prepared as a 112 mg/ml stock solution in ethanol.

II.C Cloning and transfection procedures

1. Cloning of cyclin D1 mutants

Flag-tagged *wild type* (*wt*) cyclin D1 and Thr286Ala (**T286A**) mutant cyclin D1 constructs were kind gifts from Dr. Charles Sherr (St Jude Children's Research Hospital, Memphis, TN). These constructs were generated by cloning mouse cyclin D1 cDNA sequence into the EcoR I site downstream of the twelve-amino-acid "flag-tag" sequence in the pFlex vector which was derived from a pBJ5 plasmid (Diehl et al., 1997). R29Q and L32A "*destruction box*" mutants were generated from the *wt* cyclin D1 construct using the Quick-Change Site-directed Mutagenesis Kit and appropriate primers (Stratagene; La Jolla, CA). For the R29Q mutant, the primer used was 5'-GGG TGC TGC AGG CCA TGC TCA AG-3', while 5'-GCG AGC CAT GGC CAA GAC GGA G-3' was used for the L32A mutant. The PCR products were then subjected to *Dpn I* digest, which cuts the DNA at methylated sites, to eliminate the *wt* parental plasmid. Following *Dpn I* digest, the linear plasmids were transformed into super competent cells using NYZ⁺ medium [prepared by dissolving 1 g NZamine, 0.5 g yeast extract, and 0.5 g NaCl in 100 ml ddH₂O and adjusting the pH to 7.5 with NaOH. The stock solution was filtered with a 0.2 µm filter membrane in a sterile hood. Immediately before use, MgCl₂ and MgSO₄ (final concentration of 12.5 mM), and glucose (final concentration of 0.2%) were added]. Several colonies were obtained after plating each transformation reaction onto agar plates with ampicillin (75 mg/L). Minipreps (Qiagen; Valencia, CA) were performed on 4 of the colonies obtained. Following isolation of plasmid DNA from each miniprep, a total of 4 samples for each mutation were sequenced by the RPCI Biopolymer Facility.

All four samples contained the targeted mutation at the R29 or L32 site. Only one of the four DNA samples for each mutation was arbitrarily chosen to expand in a maxiprep for the transfection assays (Qiagen; Valencia, CA).

2. Transfection of IEC-18 cells

90-95% confluent IEC-18 cell populations in 60 mm- tissue culture dishes were transiently transfected with 5 µg cyclin D1 *wt* or mutant constructs using Lipofectamine 2000 reagent (Invitrogen). In the case of the T286A mutant, only 500 ng of the construct were used in transfections to avoid excessive accumulation of this degradation-defective protein in IEC-18 cells. Two plates were used for each construct. 250 µL opti-mem solution were incubated with 10 µL Lipofectamine reagent for 10 min while another 250µL aliquot of opti-mem solution was mixed with the DNA in 50 ml conical tubes. After combining the lipofectamine + opti-mem mix with the DNA + opti-mem mix in a 50 ml conical tube, 3 ml of CGM per plate were added and mixed to obtain the final transfection cocktail for two 60 mm plates. After addition of transfection cocktail, the cells were returned to the incubator. The transfection cocktail was discarded 4-5 h later, cells were washed twice with CGM, and cultured overnight. On the next day, cells were trypsinized and re-plated into two 100 mm-culture dishes for PMA treatments (2 hour) on the following day.

II.D Polyribosome Analysis

Subconfluent cell populations (8 100 mm culture dishes per condition) were treated with 100 nM PMA or vehicle for 45 min before the addition of 50 µg/ml CHX for

5 min. Cells were then rinsed twice with ice-cold sterile phosphate-buffered saline (PBS) containing CHX (50 µg/ml), harvested by trypsinization, and washed in PBS/CHX. Cells were pelleted by centrifugation at 1000 RPM for 10 min and lysed (10 min on ice) in 5 cell volumes (1 ml) of hypotonic lysis buffer [50 mM Tris-HCl pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, freshly added 5 mM DTT, 1% Triton X-100, 1% deoxycholate, and freshly added 20-40 U/ml of RNase Inhibitor (Sigma)]. Insoluble material was removed by centrifugation at 10,000 xg for 10 min at 4 °C. The supernatant was divided into two aliquots, one of which (470 µL) was adjusted to 30 mM EDTA by addition of 30 µL 0.5 M EDTA (pH 8.5) to disrupt polyribosomal complexes. Polysome distribution of cyclin D1 mRNA in these aliquots was determined by 10-40% sucrose gradient centrifugation as described by Black and Dolnick (1996).

Sucrose gradients were prepared using a gradient-maker. The instrument was washed once with RNase-away (VWR), and then rinsed with nuclease-free dd H₂O several times. Using this apparatus, a 1 ml 80% sucrose cushion was overlaid with a continuous 40% and 10% sucrose gradient. All solutions were freshly prepared using RNase-free sucrose dissolved in lysis buffer and poured into open-top, konical™, polyallomer, 14.5 ml tubes [recommended by Beckman for swinging bucket, 6 x 17 ml, 41,000 RPM (SW41 Ti) rotors]. Samples were loaded on top of the gradients (i.e., Cont, PMA, Cont/EDTA, and PMA/EDTA), and subjected to centrifugation at 36,000 RPM for 75 min at 4 °C. 600 µL aliquots were collected using the gradient maker and mixed with 150 µL of 10% SDS.

Each 750 μ L fraction was subjected to RNA isolation as follows: an equal volume (750 μ L) of phenol-chloroform-isoamylalcohol mix (25:24:1) and 60 μ L of sodium acetate (pH 5.2) were added to each sample, prior to vortexing for 10 seconds and centrifugation for 3-5 min at 10,000 $\times g$ at room temperature. The aqueous top layer was transferred into a fresh 2.2 ml microfuge tube, and mixed with 2.5 volumes of pre-chilled 100% ethanol and RNase-free glycogen (to a final concentration of 50 ng/ml) before being stored at -80 °C overnight. On the following day, samples were warmed to room temperature, and RNA was precipitated by centrifugation (5 min, 10,000 $\times g$). The pellet was washed once in 70% ethanol prepared in nuclease-free dd H₂O. After briefly drying the pellet by vacuum-centrifugation at a low-setting, the material was dissolved in 20 μ L nuclease-free dd H₂O. Note that all RNA samples were kept on ice during the remaining steps of the protocol to prevent degradation.

Cyclin D1 mRNA levels were assessed by RT-PCR using the Titanium One-step RT-PCR kit (Clontech) and specific primers (forward primer, 5'-GTC TTC CCG CTG GCC ATG AAC TAC-3'; reverse primer, 5'-AAG AAA GTG CGT TGT GCG GTA GCA-3') in the following RT-PCR program: RT step was 1 hour at 50 °C and then RT was inactivated at 94 °C for 5 min. Amplification of the cDNAs was achieved in 35 cycles of denaturation (1 minute at 94 °C), annealing of primers (1 minute at 59 °C), and extension (2 min incubation at 68 °C). Following a final extension step of 10 min at 68 °C, the RT-PCR products were stored at 4 °C. 30 μ L RT-PCR product were mixed with 3 μ L 10X loading Dye (Invitrogen), resolved on 1.2% agarose gels, and visualized by UV transillumination.

II.E In vitro Cap-affinity Assay

Cap-affinity chromatography was performed essentially as previously described (Caron et al., 2004). Subconfluent cells (2 100 mm plates per condition) were treated with vehicle or 100 nM PMA for the indicated times (see Results section), washed twice in cold PBS, and harvested in 140 μ L lysis buffer [50 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, and 1% Triton X-100, containing Phosphatase Inhibitor cocktail I (Sigma), Phosphatase Inhibitor cocktail II (Sigma) and Protease Inhibitor cocktail (Sigma) at a final concentration of 30 μ L/ml]. After centrifugation at 10,000 $\times$ g for 20 min at 4 °C (12 min followed by 8 min in a fresh tube), cell lysates (350-400 μ g protein) were incubated with 50 μ L 7-methyl GTP-Sepharose 4B slurry (Amersham Biosciences) for 16 h at 4 °C, with rotation, to capture eIF4E and its binding partners. Prior to addition to the lysates, beads were equilibrated in lysis buffer by three washes (10-15 min, 4 °C) in a volume of 0.75 ml. Following 16 h incubation, a 60-90 μ L aliquot of cap-supernatant was saved for Western blot analysis and beads were washed THREE times with lysis buffer. For each wash, beads were gently resuspended in 0.75 ml lysis buffer (1 or 2 min with rotation), and pelleted by centrifuging at 1000 RPM for 3 min at 4 °C. After the final wash, cap-bound protein was eluted by resuspending the beads in 36 μ L 2X Laemmli sample buffer and vortexing vigorously, prior to brief centrifugation and boiling for 5 min. Both cap-bound and cap-supernatant fractions were analyzed for 4EBP-1 and eIF4E levels by Western blot analysis (see below).

II.F Immunoprecipitation-reverse transcriptase-polymerase chain reaction (IP-RT-PCR) assay

This assay was adapted from a protocol which was kindly provided by Dr. Timothy Fisher. Subconfluent cells (3-4 100 mm plates per condition) were treated with vehicle or 100 nM PMA for the indicated times (see Results section), washed twice in ice-cold PBS, and harvested in 150 μ L detergent-free lysis buffer [50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM $MgCl_2$, and 10% Glycerol containing freshly added Phosphatase Inhibitor cocktail I (Sigma), Phosphatase Inhibitor cocktail II (Sigma), Protease Inhibitor cocktail (Sigma) (40 μ L/ml each), and 56 U/ml RNase Inhibitor (Sigma)]. Cells were scraped from the dishes, transferred to microfuge tubes, and incubated with rotation for 20 min at 4 °C for complete lysis. Following centrifugation at 10, 000 xg for 28 min (20 min followed by 8 min in fresh tubes) at 4 °C, a 25 μ g aliquot was removed for WCL RNA isolation.

For immunoprecipitation of 4E-BP1, cell lysates (400-450 μ g) were supplemented with Tween-20 (0.5%), RNase inhibitor (53 U/ml), DTT (0.1 mM), and 1.8 μ g 4E-BP1 rabbit polyclonal antibody (Cell Signaling, #9452), and incubated with rotation for 16 h at 4 °C. A total of 480 μ L Protein A Dynabeads (Dyna) were equilibrated with lysis buffer containing 0.5% Tween-20 and 0.1 mM DTT in two 5-min-incubations in 1 ml lysis buffer with rotation at 4 °C. After resuspending the equilibrated beads in 480 μ L lysis buffer, 80 μ L aliquots of slurry were added to each IP reaction for 4h at 4°C (with rotation). Beads were collected using a magnet (1 min) and 25 μ g and 90 μ L aliquots of

the supernatant were saved for RNA (IP/SUP/RNA), and protein analysis (IP/SUP/protein), respectively. The beads were washed THREE times as described above. It should be noted that the first two washes were in lysis buffer with 0.5% Tween-20 and the last one was in lysis buffer without Tween-20. After the final wash, beads were resuspended in 45 μ L lysis buffer without Tween-20 by gentle pipetting several times, and 25 μ L and 20 μ L aliquots were saved for RNA isolation (IP/RNA) and Western blot analysis (IP/protein), respectively.

IP/protein samples were boiled in 2X sample buffer, while IP/RNA samples (together with their WCL and IP/SUP RNA counterparts) were subjected to Proteinase K digestion at 37 °C for 30 min in 250 μ L of Proteinase K solution [200 μ g/ml Proteinase K, 10 mM Tris-HCL pH 8.0, 10 mM EDTA, 1% SDS, and 100 mM NaCl]. Following Proteinase K digestion, 275 μ L of phenol:chloroform:isoamyl alcohol (25:24:1) mix and 48.3 μ L 3 M sodium acetate pH 5.2 were added, samples were vortexed for 10 seconds, and centrifuged at 10, 000 xg for 5 min at room temperature. The aqueous layer from each sample was transferred into a fresh tube, and mixed with 732.5 μ L (2.5X volume) pre-chilled 100% ethanol and 25.63 μ L 2 μ g/ml RNase-free glycogen (final concentration of 50 ng/ml) before being stored at -80 °C overnight. On the next day, samples were warmed to room temperature and centrifuged at 10,000 xg for 10 min at room temperature. Supernatants were decanted and the pellets were washed once in 70% ethanol (prepared in nuclease-free dd H₂O). Pellets were briefly dried by vacuum centrifugation, and resuspended in 20 μ L of nuclease-free dd H₂O. Note that all RNA

samples were kept on ice during the remaining steps of the protocol to prevent degradation.

A 2 μ L aliquot from each sample was used to amplify the Cyclin D1 message in a RT-PCR reaction as described above, using the following RT-PCR program: after a 1 hour RT step at 50 °C, RT enzyme was inactivated at 94 °C for 5 min. To ensure detection of differences in the amount of cyclin D1 mRNA in each sample, only 27 cycles of denaturation (1 min at 94 °C), annealing of primers (1 min 59 °C), and extension (2 min 68 °C) were performed without including the final extension step. To confirm that 27 cycles of amplification under these RT-PCR conditions were in the linear range, 2 μ L aliquots of an IP/SUP RNA sample were subjected to RT-PCR with increasing number of cycles (10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32), and the amount of cyclin D1 RT-PCR product obtained with each cycle number was determined (data not shown).

II.G [S^{35}]-Met/Cyst pulse-chase analysis

To examine cyclin D1 turnover rates, subconfluent cell populations (two 100 mm culture dishes per condition) were incubated in labeling medium for 1 h. The labeling medium consisted of meth/cyst-free DMEM (GIBCO) containing 4 mM glutamine, 5 μ g/ml insulin, and 5% dialyzed fetal bovine serum (dFBS), and [S^{35}]-Methionine/Cysteine protein labeling mix (NEN) (at a final concentration of 94 μ Ci/ml). Cells were then treated with vehicle or 100 nM PMA for 1 h and chase was initiated by changing the labeling medium to label-free CGM containing ethanol or 100 nM PMA.

Cells were harvested in 1% SDS lysis buffer at 15 min intervals (as described below), and cyclin D1 was immunoprecipitated from the cell lysates collected at each time point with rabbit polyclonal anti-cyclin D1 antibody (sc-753) (see below). Immunoprecipitates were resolved on 20% SDS-PAGE gels, transferred onto nitrocellulose membrane, and exposed to phosphorimager screens. Signal from cyclin D1 was quantified using ImageQuant software.

II.H Biosynthetic labeling experiments

Subconfluent cell populations (two 100 mm culture dishes per condition) were starved of methionine and cysteine coincident with the initiation of 100 nM PMA or ethanol treatment for 30 min. Cells were then incubated with [S^{35}]-Methionine/Cysteine (0.38 mCi/plate) in the continued presence of vehicle or PMA for the indicated times (see Results section). At each timepoint, cells were harvested under cold lysis conditions (see below, Immunoprecipitation section), and cyclin D1 was immunoprecipitated using monoclonal anti-cyclin D1 (SC-450) antibody (see below). Immunoprecipitates were resolved on 20% SDS-PAGE gels, transferred onto PVDF membrane, and exposed to phosphorimager screens. Signal from cyclin D1 was quantified using ImageQuant software.

II.I Cell Lysate Preparations

1. Protein isolation

Subconfluent IEC-18 cell populations (1-2 100 mm plates per condition) were rinsed twice with nonsterile PBS. After aspirating excess PBS, 100 μ L boiling 1% SDS lysis buffer (10 mM Tris, pH 7.4, 1% SDS) were added to each plate. Cells were scraped from the dish and transferred into microfuge tubes. The WCL was boiled for 5 min, and sheared by 5-10 passes through a 27 g needle, avoiding excessive foaming. DNA was pelleted by centrifugation (10-25 min; at 10,000 xg at room temperature). The supernatants, i.e., WCL protein samples, were transferred to fresh microfuge tubes, and centrifuged at 10,000 xg for 8 min prior to quantification of protein concentration.

Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Company; Rockford, IL): 2.5, 5.0, 7.5, and 10 μ L of BSA standard (1 or 2 mg/ml) and 5-7.5 μ L of protein samples were added per well in quadruplicate in a 96-well plate, and combined with 200 μ L (per well) BCA mix [20 μ L reagent B was pre-mixed per 1 ml of reagent A and vortexed briefly before mixing with the samples]. After shaking the 96-well plate in a plate-shaker at the lowest setting for 1 min, it was incubated at 37 °C for 30 min. Absorbance readings at λ =570 nm were determined with a microplate reader (ELx808_{II} model, BioTek; Winooski, VT). Average concentration for each protein sample was calculated using the KC4 software system (BioTek; Winooski, VT). The sample was then boiled with 4X Laemmli sample buffer

[50 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2.5% SDS, 2 mM EDTA, 10 mM DTT, and 0.05% (w/v) Bromophenol Blue (Laemmli, 1970)] for 5 min before being subjected to Western blot analysis.

2. Subcellular Fractionation

IEC-18 cells were partitioned into soluble (cytosolic) and particulate fractions essentially as previously described (Saxon et al., 1994). Briefly, cells were washed twice in cold PBS and scraped in a digitonin-based extraction buffer containing 20 mM Tris, pH 7.5, 2 mM EGTA, 2 mM EDTA, 0.5 mg/ml digitonin, 20 μ L/ml Phosphatase Inhibitor cocktail I (Sigma), Phosphatase Inhibitor cocktail II (Sigma), and Protease Inhibitor cocktail (Sigma). Digitonin-soluble (cytosolic) and -insoluble (particulate) fractions were separated by ultracentrifugation at 100,000 $\times g$ for 40 min at 4 °C. The cytosolic proteins in the supernatant were precipitated with a freshly prepared 10% TCA solution, incubated on ice for 10 min, pelleted, washed in acetone, dried briefly, solubilized in 50 μ L of 100 mM NaOH, and neutralized by addition of 50 μ L 100 mM HCl. The particulate pellet was incubated on ice for 30 min in 100 μ L digitonin buffer containing 1% Triton X-100 (Triton buffer). The membrane sample was then cleared by centrifugation (10,000 $\times g$) for 30 min at 4 °C. Cytosolic and membrane fractions were boiled in Laemmli sample buffer for 5 min before being subjected to Western blot analysis.

3. Total RNA isolation

Subconfluent cell populations (2-3 100 mm plates per condition) were treated with vehicle or 100 nM PMA for the indicated times (see Results section). At each time point, cells were washed twice in cold sterile PBS and lysed in 300 μ L RLT buffer (provided by Qiagen Rneasy kit) containing β -mercaptoethanol (**BME**) (10 μ L/ml). Cells were scraped into a microfuge tube and immediately stored at -80°C until collection of all time points was completed. The remaining steps of the isolation were carried out as instructed by the manufacturer. After quantifying the RNA samples by measurement of absorbance at $\lambda=260$ nm, 10 μ g of each RNA sample were subjected to Northern blot analysis as described below.

4. RNA fractionation assay

Subconfluent cell populations (3 100 mm culture plates per condition) were treated with vehicle or 100 nM PMA for the indicated times (see Results section). At each time point, plates were rinsed once in ice-cold sterile PBS, excess PBS was aspirated by vacuum, and cells were collected by trypsinization (in CGM). Cell pellets were gently resuspended in 250 μ L RLN buffer [50 mM Tris pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% NP-40] and centrifuged at 300 $\times g$ for 2 min at 4 °C. 175 μ L supernatant were transferred to a fresh tube and labeled the “cytosolic fraction”. The remaining supernatant was removed and the pellet was saved for nuclear RNA isolation. The Qiagen Rneasy kit and the protocol provided by the manufacturer were used to isolate RNA from each subcellular fraction. 10 μ g RNA sample was then subjected to Northern blot analysis.

5. Immunoprecipitation (IP)

To immunoprecipitate cyclin D1, cells were lysed in boiling 1% SDS lysis buffer. WCL were boiled for 5 min, and DNA was sheared by 5-10 passes through a 27 g needle. Extracts were cleared by centrifugation (10 min; 10,000 xg, at room temperature). Protein concentrations were determined by the BCA microplate method as described above. 400-600 µg of each sample were made up to 1 ml using 2X DN IP buffer [10 mM Tris, pH 7.4; 150 mM NaCl, 5 mM EDTA, 100 mM NaF, 1% Triton X-100, 0.5% NP-40 with fresh addition of phosphatase and protease inhibitor cocktails from Sigma], and pre-cleared by sequential incubation (at 4 °C, with rotation) with 10-15 µL normal rabbit serum for 1 h, followed by 45-50 µL Protein A/G-plus agarose slurry (Santa Cruz Biotechnology) for 1 h. Pre-cleared lysates were then incubated with 2-4 µg rabbit polyclonal anti-cyclin D1 antibody (H295) overnight at 4 °C with rotation. Immunocomplexes were collected by adding 45 µL of Protein A/G-plus agarose slurry for 2 h at room temperature. Beads were washed 6 times with 500 µL RIPA buffer [10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 25 mM KCl, 5 mM MgCl₂, 0.2 mM CaCl₂, 1% NP-40, 1% Deoxycholate, and 0.1% SDS]. After the final wash, the beads were resuspended in 25 µL 2X Laemmli sample buffer, vortexed, spun down briefly and boiled for 5 min.

In an alternative cyclin D1 immunoprecipitation protocol (obtained from Dr. Martine Roussel and used in biosynthetic labeling experiments), cells were rinsed twice with ice-cold PBS and lysed in Nonidet P-40 (NP-40) cold lysis buffer [20 mM Tris-HCl

pH 7.6, 150 mM NaCl, 1% vol/vol NP-40, and 0.5% wt/vol sodium deoxycholate with fresh addition of inhibitor cocktails from Sigma at 10 μ L/ml of lysis buffer] for 20 min at 4 °C with slow rocking. After centrifugation at 10,000 xg for 12 min, protein lysates were transferred to a fresh tube and centrifuged for 8 min at the same speed prior to protein quantification as described above. 450-600 μ g of each sample was made up to 1 ml with lysis buffer and pre-cleared by sequential incubation with 12.5-15 μ L Normal Mouse IgG (Santa Cruz Biotechnology) for 45 min, followed by 45-50 μ L Protein A/G-plus agarose slurry for 1 h at 4 °C with rotation. After centrifugation at 2000 RPM for 4 min at 4 °C, the supernatant was transferred into fresh tubes and incubated with 2-3 μ g mouse monoclonal anti-cyclin D1 (SC-450) antibody overnight at 4 °C with rotation. Immunocomplexes were collected by adding 45-50 μ L Protein A/G-plus agarose bead-slurry for 2-4 h at 4 °C with rotation. Beads were washed 5 times with 1 ml NP-40 cold lysis buffer. Following the final wash, beads were resuspended in 25 μ L 2X Laemmli sample buffer, vortexed, pelleted briefly and boiled for 5 min.

To immunoprecipitate exogenously expressed cyclin D1 mutants, cells were harvested in 130 μ L lysis buffer [20 mM Tris pH 7.6, 120 mM NaCl, and 0.5% NP-40 buffer with fresh addition of phosphatase and protease inhibitor cocktails from Sigma (10 μ L/ml)]. After clearing the lysates by centrifugation at 10,000 xg for 10 min, 400-600 μ g WCL were incubated overnight with 50 μ L Anti-FLAG M2 Affinity Gel conjugated beads (Sigma, A2220). On the next day, a 120 μ L aliquot of IP-supernatant was saved for analysis before beads were washed 6 times in lysis buffer without inhibitors as described

above. Following the final wash, beads were resuspended in 40-50 μ L 2X Laemmli sample buffer and boiled for 5 min before Western blot analysis for cyclin D1.

II.J Western Blot Analysis

WCL (30 μ g), subcellular fractions or IP samples were electrophoretically resolved using 7.5% (for phospho-Thr56-eEF2, total eEF2, and phospho-Ser366-eEF2K), 10% (for PKC isozymes), or 20% (for cyclin D1, phospho-Ser209-eIF4E, total eIF4E, eIF2 α , 4E-BP1) polyacrylamide minigels.

Gels were run at 200 V in a Tris-glycine running buffer [25 mM Tris, 192 mM Glycine, 0.1% SDS] until the dye-front passed the edge of the plate (approximately 1-1.25 h). Proteins were electrophoretically transferred onto nitrocellulose membrane (0.2 μ m pore-size, Bio-Rad Laboratories, Hercules, CA) in transfer buffer [10 mM Tris-acetate, pH 8.3, 20% isopropanol, and 0.1% SDS] at 60 V, on ice, for 1-1.25 h. Blots were routinely stained with 0.1% fast green (Sigma) solution [made in 20% methanol and 5% acetic acid] to confirm equal loading and even transfer.

Membranes were blocked in blocking buffer (**TBSt/milk**), [Tris-buffered saline, **TBS**; 20 mM Tris-HCl, 137 mM NaCl, pH 7.6, containing 5% non-fat dried milk (Carnation) and 0.1% Tween-20], for 30 min at 37 °C. Membranes were then incubated with primary antibody in TBSt/milk overnight at 4 °C, followed by one rinse and three slow-5 min washes in TBS/0.1% Tween-20 (**TBSt**). Blots were then incubated with secondary HRP-conjugated antibody in TBSt/milk for 45 min at room temperature. After

four 10-15-min fast-washes in TBSt and one 5 min-wash in 1X TBS, bound HRP was detected using the SuperSignal ECL system (Pierce Chemical Company, Rockford, IL). Primary antibody dilutions were as follows: 1:1000 for PKC α , PKC ϵ , cyclin D1, phospho-Ser9-GSK-3 β , phospho-(Ser21/9)-GSK-3 α/β , phospho-Ser209-eIF4E, phospho-Ser51-eIF2 α , phospho-Thr56-eEF2; 1:2000 for PKC δ , and 4E-BP1. Secondary antibodies were used at 1:2000.

II.K Northern Blot Analysis

RNA samples (10 μ g) were prepared in a total 40 μ L cocktail containing 50% deionized formamide, 13.5% formaldehyde, 1X MOPS buffer [40 mM MOPS, 10 mM Na Acetate, 2 mM EDTA adjusted to neutral pH diluted from a 20X stock], 1X loading dye, and 12.5 μ g/ μ L Ethidium Bromide. Samples were incubated at 55 $^{\circ}$ C for 15 min immediately before loading.

RNA cocktails were loaded on 1-1.2 % agarose-formaldehyde gels [per gel tray, 180 ml of gel mix was prepared by melting 2.16 g of SeaKem agarose in 130 ml of dd H₂O in pre-baked erlenmeyer flasks, before the addition of 32 ml of formaldehyde (Sigma) and 18 ml 10X MOPS stock]. Samples were resolved at 80-90 volts for 6-8 h in 1X MOPS buffer, and then transferred onto Genescreen-plus (NEN) membranes in 10X SSC buffer [87.65 g NaCl and 44.1 g sodium citrate] using the downward capillary-action technique overnight. Membranes were visualized under UV light to mark the positions of the 18 S and 40 S ribosomal bands and crosslinking was performed using a UV-crosslinker (Stratagene) at 1200 joules. Following three washes with dd H₂O to

eliminate the salts carried from the blotting step, membranes were prepared for probing. Membranes were pre-hybridized in 15 ml of Quick-Hyb solution (Stratagene) at 68 °C for at least 1 h in a revolving hyb-oven to ensure that the entire membrane was well-coated with a thin film of QuickHyb hybridization buffer.

To radioactively label mouse cyclin D1 cDNA with αP^{32} -deoxycytosinetriphosphate (αP^{32} -dCTP) in a 30 μ L-Klenow reaction, 50-60 ng cyclin D1 cDNA were boiled with 10 μ L nuclease-free ddH₂O at 95 °C for 5 min and cooled on ice for 1 minute. 50 μ Ci (αP^{32} -dCTP), 6 μ L of 5X OLB buffer [250 mM Tris-HCl pH 8.0, 25 mM MgCl₂, 5 mM BME, 2 mM dATP, dTTP, dGTP, 1 M Hepes pH 6.6, and 1 mg/ml pd(N)₆ 5'-PO₃, Na⁺ salt, random primers (cat # 27-2166, Amersham Pharmacia)], 3 μ L of 10X Klenow buffer, 2 μ L of BSA, and 2 μ L of Klenow enzyme were then added and the reaction was incubated at room temperature overnight. The radiolabeled probe was purified using G₅₀ columns (Amersham Pharmacia) based on the instructions of the manufacturer and boiled for 3-5 min at 95 °C before being mixed with pre-hybridization buffer. After a three-hour hybridization step at 65 °C, the membrane was washed once with 1X SSC, 0.1% SDS wash buffer at room temperature for 15 min and once with 0.1X SSC and 0.1% SDS stringent wash buffer at 60 °C for 20-30 min. The signal was detected after exposing the membrane wrapped in Saran Wrap to Phosphoimager Screens (Molecular Dynamics) using ImageQuant software.

II.L Immunofluorescence staining of Apc^{min} mouse adenomas for cyclin D1

Immunofluorescence analysis of Apc^{min} mouse adenomas was performed essentially as described in Frey (2000a and 2000b). Duodenal tissue from Apc^{min} mice (Jackson Laboratories) was removed and flushed with ice-cold PBS. Tissue used for cyclin D1 immunostaining was fixed immediately after removal in 2% freshly depolymerized paraformaldehyde/PBS for 2 h at 4 °C, washed 3 times (15 min) in 50 mM NH_4Cl /PBS, cryoprotected in 30% sucrose/PBS for at least 5 h, and frozen in liquid nitrogen-cooled 2-methylretane. Sections (4-6 μM) were cut on a cryostat microtome (Reichert-Jung, Rochester, NY), and thaw-mounted onto gelatin-coated coverslips. Non-specific binding of antibodies to tissue sections was blocked by preincubation in PBS containing 0.03% casein and 0.05% Tween-20 for 30 min at room temperature. Sections were then immunostained by incubation with primary antibody in PBS containing 0.2% Triton X-100 (Sigma) for 60 min, followed by a 15 min PBS/Triton wash, 40 min in secondary antibody in PBS/Triton, and a 15 min PBS wash. Primary antibody dilution was: 1:10 for anti-cyclin D1. TRITC-conjugated secondary antibodies were used at 1:100 dilution. Coverslips were rinsed in ddH₂O and mounted onto slides with Aqua Polymount (Polysciences, Inc.; Warrington, PA). Fluorescence was viewed with a Zeiss epifluorescence microscope (OberKochen, Germany) equipped with appropriate optics and filter modules.

II.M Immunohistochemical analysis of PKC α expression in Apc^{min} mouse adenomas

Formalin-fixed, paraffin-embedded Apc^{min} mouse intestinal tissues were sectioned (4-6 μ m), deparaffinized, and rehydrated. Following a 15-min-incubation in 3% H₂O₂, slides were washed once in PBS (5 min). Antigen retrieval was performed by microwaving the sections twice (10 min each) in citrate buffer. After cooling to 20 °C, sections were blocked with 0.03% casein for 30 min and then incubated with primary polyclonal anti-PKC α antibody (1 μ g/ml, sc-208) diluted in PBS-Tween-20 buffer (PBST) for 1 h. Slides were washed twice in PBS (5 min each), and secondary anti-rabbit antibody (at 1:250 dilution) incubation was performed for 30 min. Sections were washed twice in PBS (5 min each) before being subjected to a 30-min treatment with freshly prepared Elite-Kit ABC solution. Following two 5-min PBS washes, DAKO DAB-K3466 was applied for 5 min. Slides were first rinsed under tap water for 3 min, and then with ddH₂O for 3 min, before the sections were counterstained with hematoxylin for 2 min.

II.N Silencing of PKC α , PKC δ and PKC ϵ Using RNA interference (RNAi) Technology

Targeted knock down of PKC α , PKC δ , or PKC ϵ expression was achieved using RNAi technology essentially as described in Clark and Black et al. (2004). The following sequences were used to target rat PKC isozymes: for PKC α 5'-(GGCAGAGGUCACAGAUGAA)d(TT)-3' and 5'-UUCAUCUGUGACCUCUGCC)d(TT)-3', for PKC δ 5'-AGATTATCGGCCGCTGCACT-3', and for PKC ϵ 5'-AAGTGCGCTGGGCTAAAGAAA-3' (Irie et al., 2002). High pressure liquid chromatography-purified and annealed double-stranded short interfering RNA sequences with d(TT) overhangs at the 3'-end (siRNAs) were obtained from Qiagen, Inc. siRNAs were transfected into IEC-18 cells at 30% confluence using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. siRNA resuspension buffer was used in place of siRNA for control transfections. 48 h after transfection, cells were harvested in boiling 1% SDS lysis buffer. Selective silencing of the appropriate PKC isozyme was confirmed by subjecting parallel WCL to anti-PKC α , anti-PKC δ , and anti-PKC ϵ immunoblot analysis, and samples were then immunoblotted for cyclin D1.

III. RESULTS

III.A PKC-induced cell cycle arrest in cultured intestinal epithelial cells involves early loss of cyclin D1 protein.

III.A.1 PMA induces an early loss of cyclin D1 protein

As discussed in the Introduction, activation of PKC in IEC-18 promotes downregulation of cyclin D1 protein. To gain insight into the precise timing of PKC-induced cyclin D1 loss, we treated asynchronously growing IEC-18 cells with 100 nM PMA or vehicle (ethanol) for the indicated times (Figure 18). Western blot analysis of the samples demonstrated a decrease in cyclin D1 protein as early as 30 min following PKC activation and levels remained minimal up to 2 h of PMA treatment.

Figure 18

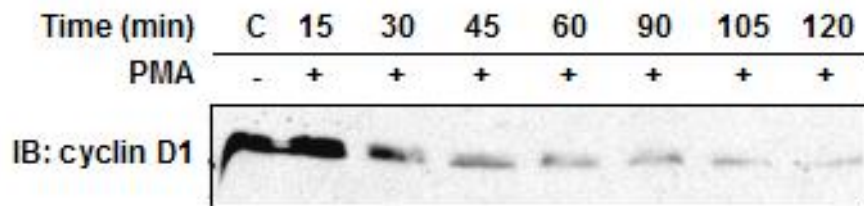


Figure 18 Changes in cyclin D1 expression in response to PKC-activation with PMA

Subconfluent populations of IEC-18 cells were treated with 100 nM PMA for the indicated times. All conditions were harvested in boiling 1% SDS lysis buffer. 30 µg of each protein lysate were resolved on 20% SDS-PAGE gels and immunoblotted for cyclin D1 using a rabbit polyclonal antibody (SC-753). Data are representative of 15 independent experiments.

III.A.2 Other PKC agonists also induce early loss of cyclin D1 protein

To determine whether PKC agonists other than PMA can also promote loss of cyclin D1 protein, we treated IEC-18 cells with a panel of PKC agonists including the phorbol ester PdBu, the macrocyclic lactone Bryo, and the DAG-analogue DiC₈ for 2 h. Protein lysates isolated from each condition were immunoblotted for cyclin D1. As shown in Figure 19, each of these PKC agonists induced downregulation of cyclin D1 levels by 2 h of treatment, paralleling the effects of PMA.

Figure 19



Figure 19 Changes in cyclin D1 expression in response to PKC activation by various PKC agonists Subconfluent populations of IEC-18 cells were treated with 100 nM PMA (P), 1 μ M PdBu (PB), 100 nM Bryo (B), or 20 μ g/ml DiC₈ (D) for 2 h. Cells were harvested in boiling 1% SDS lysis buffer and 15 μ g WCL protein sample from each condition were analyzed for cyclin D1 by Western blotting. Data are representative of 4 independent experiments.

III.A.3 Depletion of PKC isozyme protein or inhibition of PKC activity blocks PMA-induced cyclin D1 downregulation in IEC-18 cells

As reviewed in Kazanietz (2000), although classical and novel PKC isozymes are well-characterized major receptors for phorbol esters and DAG analogs, other proteins containing the activator-binding conserved C1 domain could also be secondary targets for these agents. Therefore, two different approaches were taken to confirm that PMA-induced downregulation of cyclin D1 is PKC-dependent.

First, we depleted phorbol ester responsive PKC isozymes (α , δ , and ϵ) from IEC-18 cells by treatment with 1 μ M PdBu for 24 h and PKC-depleted cells were subsequently treated with PMA for 2 h. WCL samples were immunoblotted for cyclin D1 (Figure 20). It has been established by our laboratory that long-term treatment of IEC-18 cells with PdBu results in complete disappearance of the PKC α , δ , and ϵ isozymes, without affecting the levels of PKC ζ or ι (Frey 2000b). This well-accepted approach for PKC depletion relies on the fact that administration of high concentrations of phorbol esters for prolonged periods promotes proteolytic degradation of PKC isozymes. This experimental approach is widely used to test whether the effect of a given PKC agonist is due to its action on targets other than PKC.

In a second approach, we pretreated the cells (30 min) with a general inhibitor of PKC activity, BIM I, to block PKC signaling in IEC-18 cells. PMA was then added for

2 h and WCL were immunoblotted for cyclin D1 protein (Figure 20). Both PKC-depletion and BIM I-pretreatment blocked PMA's ability to downregulate cyclin D1 protein, confirming that PMA-induced cyclin D1 loss is dependent on PKC activation (lanes 4 and 6). Interestingly, we also detected a marked induction of cyclin D1 in cells treated with PdBu and BIM I alone (lanes 3 and 5), suggesting that depletion of PKC protein or inhibition of endogenous PKC activity can lead to aberrant elevation of cyclin D1 expression.

Figure 20

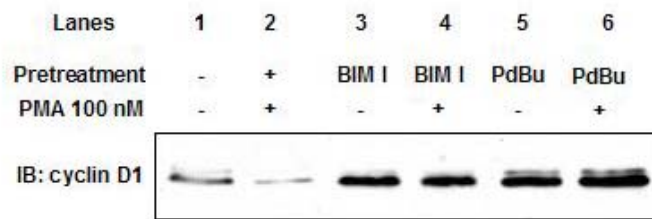


Figure 20 PMA-induced changes in cyclin D1 expression is PKC-dependent

Subconfluent cell populations were pretreated with either 1 μ M PdBu for 24 h or 5 μ M BIM I for 30 min before the addition of 100 nM PMA for 2 h. Cells were harvested in boiling 1% SDS lysis buffer. 30 μ g of WCL protein were analyzed for cyclin D1 by Western blotting. Data are representative of at least three independent experiments.

III.A.4. Inhibition of PKC α signaling blocks PMA-induced loss of cyclin D1

IEC-18 cells express three PKC isozymes that are responsive to the PKC agonists used in this study: PKC α , δ , and ϵ . Our previous studies demonstrated that PKC α -signaling is sufficient to promote cyclin D1 downregulation in this system (Frey et al., 2000a). In order to determine the requirement for PKC α in mediating PKC agonist-induced loss of cyclin D1, asynchronously growing cells were treated with the PKC α -selective inhibitor Gö6976. This inhibitor is specific for the classical PKCs; since PKC β I, β II, and γ are not expressed in IEC-18 cells, PKC α is the only isozyme that would be affected by Gö6976. Following pretreatment with Gö6976, cells were treated with PMA for the indicated times (Figure 21). Like BIM I, Gö6976 blocked PMA-induced downregulation of cyclin D1. Furthermore, Gö6976 alone also led to upregulation of cyclin D1 (Figure 21), pointing to a key role for PKC α -associated activity in control of cyclin D1 levels in IEC-18 cells.

Figure 21



Figure 21 The PKC α -selective inhibitor Gö6976 blocks PMA-induced loss of cyclin D1 Following a 30 minute-pretreatment with Gö6976, subconfluent cells were treated with PMA for 2 h. Control plates were treated with DMSO. Each condition was harvested in boiling 1% SDS lysis buffer, and 15 μ g of each WCL protein sample were immunoblotted for cyclin D1. Data are representative of six independent experiments. (Studies by Pysz, M.A. and Hizli, A.A.)

III.B. Analyses of cyclin D1 regulation in response to PKC/PKC α signaling

III.B.1 PKC/PKC α -mediated downregulation of cyclin D1 protein does not involve changes in cyclin D1 gene expression or stability of its mRNA

To determine whether the observed decrease in cyclin D1 protein levels in response to PKC activation is due to a transcriptional or post-transcriptional mode of regulation, parallel plates of control and PMA-treated cells were analyzed for cyclin D1 protein and message by Western and Northern blot analysis (Figure 21 (a) and (b), respectively). While a decrease in cyclin D1 protein levels could be detected as early as 30 min following activation of phorbol ester-responsive PKC isozymes (α , δ , and ϵ), no change was detected in cyclin D1 mRNA up to 2 h of PMA treatment, excluding alterations in transcription of the cyclin D1 gene or changes in cyclin D1 mRNA stability in PKC-induced loss of cyclin D1.

Figure 22

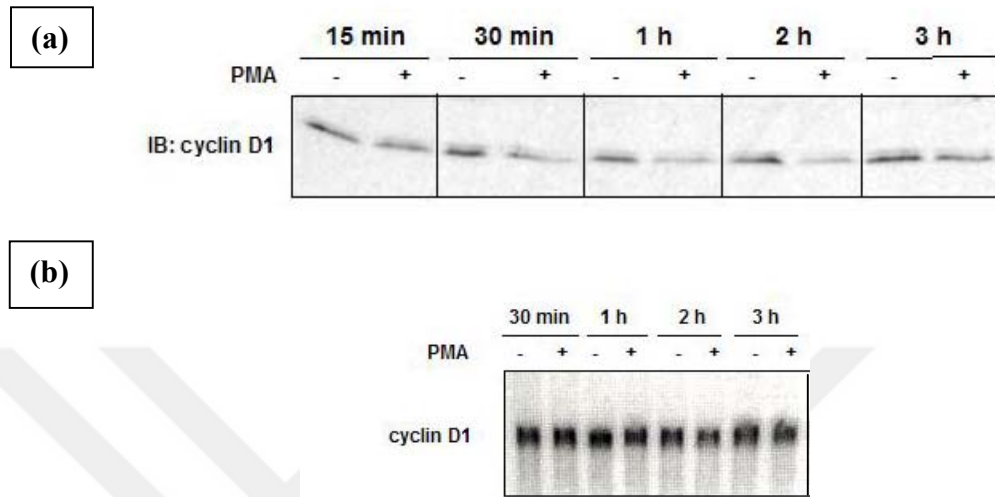


Figure 22 PKC-induced downregulation of cyclin D1 protein is not due to changes in regulation of cyclin D1 message (a) Subconfluent cell populations were treated with 100 nM PMA for 15 and 30 min, 1, 2, and 3 h. A control treated with ethanol was collected for each time point. 20 μ g of each protein sample were immunoblotted for cyclin D1 protein as described previously. **(b)** 10 μ g of each RNA sample was analyzed for cyclin D1 message by Northern blot analysis. Data are representative of at least five independent experiments.

III.B.2. PKC/PKC α activation does not alter the transport of cyclin D1 message

Although levels of cyclin D1 mRNA were not changed in response to PKC/PKC α activation, these data did not rule out the possibility of alterations in the transport of cyclin D1 mRNA. In fact, there have been reports in the literature showing that enhanced transport of cyclin D1 message resulted in higher cyclin D1 protein expression in NIH-3T3 cells overexpressing the translation initiation factor eIF4E (Rosenwald et al., 1995).

To test the possibility that PKC/PKC α signaling leads to inhibition of cyclin D1 mRNA transport out of the nucleus, the amount of D1 message in nuclear and cytosolic fractions of vehicle and PMA-treated cells was compared (Figure 23). No significant change (i.e. accumulation) in nuclear versus cytosolic cyclin D1 mRNA content was detected at any time point examined. These data indicated that the PMA-induced decrease in cyclin D1 protein could not be due to alterations in cyclin D1 mRNA nucleocytoplasmic transport.

Figure 23

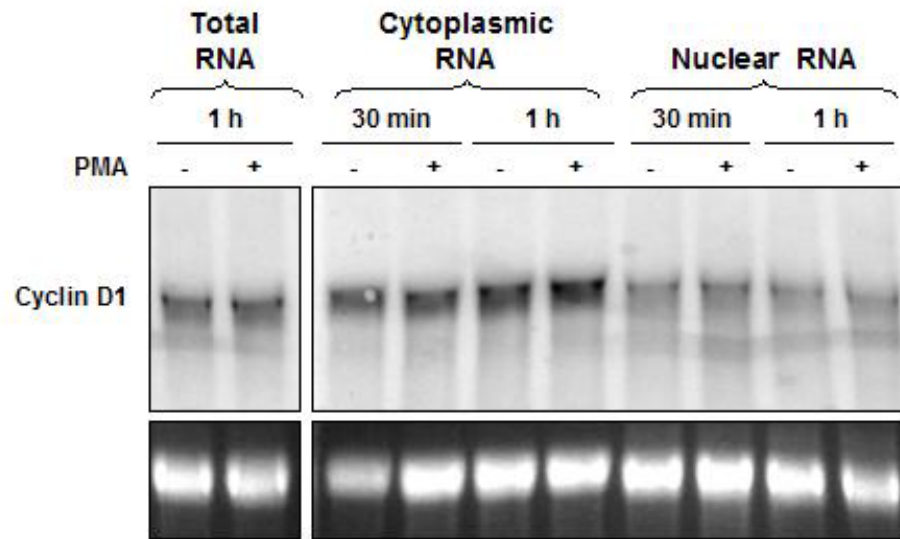


Figure 23 PKC signaling does not alter nucleocytoplasmic transport of cyclin D1

message Subconfluent cell populations were treated with 100 nM PMA for 30 min and 1 h. Cells were collected by trypsinization and lysed in cold lysis conditions. Cell lysates were partitioned and enriched for nuclear and cytosolic fractions. RNA isolated from each fraction was analyzed for the amount of cyclin D1 RNA by Northern blot analysis. Data are representative of three independent experiments.

III.B.3 PKC/PKC α signaling does not alter the stability of cyclin D1 protein

Pulse-chase analysis was performed to determine whether PKC activation induces changes in the stability of cyclin D1 protein (Figure 24). Cells were radioactively labeled with labeling medium containing [S^{35}]-Methionine/Cysteine protein labeling mix for 2 h, with the addition of PMA in the second hour. The cells were then returned to CGM and disappearance of the label incorporated into cyclin D1 protein was assayed by collecting cells at the indicated times. Although PMA-treated lysates displayed less signal incorporated into cyclin D1 compared with their corresponding controls, there was no difference in the rate of signal loss for cyclin D1 protein between PMA and control cells. Thus, PKC activation does not lead to altered stability of cyclin D1 protein in IEC-18 cells accumulating in G0.

Figure 24

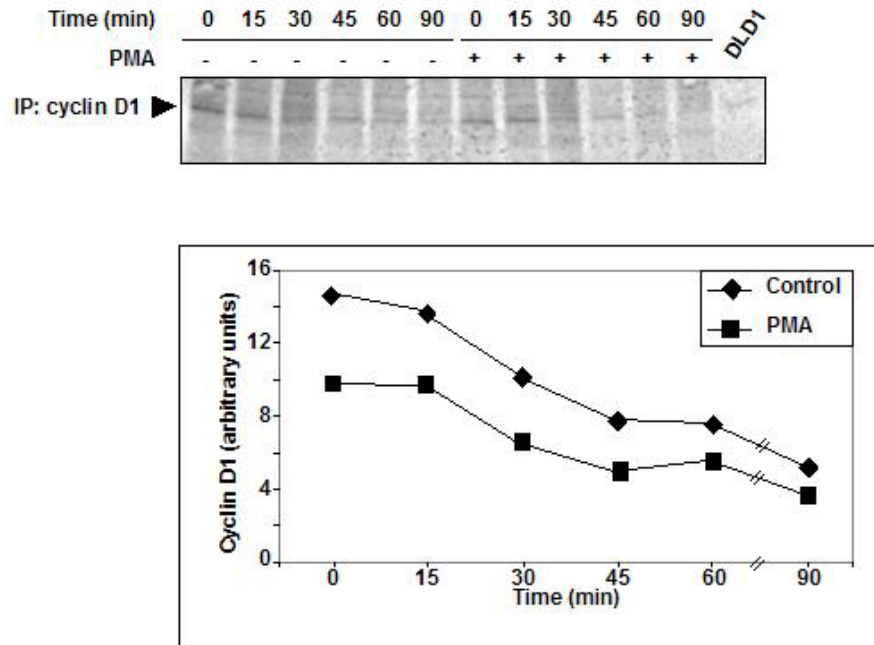


Figure 24 PKC-activation does not alter stability of cyclin D1 protein

Asynchronously growing subconfluent cells were incubated in labeling medium containing [S^{35}]-Methionine/cysteine for 1 h, and 100 nM PMA or vehicle was then added for another one-hour period. Chase was initiated by returning the cells to CGM without label for the indicated intervals (0, 15, 30, 45, 60, and 90 min). Cyclin D1 was immunoprecipitated from the cell lysates collected at each time point using a polyclonal rabbit anti-cyclin D1 antibody (SC-753) and the immunoprecipitates were resolved on 20% SDS-PAGE gels, transferred onto nitrocellulose membranes, and exposed to phosphorimager screens (Molecular Dynamics). Data are representative of 3 independent experiments. Data are representative of at least three independent experiments.

It should be mentioned here that pulse-chase analysis experiments for determination of cyclin D1 protein half-life in IEC-18 cells were technically difficult because (a) these cells express cyclin D1 at very low levels (note the comparison of cyclin D1 levels in IEC-18 cells with those in human and rodent colon carcinoma cell lines in Figure 60), and (b) cyclin D1 steady state levels are rapidly reduced by methionine/cysteine-starvation (Figure 25). This may reflect the high percentage of methionines and cysteines in rat cyclin D1 (12 methionines and 14 cysteines out of 295 residues, giving a total of 8.8% methionine+cysteine content) as well as the sensitivity of both translation initiation (via hypophosphorylation of 4E-BP1) and elongation (through increased phosphorylation of eEF2) to amino acid starvation [a well known cellular response to spare energy from biosynthetic pathways under sub-optimal growth conditions (Proud 2002; Richardson et al., 2004)]. Therefore, to confirm our findings, we addressed the involvement of known pathways of cyclin D1 proteolysis including GSK-3 β -mediated phosphorylation of Thr286 and “destruction-box”-mediated degradation of the protein.

Figure 25

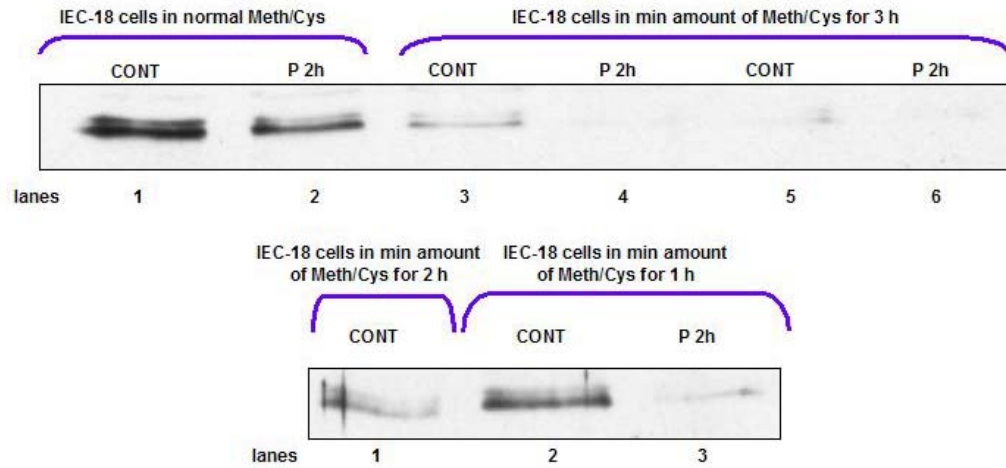


Figure 25 Adverse effects of prolonged methionine/cysteine starvation on cyclin D1 steady state levels in IEC-18 cells Asynchronously growing cells were incubated in CGM with (*upper panel* lanes 1 and 2) or without Methionine/Cysteine (**Meth/Cys**) for the indicated times. Lanes 3-6 in the *upper panel* show samples from cells which were Meth/Cys-starved for 1 h, mock Meth/Cys-labeled for 2 h with the addition of vehicle or PMA during the final hour, and chased with CGM containing vehicle or PMA, making the total incubation period under Met/Cys-free conditions 3 h. Note that vehicle or PMA was not included in the chase in the case of samples presented in lanes 5 and 6. In the *lower panel*, lane 1 shows a control sample from cells which were subjected to the same order of manipulations as described above except for the omission of the Meth/Cys-starvation step, making the total incubation period under Met/Cys-free conditions 2 h and recapitulating the conditions used for the pulse-chase analysis (Figure 24). Lanes 2 and 3 show cells which were directly mock-labeled for 1 h, followed by chase and vehicle/PMA treatment for 2 h before harvest. Note that the three scenarios depict levels of cyclin D1 at 2 h of PMA treatment assayed in response to varying periods of Meth/Cys-starvation.

III.B.4a. Addressing the possible involvement of GSK-3 β in PKC/PKC α -induced downregulation of cyclin D1

As discussed in the Introduction, Diehl and colleagues (1997 and 1998) determined that when a C-terminal site, Thr 286, becomes phosphorylated by GSK-3 β , cyclin D1 is primed for polyubiquitination and proteasomal degradation. Mutation of this site to alanine yielded a degradation mutant that failed to be polyubiquitinated and displayed an unusually prolonged half-life (3.5 hrs compared with 30 min of the wild type). A number of studies subsequently reported involvement of GSK-3 β -dependent turnover of cyclin D1 in response to differentiation or apoptotic signals (Spinella et al., 1999 and Shao et al., 2000).

Conversely, several mitogenic stimuli have been shown to promote the inhibition of the GSK-3 β . Inhibition of GSK-3 β by mitogenic signals that activate the PI3K/Akt pathway is achieved by direct phosphorylation of GSK-3 β at the Ser9 residue by Akt *in vivo* (Cross et al., 1995 and see Figure 27). As shown in Figure 26, analysis of the effects of PKC activation on Akt signaling in IEC-18 cells demonstrated rapid loss of phospho-Ser473-Akt, indicative of Akt inactivation. Based on these findings, we next used several approaches to test the hypothesis that PKC α -induced loss of Akt activity promotes activation of GSK-3 β and Thr286-phosphorylation of cyclin D1, resulting in increased turnover of the protein in IEC-18 cells.

Figure 26

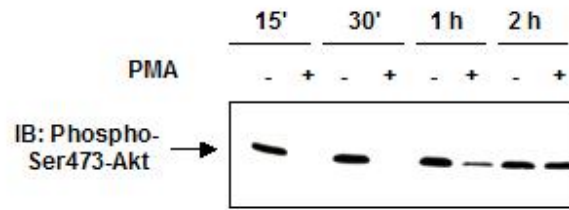


Figure 26 Rapid and transient loss of Akt signaling upon PKC activation in IEC-18 cells Subconfluent cell populations were treated with 100 nM PMA for 15 and 30 min, 1 and 2 h. A control treated with ethanol was collected for each time point. 20 μ g of each protein sample were immunoblotted for phospho-Ser473-Akt with a polyclonal antibody. Data are representative of at least five independent experiments. (Studies by Frey, M.R., Hizli, A.A., and Guan, L.J.)

Figure 27

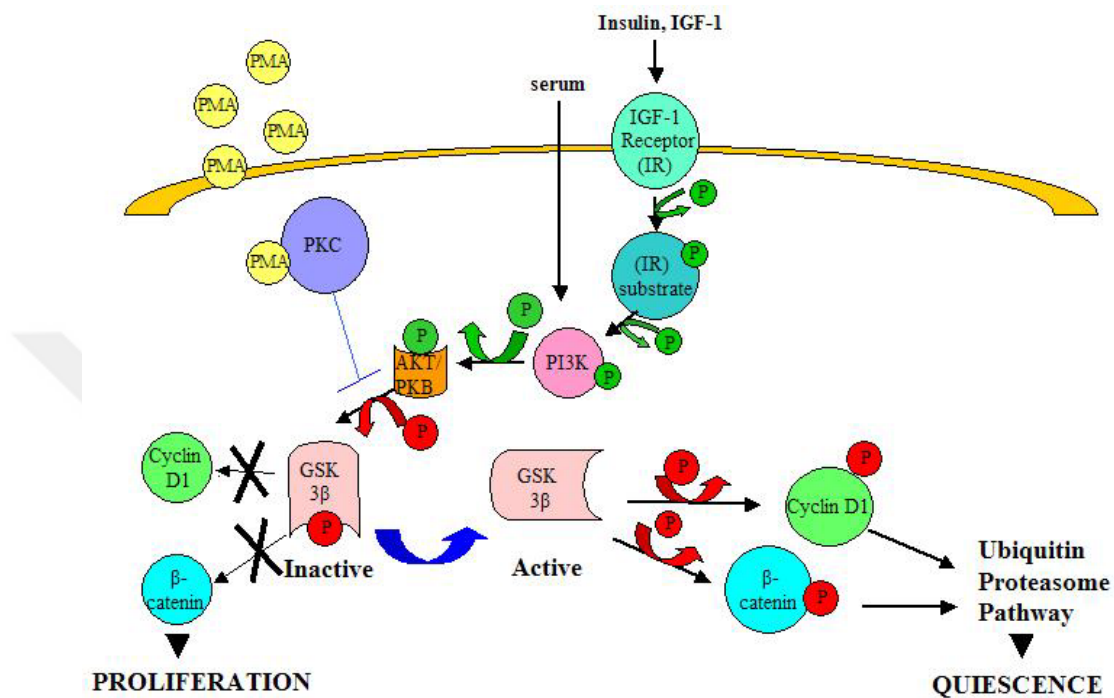


Figure 27 Crosstalk between PKC and PI3K/Akt/GSK-3β signaling pathways

Schematic representation of the hypothesis that PKC α -mediated attenuation of Akt signaling could lead to activation of GSK-3 β to account for enhanced turnover of cyclin D1. Another substrate of GSK-3 β , β -catenin, which also becomes ubiquitinated and degraded as result of GSK-3 β -mediated phosphorylation, is also depicted in this illustration.

III.B.4a.I. Inhibition of GSK-3 β activity does not block PKC α -mediated loss of cyclin D1

First, we used the GSK-3 β inhibitor, Li⁺, to address the question whether blockade of GSK-3 β activity could abolish PMA-induced downregulation of cyclin D1 (Stambolic et al., 1996). As can be seen in Figure 28, Li⁺ did not inhibit PMA-induced loss of cyclin D1, providing the first line of evidence that PKC/PKC α -induced loss of cyclin D1 occurs via GSK-3 β -independent mechanisms. Nonetheless, an accumulation of cyclin D1 was noted in samples treated with Li⁺ alone, pointing to a role for GSK-3 β in normal turnover of cyclin D1 in IEC-18 cells.

Figure 28

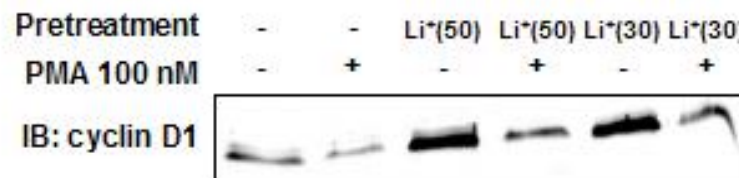


Figure 28 Inhibition of GSK-3 β by Li⁺ does not prevent PKC-induced loss of cyclin D1 Subconfluent cell populations were treated with Li⁺ overnight or for 3 h (data not shown). 100 nM PMA was then added for 2 h, and cells were harvested in boiling 1% SDS lysis buffer. 30 μ g of each protein lysate were immunoblotted for cyclin D1 as described previously. Data are representative of at least three independent experiments.

III.B.4a.II. PKC/PKC α activation does not lead to changes in GSK-3 β phosphorylation on Ser9

Next, we analyzed samples from early time points of PMA treatment using phospho-specific antibodies for GSK-3 β . There are two commercially available phospho-specific antibodies that can recognize phosphorylated GSK-3 β at the Ser9 position (see Appendix C). Both of these antibodies failed to detect a decrease in the amount of phospho-Ser9-GSK-3 β in response to PMA, indicating that, despite its ability to attenuate Akt activity, PKC activation does not lead to changes in the activity of GSK-3 β in IEC-18 cells (Figure 29 and see Appendix C). As we discuss in Appendix C, the inability of loss of Akt signaling to promote activation of GSK-3 β may reflect differences in the molecular background of IEC-18 cells compared with other cell lines.

Figure 29

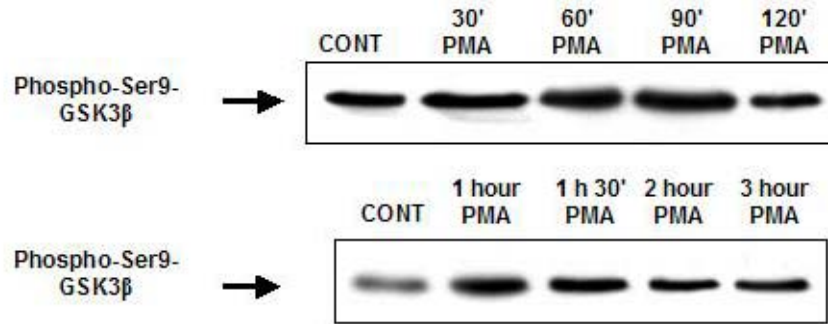


Figure 29 PKC activation does not decrease the levels of inhibitory phosphorylation of GSK-3 β Subconfluent cell populations were treated with 100 nM PMA for the indicated times. Cells were harvested in boiling 1 % SDS lysis buffer. 20 μ g of each protein sample were immunoblotted for phospho-Ser9-GSK-3 β . Blots presented in the upper and lower panels are representative of two independent experiments.

III.B.4a.III. PMA downregulates a GSK-3 β non-phosphorylatable cyclin D1 mutant

Third, we transiently transfected flag-tagged-*wt* or -T286A mutant cyclin D1 constructs into IEC-18 cells to test the ability of 100 nM PMA to downregulate a cyclin D1 mutant which cannot be phosphorylated by GSK-3 β (Figure 30a). At 2 h of PMA treatment, there was a clear decrease in the band intensity for the exogenously expressed flag-tagged-T286A mutant, which typically migrated just above the band for endogenous cyclin D1 due to the 12-amino acid-long flag motif (Figure 31a). We confirmed the identity of this slower migrating band by immunoprecipitating exogenously expressed cyclin D1 using flag-specific M2 monoclonal antibody conjugated to agarose beads and subjecting the immunoprecipitates to Western blot analysis for cyclin D1 (Figure 30a and b). It should be noted that only 500 ng of the T286A cyclin D1 mutant (1/10 of the amount of the *wt* construct) was used in transfections in order to visualize PMA-induced downregulation clearly. When higher amounts were used, the defective turnover the T286A mutant led to such high levels of accumulation of this construct that PMA-induced decreases could no longer be detected within the saturation limits of chemiluminescence (data not shown).

Figure 30

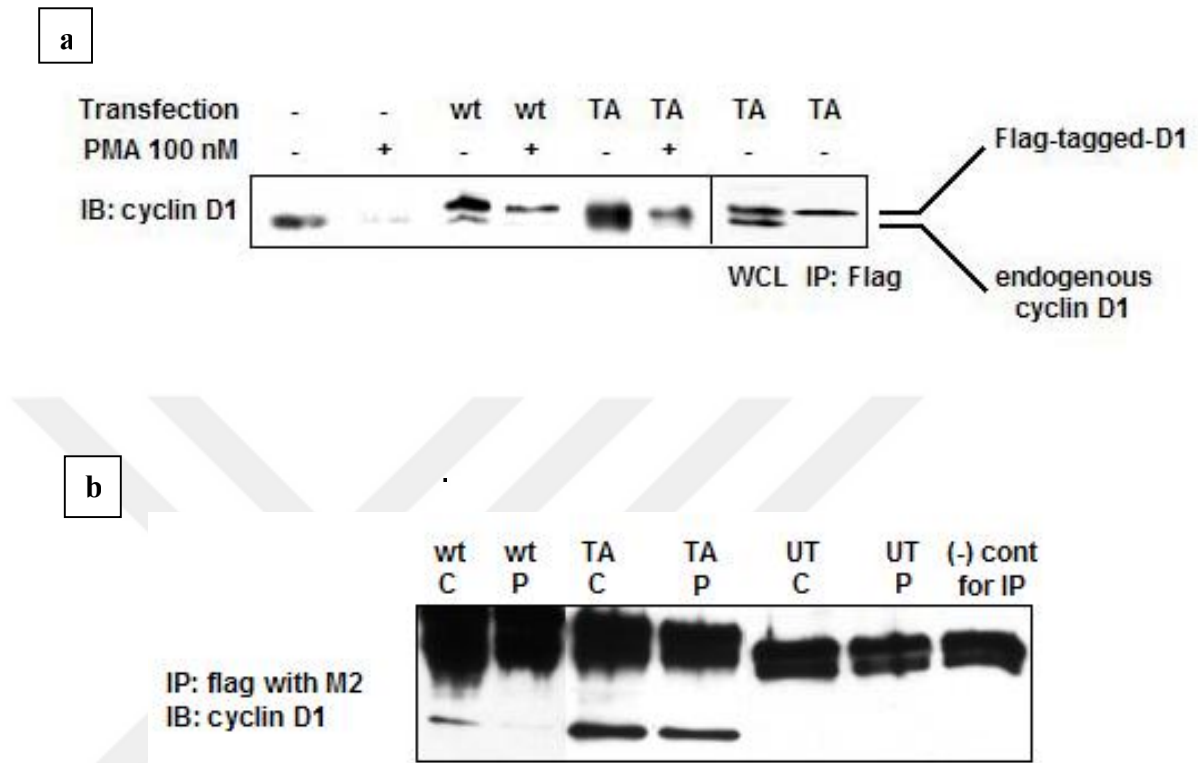


Figure 30 PMA downregulates a Thr286A cyclin D1 mutant in IEC-18 cells (a) Cells were transiently transfected with *wt* or flag-tagged-T286A cyclin D1 mutant (TA). Following treatment of transfectants with 100 nM PMA (P) for 2 h, cells were harvested in boiling 1% SDS lysis buffer, and lysates were subjected to anti-cyclin D1 immunoblotting **(b)** Cells transfected with *wt* or TA cyclin D1 mutant and untransfected controls (UT) were treated with 100 nM PMA for 2 h. Cells were harvested in 1 % NP-40 cold lysis buffer in the presence of protease and phosphatase inhibitor cocktails. Exogenously expressed cyclin D1 constructs were immunoprecipitated with agarose-conjugated M2-monoclonal antibody and immunocomplexes were analyzed for cyclin D1 in Western blots. Data are representative of at least three independent experiments.

Based on the findings that (a) GSK-3 β inhibition with Li⁺ failed to block PKC-induced loss of cyclin D1 (Figure 28), (b) PMA did not lead to activation of GSK-3 β (Figure 29), and (c) PKC activation was able to downregulate the T286A cyclin D1 mutant (Figure 30a and b), we ruled out a GSK-3 β -dependent mechanism in PKC-mediated loss of cyclin D1. These results were, therefore, consistent with data from pulse-chase experiments showing no change in cyclin D1 protein stability.

III.B.4b. PKC/PKC α -mediated loss of cyclin D1 does not involve engagement of “destruction-box” motif-mediated degradation pathways

For full confirmation of the cyclin D1 stability experiments, we tested the involvement of the N-terminal, “destruction-box” motif which was shown to mediate proteolysis of cyclin D1 in response to genotoxic stress (Bernards and Agami, 2000). Flag-tagged-R29Q and L32A cyclin D1 destruction-box mutant constructs (Figure 31) were transiently transfected into IEC-18 cells. Transfectants were treated with 100 nM PMA for 2 h and harvested in boiling 1% SDS lysis buffer. Like the T286A mutant, the flag-tagged destruction-box mutants also migrated just above the endogenous cyclin D1 in 20 % SDS-PAGE gels, and a clear downregulation of both of mutants was observed with PMA treatment (Figure 32a). We confirmed these results by harvesting transfected cells in cold lysis buffer, immunoprecipitating exogenous cyclin D1 protein with anti-Flag M2-conjugated agarose beads, and immunoblotting the immunoprecipitates for cyclin D1 (Figure 32b).

Figure 31

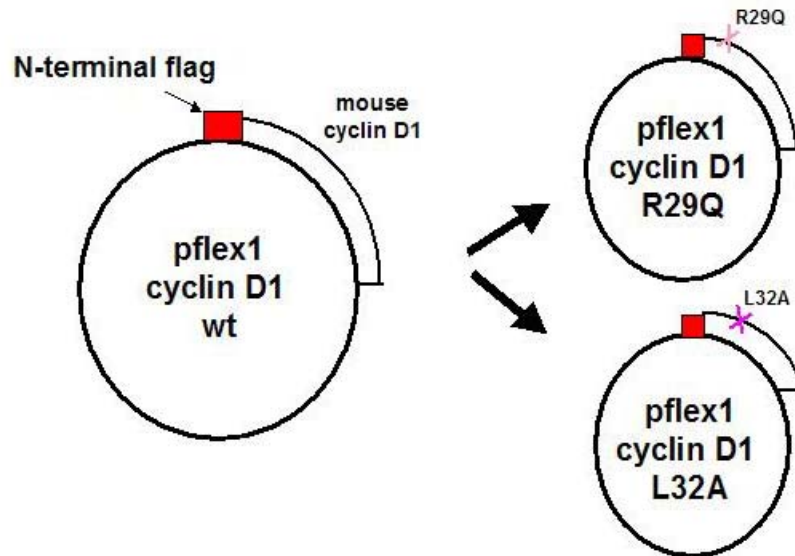


Figure 31 Generation of the cyclin D1 “destruction-box”-mutants Using the Stratagene Quickchange site mutagenesis kit, we performed single mutations in the “destruction-box” motif of *wt* mouse cyclin D1 in the flag-tagged-cyclin D1 (*wt*) construct (from Dr. Sherr) to generate flag-tagged-R29Q and flag-tagged-L32A cyclin D1 mutants.

Figure 32

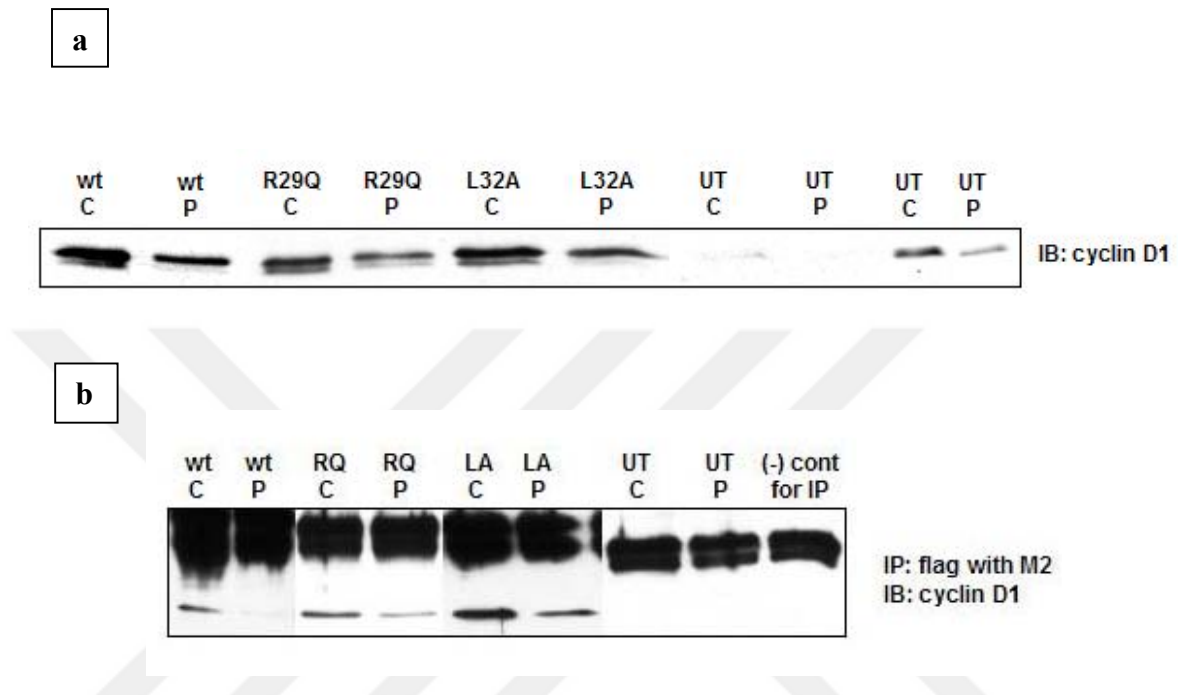


Figure 32 PMA downregulates the “destruction-box” cyclin D1 mutants (a) Cells were transiently transfected with flag-tagged-*wt*, R29Q (RQ), or L32A (LA) cyclin D1 constructs as described above. Transfectants were treated with 100 nM PMA for 2 h and samples were subjected to anti-cyclin D1 immunoblotting. UT: Untransfected cells **(b)** Cells were transiently transfected with flag-tagged-*wt*, RQ, or LA cyclin D1 constructs as described above, and treated with 100 nM PMA for 2 h. Cells were harvested in 1 % NP-40-cold lysis buffer in the presence of protease and phosphatase inhibitor cocktails. The exogenously expressed cyclin D1 constructs were immunoprecipitated with anti-flag M2-monoclonal antibody conjugated to agarose beads. Immunocomplexes were analyzed for cyclin D1 in Western blots. Data are representative of at least three independent experiments.

Together these lines of evidence confirmed data from pulse-chase analysis and led to the conclusion that PKC-induced G1/G0 growth arrest in IEC-18 intestinal crypt cell model does not involve loss of cyclin D1 protein via alterations in protein turnover.

III.C. Analyses of regulation of cyclin D1 translation in response to PKC/PKC α signaling

III.C.1 PKC/PKC α activation leads to inhibition of cyclin D1 synthesis

To address the impact of PKC signaling on synthesis of cyclin D1 protein, we performed [S^{35}]-methionine biosynthetic labeling experiments. As shown in Figure 34, levels of radiolabeled cyclin D1 protein were significantly lower in PMA-treated samples at each time point examined, indicating that PKC signaling impacts cyclin D1 translation IEC-18 cells.

Figure 33

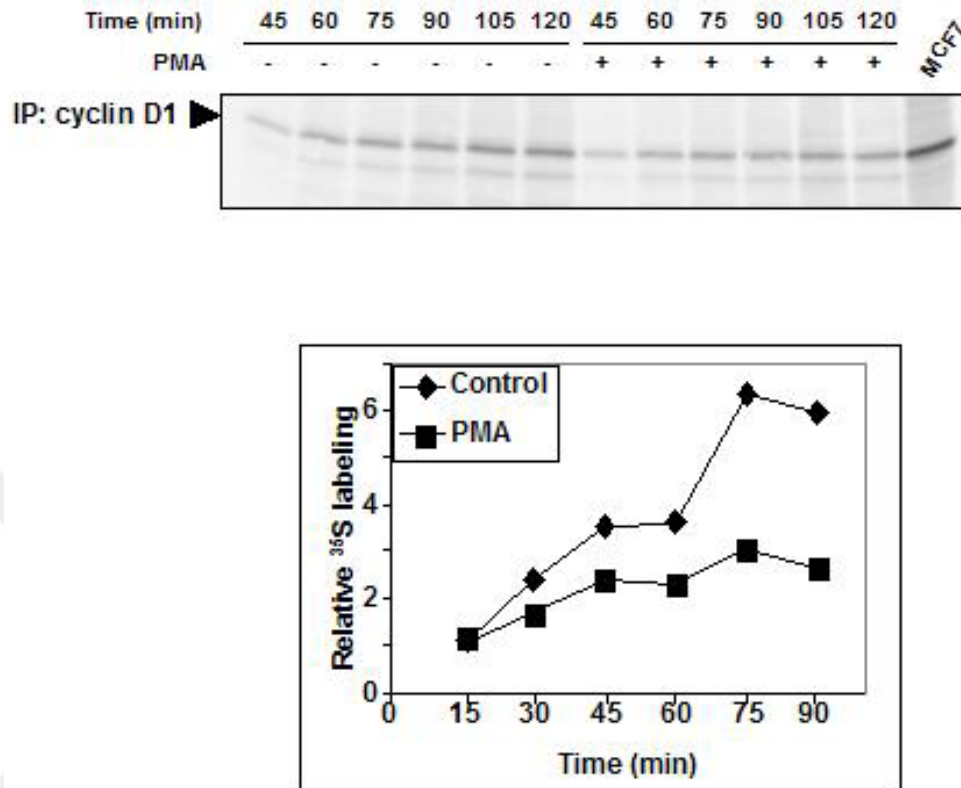


Figure 33 PKC signaling suppresses cyclin D1 synthesis in IEC-18 cells

Asynchronously growing cell populations were incubated in Meth/Cys-free starvation medium containing either 100 nM PMA or ethanol (the vehicle) for 30 min. Cells were then incubated in labeling medium [starvation medium containing [³⁵S]-Meth/Cys Protein labeling mix (0.38 mCi/plate) with 100 nM PMA or ethanol] for the indicated times. A mouse monoclonal anti-cyclin D1 antibody (sc-450) was used to immunoprecipitate cyclin D1 from the cell lysates collected at each time point. Note that the polyclonal anti-cyclin D1 antibody, sc-753 (H295), was used for the immunoprecipitation of cyclin D1 from MCF-7 cells. Immunoprecipitates were resolved on a 20% SDS-PAGE gel and transferred onto PVDF membrane, that was subsequently exposed to phosphorimager screens. The signal from cyclin D1 protein in each condition was quantified and graphed using ImageQuant software. Data are representative of three independent experiments.

III.C.2 PKC/PKC α -activation leads to major changes in the translation machinery

In the following sections, we describe changes induced in key factors of the translation machinery in response to PKC/PKC α activation and address whether these events could account for the observed decrease in cyclin D1 protein levels in IEC-18 cells. First, we describe PKC/PKC α -induced changes in key regulators of cap-dependent translation initiation, i.e., eIF4E, and its inhibitory binding protein 4E-BP1. Second, we report the effects of PKC-signaling on control of eIF2 α , another key regulator of translation initiation (Clemens, 2004; Brewer et al. 1996, Campo et al., 2002). Finally, we demonstrate the ability of PKC-signaling to promote rapid and transient inhibition of eEF2 through increased phosphorylation at Thr56.

III.C.2.1 PKC/PKC α signaling leads to inhibition of cap-dependent translation initiation

The tight correlation between expression of eIF4E and levels of cyclin D1 protein is an extensively studied aspect of cyclin D1 regulation. Thus, we first addressed possible alterations in the function of eIF4E in response to PKC/PKC α activation. Based on current understanding of eIF4E regulation, we hypothesized that PKC/PKC α activation could (a) lead to a decrease in the levels of eIF4E, (b) alter eIF4E phosphorylation at Ser209, or (c) alter the phosphorylation status of 4E-BP1 to increase its binding to eIF4E.

III.C.2.2 Impact of PKC/PKC α -activation on levels of eIF4E

To address possible PKC-induced changes in the levels of eIF4E, we subjected PMA-treated samples to anti-eIF4E immunoblotting using a rabbit polyclonal anti-eIF4E antibody (Figure 34). Based on this Western blot analysis, we concluded that levels of eIF4E remain essentially unchanged during PKC activation in IEC-18 cells.

Figure 34

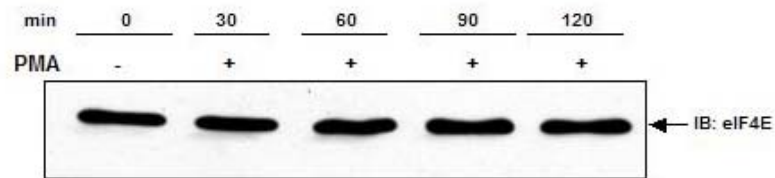


Figure 34 Effect of PKC signaling on levels of eIF4E Subconfluent cell populations were treated with 100 nM PMA for the indicated times. Cells were harvested in boiling 1 % SDS lysis buffer. 20 μ g of each lysate were immunoblotted for eIF4E using a polyclonal antibody.

III.C.2.3 PKC/PKC α -activation leads to rapid, strong and sustained phosphorylation of eIF4E

Analysis of the changes in Ser209 phosphorylation of eIF4E in response to activation of PKC revealed a marked increase in the amount of phospho-Ser209-eIF4E both early and late in PMA treatment (Figure 35a and b). Phosphorylation of eIF4E at Ser209 occurs rapidly in response to PKC activation and is sustained for up to 10 h (Figure 35b). Furthermore, pre-treatment of cells with inhibitors of the MAP Kinase pathway, (e.g., UO126) prior to the addition of PMA, blocked PMA-induced phosphorylation of eIF4E, indicating that this event is MAP Kinase pathway-dependent as shown in other systems (Figure 36).

Figure 35

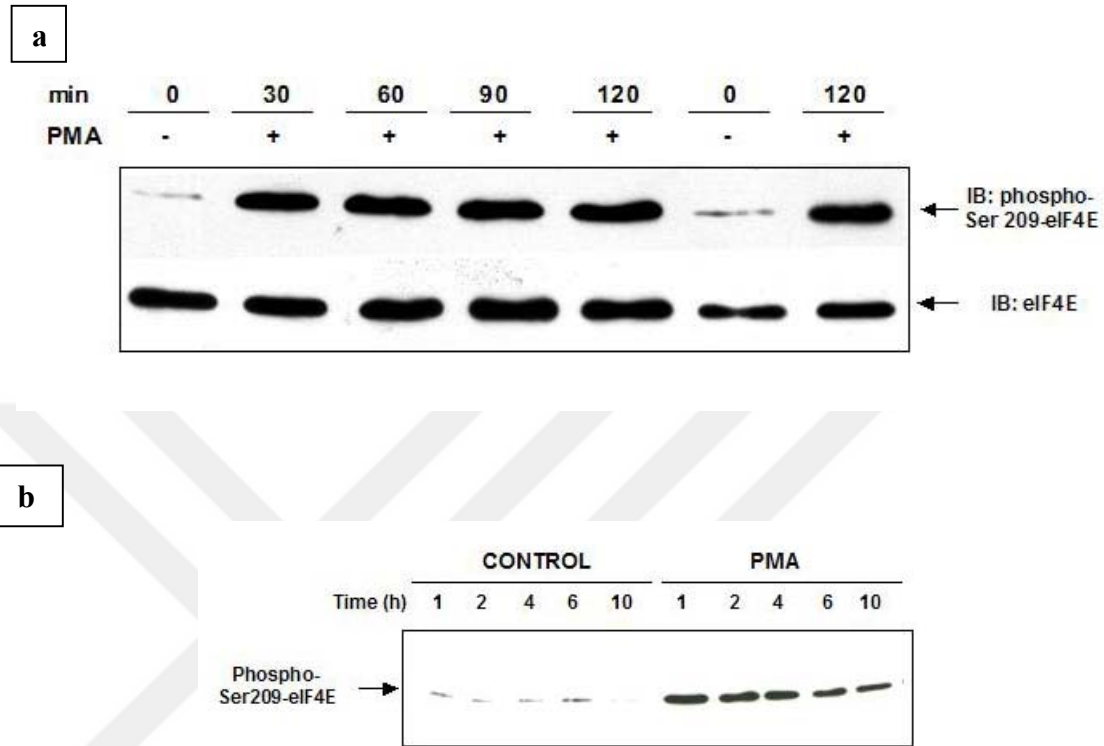


Figure 35 Impact of PKC activation on eIF4E phosphorylation at Ser209

(a) Subconfluent cell populations were treated with 100 nM PMA for 30, 60, 90, and 120 min. Cells were harvested in boiling 1% SDS lysis buffer and 30 and 20 μ g of each sample were subjected to immunoblotting for phospho-Ser209-eIEF4E and for total-eIF4E, respectively. Also shown are control and PMA-treated (2 h) samples from another experiment **(b)** Subconfluent cell populations were treated with 100 nM PMA or ethanol (control) for the indicated times. Cells were harvested in boiling 1 % SDS lysis buffer. 15 μ g of each sample were immunoblotted for phospho-Ser209-eIEF4E. Data are representative of at least five independent experiments.

Figure 36



Figure 36 PKC-induced phosphorylation of Ser209 on eIF4E is Erk/MAPK-dependent Subconfluent cell populations were pretreated with the MAPK-inhibitor UO126 (10 μ M) for 30 min prior to the addition of 100 nM PMA for 30 min. Cells were harvested and subjected to phospho-Ser209-eIF4E (30 μ g) and total-eIF4E (20 μ g) immunoblotting (Studies done by Clark, J.A. and Hizli, A.A.).

III.C.2.4 PKC/PKC α activation leads to rapid and sustained activation 4E-BP1

Using an antibody that recognizes the three characteristic phosphoforms of 4E-BP1 (α , β , and γ forms), we demonstrated that PMA induces rapid hypophosphorylation, i.e. activation, of this factor (Figure 37). Accumulation of hypophosphorylated forms of 4E-BP1 was detected as early as 15 min following addition of PMA and remained evident for up to 3-4 h. Among the three phosphoforms characterized in murine cells, the α and β forms, which are known to have full and semi-affinity for eIF4E respectively, were upregulated upon PMA addition, whereas the γ form, which has no affinity for eIF4E (Gingras et al., 1999a; Gingras et al., 2001), remained unchanged or, in some experiments, decreased to a small extent.

Figure 37

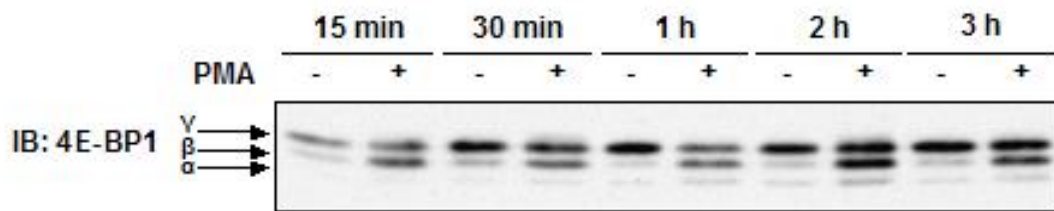


Figure 37 PKC activation promotes hypophosphorylation of 4E-BP1 Subconfluent cell populations were treated with 100 nM PMA or ethanol for the indicated times. 10 μ g of cell extracts were immunoblotted for total 4E-BP1. Positions of the α , β , and the γ phosphoforms, which have high, semi, and no affinity for eIF4E, are indicated with arrows. Data are representative of at least fifteen independent experiments.

III.C.2.5 Activation of 4E-BP1 is PKC α -dependent

To test whether PMA-induced activation of 4E-BP1 is dependent on PKC signaling, we depleted PKCs from IEC-18 cells by a 24-hour exposure to 1 μ M PdBu. PKC α , δ , ϵ -depletion abrogated PMA's ability to produce hypophosphorylated 4E-BP1, indicating that the PMA-induced activation of 4E-BP1 is PKC signaling-dependent (Figure 38).

Figure 38

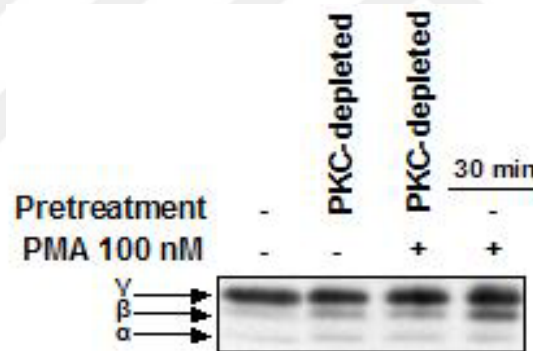


Figure 38 PMA-induced activation of 4E-BP1 is PKC signaling-dependent

Subconfluent cells were incubated with 1 μ M PdBu for 24 h to deplete PKC α , δ , and ϵ . 100 nM PMA was added for 30 min, cells were harvested in boiling 1% SDS lysis buffer, and lysates (10 μ g) were analyzed for total 4E-BP1 by Western blotting. Data are representative of three independent experiments.

Next, we also tested the ability of other PKC-agonists (presented in Figure 18) to hypophosphorylate 4E-BP1, to determine if the decline in cyclin D1 produced by these agents could also be mediated by the activation of this factor. As shown in Figure 40, PdBu, Bryo, and DiC₈ are all able to induce 4E-BP1 hypophosphorylation in IEC-18 cells (Figure 39)

Figure 39

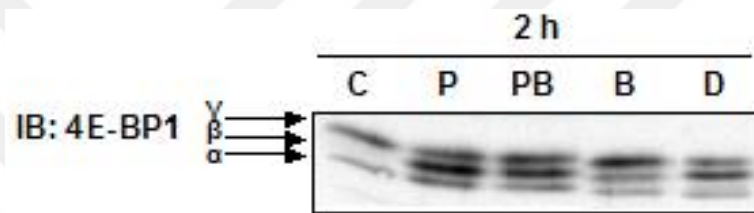


Figure 39 Other PKC-agonists can induce activation of 4E-BP1 Subconfluent populations of IEC-18 cells were treated with 100 nM PMA (P), 1 μ M PdBu (PB), 100 nM Bryo (B), or 20 μ g/ml DiC₈ (D) for 2 h. 10 μ g WCL protein samples were analyzed for 4E-BP1 in Western blots. Data are representative of four independent experiments.

We have previously demonstrated that IEC-18 cells depleted of PKC δ and ϵ , thus expressing PKC α as the only phorbol-ester-responsive isozyme, can downregulate cyclin D1 upon treatment with PMA (Frey et al., 2000a). We, therefore, predicted that PKC α signaling accounts for the modulation of 4E-BP1 by PMA. As shown in Figure 41, activation of PKC α in PKC δ/ϵ -depleted cells with PMA resulted in hypophosphorylation of 4E-BP1 by 15 min, indicating that PKC α alone can lead to activation of 4E-BP1 in this system (Figure 40).

Figure 40

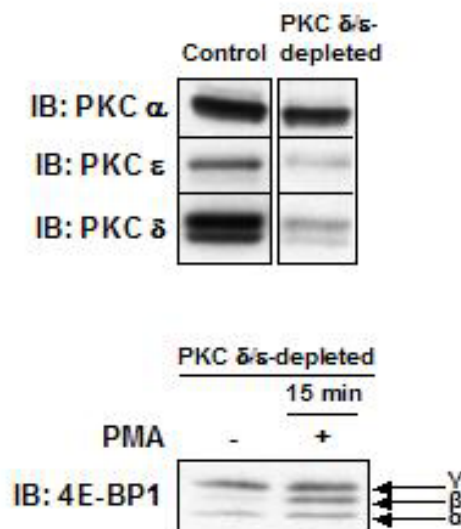


Figure 40 PMA can dephosphorylate 4E-BP1 in PKC δ/ϵ -depleted cells IEC-18 cells were pulse-treated with 10 nM PMA for 15 min and then returned to CGM for 24 h to deplete PKC δ and ϵ (Frey et al., 2000a). PMA (100 nM) was then added to activate PKC α , and cells were harvested after 15 min of treatment. Samples (10 μ g) were analyzed for PKC α , δ , and ϵ , in 10% SDS-PAGE gels (*upper panel*) and for 4E-BP1 in 20% SDS-PAGE gels (*lower panel*). Data are representative of at least two independent experiments.

To confirm the role of PKC α in modulating the phosphorylation of 4E-BP1, we tested the ability of the PKC α -selective inhibitor Gö6976 to block PMA-induced hypophosphorylation of 4E-BP1. Although Gö6976 produced a modest increase in the β -phosphoform of 4E-BP1, the inhibitor clearly decreased the amount of α and β -phosphoforms produced by the PMA in IEC-18 cells (Figure 41). Thus, PKC α signaling appears to be a major regulator of 4E-BP1 phosphorylation/activity in IEC-18 cells.

Figure 41

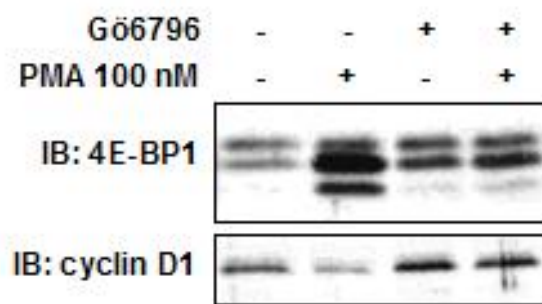


Figure 41 The PKC α selective inhibitor Gö6976 blocks PMA-induced dephosphorylation of 4E-BP1 Following a 30 min-pretreatment with Gö 6976, subconfluent cells were treated with PMA for 30 min. Control plates were treated with vehicle (DMSO). Each condition was harvested in boiling 1% SDS lysis buffer, and WCL protein samples were immunoblotted for 4E-BP1 (10 μ g) or for cyclin D1 (15 μ g). Data are representative of six independent experiments.

III.C.2.6 Increased association of 4E-BP1 with eIF4E upon PKC/PKC α activation

To determine the consequences of increases in pools of hypophosphorylated 4EBP-1 on the function of eIF4E, we conducted a cap-binding assay where eIF4E and associated factors were pulled-down by a cap analogue conjugated to sepharose beads (7-Methyl GTP-Sepharose 4B) (Figure 42). A striking increase in levels of cap-associated α and β 4EBP-1 phosphoforms was observed following PMA treatment, and this elevation was comparable to that seen in serum-starved positive control cells, demonstrating that PKC activation promotes profound inhibition of cap-dependent translation in IEC-18 cells.

Figure 42

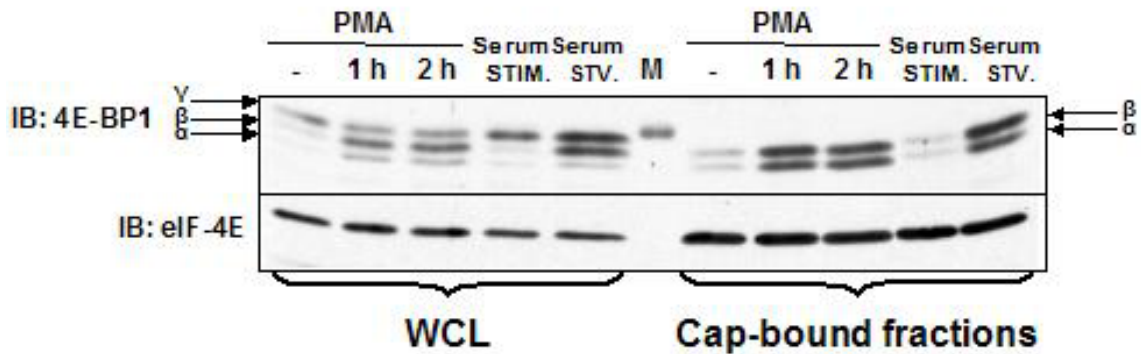


Figure 42 PKC activation results in increased association of 4E-BP1 with eIF4E

Subconfluent cells were treated with 100 nM PMA for 1 or 2 h, stimulated with 10% serum for 10 min, or serum-starved for 22 h before being harvested under cold lysis conditions. 350-400 μ g of protein lysate were incubated with the cap for 16 h. The pull-downs were resolved on 20% SDS-PAGE gels and immunoblotted for eIF4E and 4EBP-1, along with their respective WCL. M: molecular weight markers; Serum STIM.: serum stimulated; Serum STV.: serum-starved. Note that, in agreement with its inability to bind eIF4E, the γ -phosphoform of 4E-BP1, which is clearly seen in WCL samples, is not detected in the cap-bound fractions. This observation confirms the specificity of this assay for detecting eIF4E-associated factors. Data are representative of at least five independent experiments.

III.C.2.7 PKC α -activation leads to acute sequestration of cyclin D1 mRNA in 4E-BP1-associated complexes

An **IP-RT-PCR** assay was performed to determine if PKC activation leads to the accumulation of cyclin D1 mRNA in 4E-BP1-containing complexes (Figure 43 (a) and (b)). Vehicle and PMA- treated cells were collected in detergent-free lysis buffer and anti-4E-BP1 antibody was used to immunoprecipitate 4E-BP1-associated complexes. Immunocomplexes were partitioned for protein and mRNA analysis. Serum-stimulated and serum-starved conditions were included as controls for binding of 4E-BP1 to eIF4E. At 30 min of PMA treatment, we detected a substantial accumulation of cyclin D1 mRNA in 4E-BP1-associated complexes and elevated levels were sustained at 1 h and 2 h (albeit to a lesser extent) (Figure 43b). A similar accumulation of cyclin D1 mRNA was seen under conditions of serum-starvation, while levels of cyclin D1 mRNA associated with 4E-BP1 complexes in serum-stimulated samples were essentially the same as in vehicle-treated control cells. These results support the conclusion that activation of PKC leads to inhibition of cap-dependent translation in IEC-18 cells, and sequestration of cyclin D1 mRNA in 4E-BP1 complexes could account for the observed decrease in synthesis of cyclin D1.

Figure 43

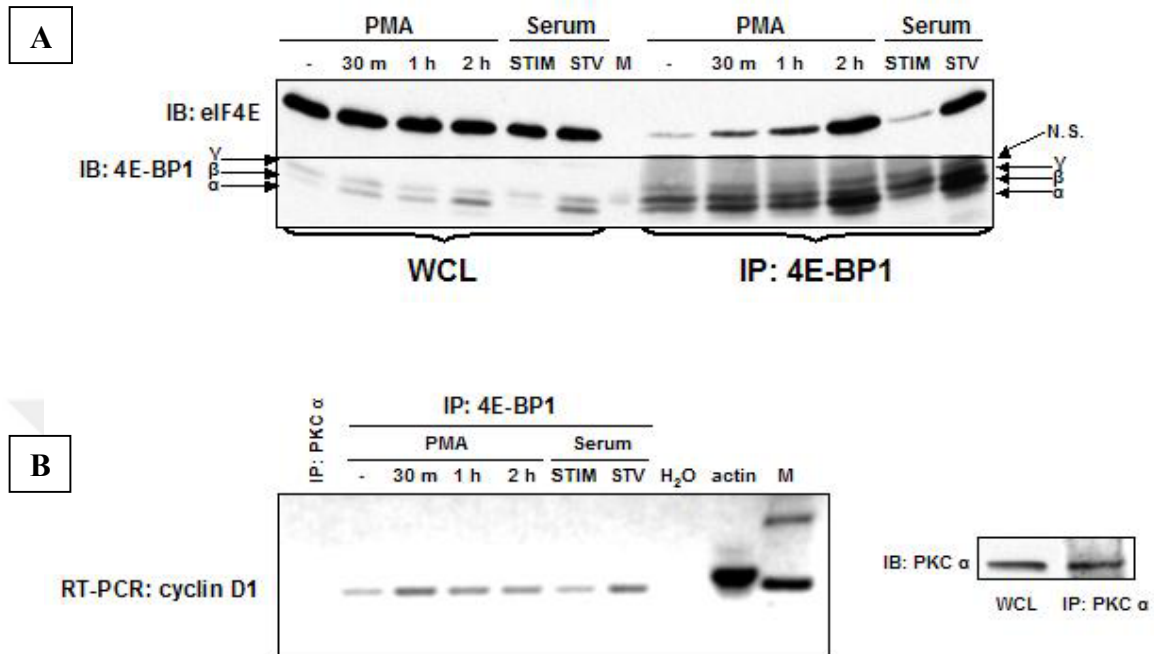


Figure 43 Activation of PKC leads to sequestration of cyclin D1 mRNA in 4E-BP1-associated complexes (a) Subconfluent cells were treated with 100 nM PMA for the indicated times, stimulated with serum for 10 min, or serum-starved for 22 h. Control plates were treated with ethanol. All conditions were harvested under detergent-free cold lysis conditions and lysates were incubated with a polyclonal anti-4E-BP1 antibody for 16 h. Immunocomplexes were collected using protein A Dynabeads. One third of each immunoprecipitate was used for protein isolation and the rest for RNA analysis. Protein samples from WCL and 4E-BP1-immunoprecipitates were immunoblotted for eIF4E and 4E-BP1. N.S.: Non-specific band **(b)** RNA samples isolated from IP reactions were assayed for cyclin D1 message in an RT-PCR reaction. To control for the specificity of the association of 4E-BP1-complexes with cyclin D1 message, a parallel IP reaction was performed using a monoclonal anti-PKC α antibody (small panel to the right). Cyclin D1 amplification with a H₂O sample and actin amplification with a mouse liver RNA sample (provided by the manufacturer) were included as controls for the RT-PCR reaction. M: DNA size marker. Data are representative of two independent experiments.

III.C.3 PKC activation does not lead to changes in the activation status of eIF2 α

eIF2 α is another regulator of translation initiation whose inhibition has been shown to lead to suppression of cyclin D1 synthesis. As described in detail in the Introduction section, phosphorylation of Ser51 in the α subunit of eIF2 leads to inhibition of eIF2 function in facilitating recognition between the start-codon in the mRNA and the anti-codon in the Met-tRNA_i (Clemens 2004). We tested the effects of PKC activation on eIF2 α phosphorylation/inactivation in IEC-18 cells using a phospho-specific antibody for eIF2 α phosphorylated at Ser51. No change was seen in the amount of phospho-Ser51-eIF2 α following PMA treatment, indicating that inhibition of this initiation factor is unlikely to be involved in PKC-mediated inhibition of cyclin D1 translation in IEC-18 cells (Figure 44).

Figure 44

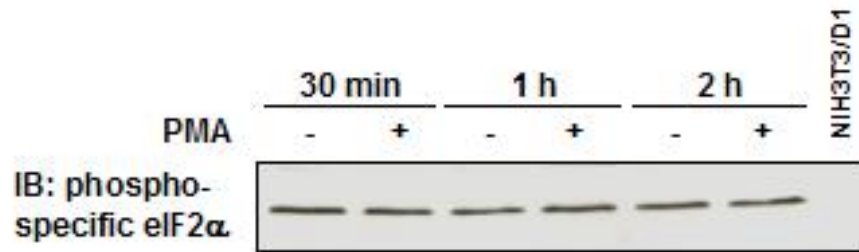


Figure 44 PKC activation does not lead to changes in phospho-Ser51-eIF2α levels

Subconfluent populations of IEC-18 cells were treated with 100 nM PMA for the indicated times. Controls were treated with ethanol. A lysate from NIH-3T3 cells overexpressing cyclin D1 (NIH3T3/D1) was included as a control [phosphorylation of eIF2α in NIH-3T3 cells was shown to be minimal in other studies (Brewer and Diehl, 2000)]. WCL (15 μg) were analyzed for phospho-Ser51-eIF2α in Western blots. Data are representative of at least five independent experiments.

III.C.4 PKC activation leads to rapid inhibition of eEF2

Polypeptide chain elongation is thought to be a crucial point in regulation of global rates of translation because this step is known to be one of the most expensive cellular processes in terms of consumption of metabolic energy (Proud 2004; Abbott and Proud, 2004; Browne and Proud, 2002). Phosphorylation of eEF2 at Thr56 is a well-known molecular switch to turn off elongation in response to sub-optimal growth conditions, including amino acid deprivation, depletion of cellular energy pools, or various cytotoxic stress stimuli. To determine if PKC-signaling promotes inhibition of eEF2 in IEC-18 cells, we analyzed levels of phospho-Thr56-eEF2 following PKC activation using a phospho-specific antibody (Figure 45). A striking and rapid increase in levels of phospho-Thr56-eEF2 was detected following PKC activation and appeared to be sustained for 1-2 h (Figure 45 and 50, and data not shown). Control reactions for phospho-Thr56-eEF2 immunoblotting are described in Appendix D. Thus, PKC activation appears to downmodulate translation elongation in intestinal epithelial cells.

Figure 45

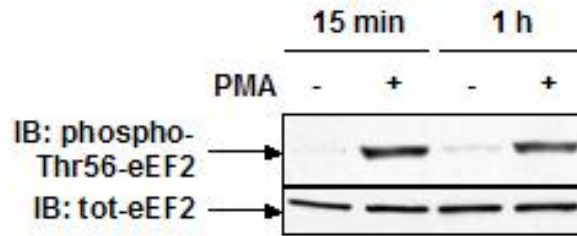


Figure 45 PKC activation promotes rapid inhibition of eEF2 Subconfluent populations of IEC-18 cells were treated with 100 nM PMA for 15 and 60 min. Controls were treated with ethanol. WCL were analyzed for phospho-Thr56-eEF2 (15 μ g) and for total eEF2 (10 μ g) by Western blots. Data are representative of at least five independent experiments.

III.C.4.a PMA-induced inhibition of eEF2 is PKC α -dependent

To establish the PKC-dependence of PMA-induced inhibition of eEF2, IEC-18 cells were depleted of phorbol ester-responsive PKC isozymes by prolonged treatment with 1 μ M PdBu. Figure 46 shows that PKC α , δ , and ϵ depletion abrogates PMA's ability to induce phospho-Thr56-eEF2 formation, confirming the PKC-dependence of the effect.

Figure 46

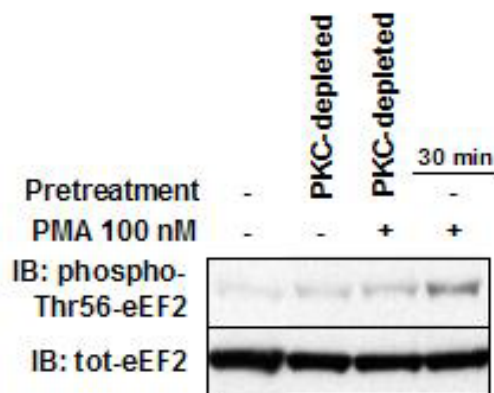


Figure 46 PMA-induced inhibition of eEF2 is dependent on PKC signaling Parental and PKC α , δ , and ϵ -depleted cells (see Materials and Methods) were treated with 100 nM PMA for 30 min. WCL were analyzed for phospho-Thr56-eEF2 (15 μ g) and total eEF2 (10 μ g) by Western blots. Data are representative of three independent experiments.

To determine if PKC α signaling can promote inhibition of eEF2, we tested the ability of PMA to induce Thr56 phosphorylation of eEF2 in PKC δ/ϵ -depleted cells (generated by pulse PMA treatment as described in the Methods section) (Figure 47). Activation of PKC α alone in PKC δ/ϵ -depleted cells led to modest Thr56 phosphorylation of eEF2 (a) confirming the ability of PKC α to inhibit this factor and (b) suggesting that PKC δ and/or ϵ -associated activities could be playing a role in promoting the stronger inhibition of eEF2 that is seen in cells expressing all three phorbol-ester-responsive isozymes (Figure 47). A complication encountered in these experiments was that PKC δ/ϵ depletion by itself appeared to promote a moderate increase in phospho-Thr56-eEF2 (Figure 47). Therefore, other approaches will be required to conclude whether PKC α -associated activity alone is responsible for the modulation of the eEF2 in IEC-18 cells.

Figure 47

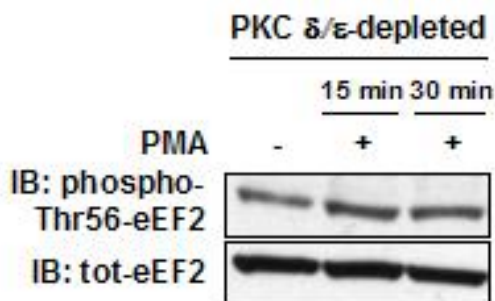


Figure 47 PKC α alone can promote modest inhibition of eEF2 PKC δ/ϵ -depleted cells were treated with PMA for the indicated times and cell extracts were subjected to anti-phospho-Thr56-eEF2 (15 μ g) and total eEF2 (10 μ g) immunoblotting. Data are representative of two independent experiments.

III.C.4.b PKC signaling does not alter the activity of eEF2K

To date, eEF2K has been the sole activity identified to mediate Thr56 phosphorylation of eEF2. As discussed in detail in the Introduction section, eEF2K itself is subject to tight regulation by a variety of growth- or stress-associated signals. To determine the involvement of eEF2K in PKC-induced Thr56 phosphorylation of eEF2, we examined the ability of PMA to relieve inhibitory phosphorylation at Ser366 in eEF2K using a phospho-specific antibody (Figure 48). The control reaction for the phospho-Ser366-eEF2K antibody is described in Appendix D. No significant change in Ser366 phosphorylation was detected in response to PKC activation. Although this result implies that PMA does not seem to act through eEF2K to induce Thr56-eEF2 phosphorylation, we should bear in mind that regulation of eEF2K is complex and determined by phosphorylation at multiple sites which are modulated by different signaling pathways. Therefore, additional studies are required to exclude the involvement of alterations in eEF2K activity in PKC-mediated inhibition of eEF2 in IEC-18 cells.

Figure 48

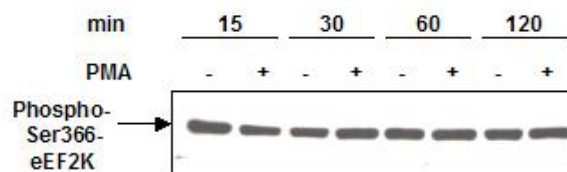


Figure 48 PMA does not alter Ser366 phosphorylation of eEF2K Subconfluent cells treated with 100 nM PMA were harvested at the indicated times. 25µg WCL were analyzed for phospho-Ser366-eEF2K by Western blotting.

III.C.5 PMA does not alter the polyribosome profile of cyclin D1 mRNA

To address the consequence of alterations in 4EBP-1 and eEF2 activity on the translation of cyclin D1 message, we conducted polyribosome analysis which provides information about the ribosome association of a given mRNA. In this analysis, polysomes are segregated by centrifugation through a continuous sucrose gradient. mRNAs complexed with fewer ribosomes sediment slower and are, thus, detected in earlier fractions, closer to the top of the gradient (lower numbers). Conversely, mRNAs associated with more ribosomes sediment faster, and are detected in later fractions, nearer to the bottom of the gradient (higher numbers). Based on its negative impact on initiation of translation, hypophosphorylation of 4E-BP1 would be expected to produce a shift of cyclin D1 mRNA toward the top of the gradient. On the other hand, inhibition of translational elongation by increased phospho-Thr56-eEF2 formation would be expected either to freeze polysomes in place or to shift cyclin D1 mRNA toward the bottom of the gradient. In untreated cells, cyclin D1 message was detected in both polysomal and non-polysomal fractions (Figure 49a, upper left panels). Although cyclin D1 mRNA was detected in all fractions, higher levels sedimented with smaller polysomes consistent with the relatively poor translation of this message (Shi et al., 2005 and Graff and De Benedetti 2004). Notably, we saw no significant change in the sedimentation of cyclin D1 message following PMA treatment for 1 h (Figure 49a, upper right panels and Figure 49b). This polysome analysis, together with our other findings, indicates that PKC-induced decreased cyclin D1 synthesis involves inhibition of both translational initiation

and elongation in IEC-18 intestinal crypt cells, as confirmed by immunoblot analysis of cell extracts from parallel plates (Figure 49c).

Figure 49

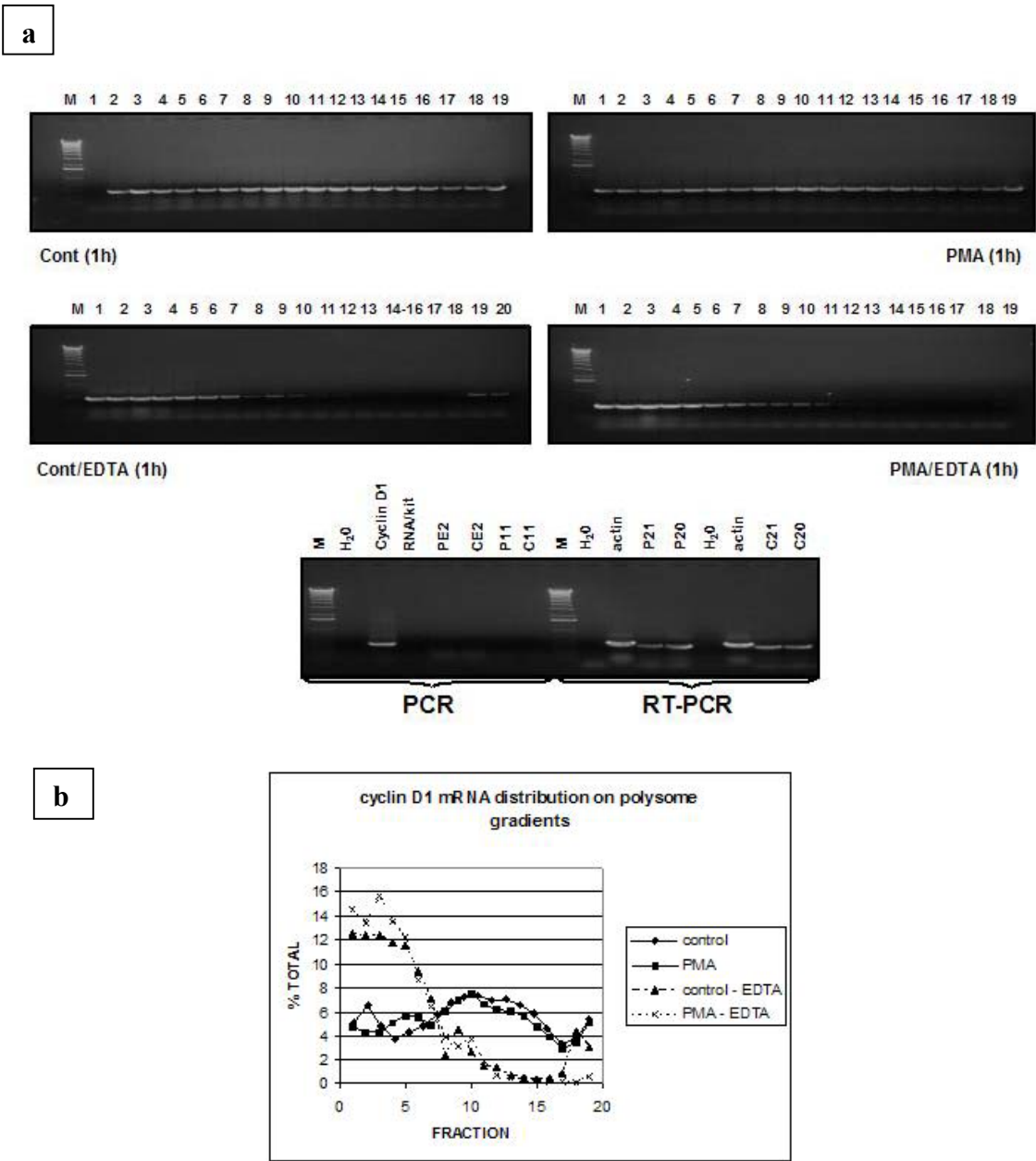


Figure 49

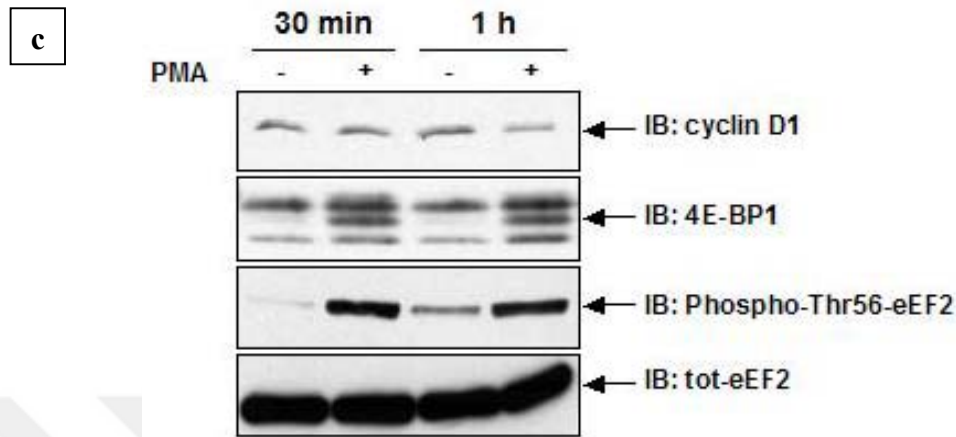


Figure 49 No change is detected in the polysome profile of cyclin D1 message 1 h after PKC activation (a) Cells treated with 100 nM PMA or vehicle (ethanol) for 1 h were collected under cold lysis conditions (see Materials and Methods). Lysates were divided into two aliquots, one of which was treated with EDTA to disrupt the ribosomal complexes. All four samples were then loaded onto sucrose gradients. Following 1 h 15 min centrifugation at 36, 000 RPM, gradients were fractionated into 0.6 ml aliquots that were collected in microfuge tubes containing 0.15 ml 10% SDS. Absorbance readings at $\lambda = 254$ nm were recorded during the fractionation. mRNA samples isolated from each fraction was assayed for cyclin D1 by RT-PCR (*upper panels*). In the *small lower panel*, RNA samples which gave the highest signal for cyclin D1 message in the RT-PCR reaction were assayed with *taq* polymerase alone to rule out the possibility of DNA contamination. **(b)** Plot of the absorbance readings obtained during the fractionation of Control (Cont), PMA, Control treated with EDTA (Cont/EDTA), and PMA treated with EDTA (PMA/EDTA) samples. **(c)** Protein lysates were collected from parallel plates at 30 min and 1 h of 100 nM PMA treatment, and immunoblotted for cyclin D1, 4EBP-1, phospho-Thr56-eEF2, and total eEF2 to confirm the effects of PKC activation on these key regulators of cell cycle progression and translation. Data are representative of three independent experiments.

III.C.6 PKC/PKC α -induced alterations in 4E-BP1 and eEF2 could account for the observed decrease in levels of cyclin D1 degradation-mutants

Lysates obtained from untreated or PMA-treated IEC-18 cells expressing cyclin D1 degradation-mutants were examined for 4E-BP1 hypophosphorylation and inhibition of eEF2 by immunoblot analysis. Hypophosphorylation of 4E-BP1 and a significant increase in the levels of phospho-Thr56-eEF2 were seen at 2 h of the PMA treatment in all conditions (Figure 50). These data support the notion that PKC-induced blockade of both translation initiation and elongation through the inhibition of eIF4E and eEF2 could account for the suppression of both endogenous as well as exogenously expressed cyclin D1 in IEC-18 cells.

Figure 50

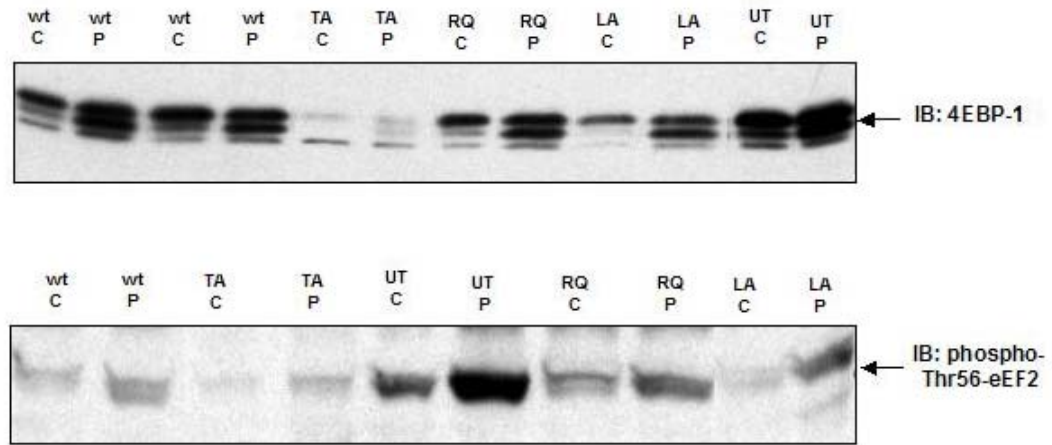


Figure 50 Hypophosphorylation of 4E-BP1 and increased phospho-Thr56-eEF2 levels are observed in samples expressing cyclin D1 degradation mutants following 2 h-PMA-treatment Samples from untreated or 100 nM PMA-treated IEC-18 cells transiently transfected with *wt* (WT) cyclin D1 or cyclin D1 degradation-mutants [T286A (TA), R29Q (RQ) and L32A (LA)] together with untransfected controls (UT) were analyzed for 4E-BP1 (10 μ g) and phospho-Thr56-eEF2 (15 μ g) by Western blotting.

III.D. Analysis of the relationship between loss of PKC/PKC α signaling and regulation of cyclin D1 expression in intestinal epithelial cells

III.D.1 Loss of PKC/PKC α signaling is associated with hyperinduction of cyclin D1 protein and accelerated IEC-18 cell growth

It has long been recognized that the members of the PKC family undergo downregulation following activation. As shown in Figure 51, PMA treatment results in progressive downregulation of all three phorbol ester-responsive PKC isozymes expressed in IEC-18 cells, i.e., PKC α , δ , and ϵ , albeit with different kinetics. PKC δ is downregulated first, followed by PKC α , and then by PKC ϵ . Figure 51 also demonstrates that during continuous PMA treatment, cyclin D1 protein expression is gradually restored in IEC-18 cells, coincident with activation-induced depletion of phorbol ester-responsive PKC isozymes. Thus, continued PKC-mediated input signal appears to be required for maintenance of the effect. Comparison of the kinetics of PKC α , δ , and ϵ downregulation demonstrates that restoration of cyclin D1 protein levels coincides more closely with the disappearance of PKC α signaling (Figure 51 3 h time point, and Figure 52 4 h time point), pointing to loss of PKC α as a pivotal event in relief of the suppressive effect of PKC signaling on cyclin D1 accumulation in IEC-18 cells. The strong correlation between loss of PKC α expression/signaling and restoration of cyclin D1 levels is clearly depicted in Figure 52. Another key observation made in these experiments is that the continued absence of PKC/PKC α signaling is associated with a marked hyperinduction of cyclin D1 protein to levels significantly higher than those observed in PKC-expressing

cycling cells (Figure 51 see 6 and 8 h and Figure 52, see time points 6-28 h). This hyperinduction is sustained for as long as PKC isozyme levels remain low (e.g., 72 h; data not shown and Fig 60).

Figure 51

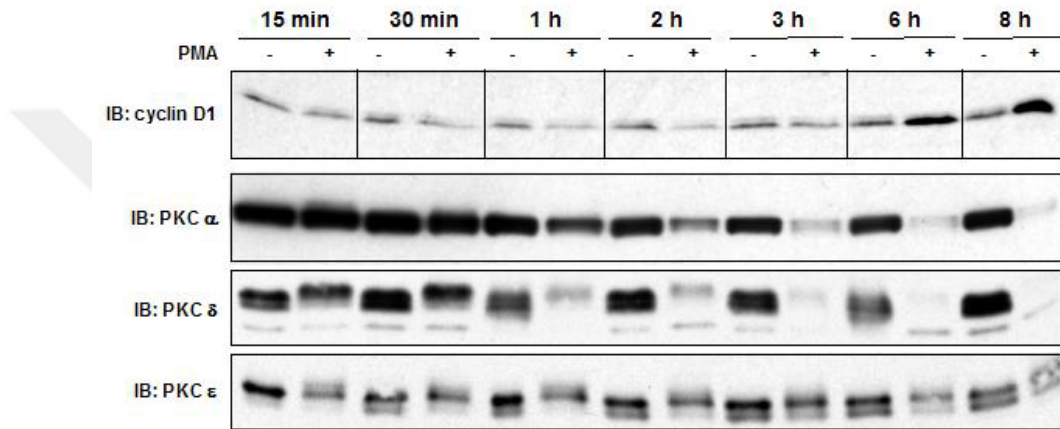


Figure 51 Restoration of cyclin D1 levels following PMA-induced downregulation coincides more closely with the disappearance of PKC α Subconfluent cell populations were treated with 100 nM PMA for the indicated times. A control treated with ethanol was collected for each time point. 20 μ g of each protein sample were immunoblotted for cyclin D1 protein. In the *lower panels*, 10 μ g of each protein sample were resolved on 10% SDS-PAGE gels and analyzed for PKC α , PKC δ , and PKC ϵ levels by Western blotting. Data are representative of at least three experiments.

Figure 53

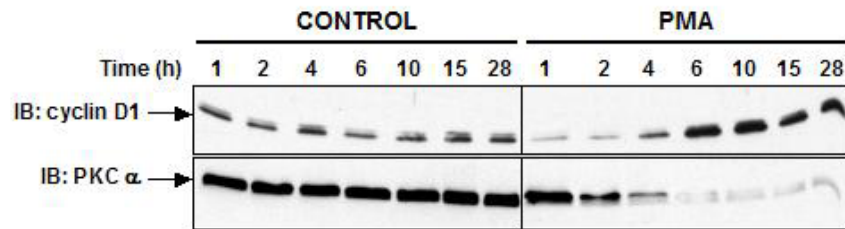


Figure 53 PKC/PKC α depletion is associated with a marked restoration/hyperinduction of cyclin D1 protein Subconfluent cells were treated with 100 nM PMA or ethanol (control) for the indicated times. At each time point, cells were harvested and subjected to immunoblot analysis for cyclin D1 and PKC α . Data are representative of at least three experiments.

The notion that modulation of cyclin D1 levels is a PKC α -specific event is supported by analysis of the effects of other PKC agonists. As shown in Figure 53, while other activators of PKC signaling can promote cyclin D1 downregulation and recovery/hyperinduction, the kinetics of these events closely correlate with the ability of these agents to sustain activation of PKC α in this system. As discussed in the Introduction section, activated PKC enzymes can be desensitized by proteolytic degradation or, in some cases, via dephosphorylation. In this regard, several studies have demonstrated that bryostatin is a potent inducer of PKC α dephosphorylation, as indicated by the accumulation of a faster-migrating 76 kDa species (Lee et al., 1996 and 1997). Comparison of the migration pattern of PKC α in IEC-18 cells treated with Bryo for 2, 4, and 8 h with their PMA- or PdBu-treated counterparts in Figure 53 provides an illustration of this phenomenon. The data in Figure 53 further show that bryostatin, which produces the most rapid desensitization of PKC α following activation, results in more modest downregulation and more rapid recovery of cyclin D1 compared with PMA or PdBu (lanes 4 vs. 2 and 3; 9 vs. 7 and 8; 14 vs. 12 and 13 in Figure 54).

Data obtained with the DAG-analogue DiC₈ provide additional support for the notion that hyperinduction of cyclin D1 expression is predominantly due to the absence of PKC α signaling. As seen in Figure 53 (lower panel), DiC₈, which transiently increases PKC α activity/membrane association but does not deplete the enzyme in IEC-18 cells (see lanes 1, 5, 10, and 15 in the upper panel of Figure 53, and Frey, Saxon et al., 1997), promotes downregulation but not hyperinduction of cyclin D1 in this system. Notably, low levels of membrane-associated/activated PKC α are detectable in control cycling cells

(Figure 53, lower panel); signaling by this pool of membrane-associated PKC α thus appears to play a repressive effect on cyclin D1 expression in proliferating cells (Figure 53, lower panel).

Figure 53

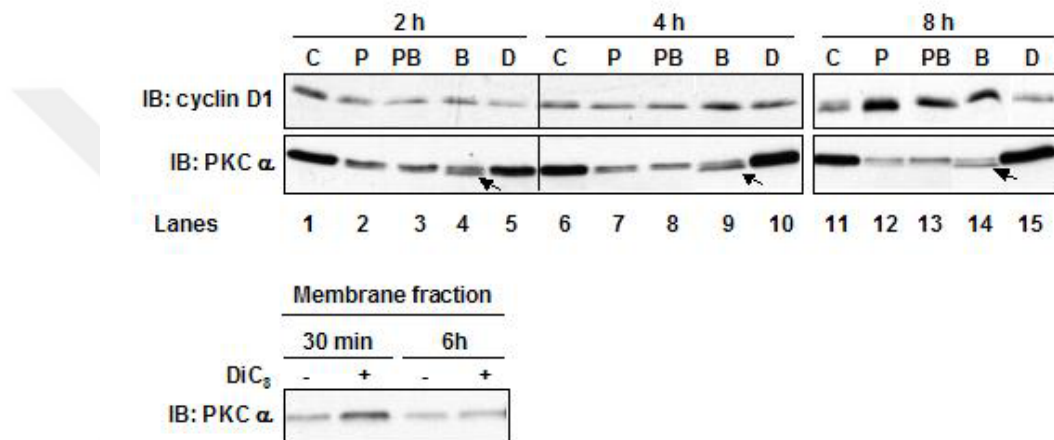


Figure 53 Regulation of cyclin D1 levels by various PKC agonists in IEC-18 cells correlates with their effects on PKC α expression/activity (*Upper panel*) Subconfluent populations of IEC-18 cells were treated with 100 nM PMA (P), 1 μ M PdBu (PB), 100 nM Bryo (B), or 20 μ g/ml DiC₈ (D) for 2, 4, 6, and 8 h. Cells were harvested in 1% SDS lysis buffer. 15 μ g of each sample was sequentially probed for cyclin D1 and PKC α on the same membrane. *Lower panel*, cells were treated with DiC₈ for 30 min and 6 h. Control plates were treated with DMSO. Cells were harvested in cold lysis buffer and fractionated into cytosolic and membrane fractions as described in the Materials and Methods section. 10 μ g of the membrane fraction samples were immunoblotted for PKC α . Note that, in cells treated with bryostatin, which promotes rapid desensitization of PKC α following activation (as indicated by the appearance of a high mobility, dephosphorylated form of the enzyme (Lee et al., 1996 and 1997), arrows), more modest downregulation of cyclin D1 at 2 h and more rapid recovery/hyperinduction of the protein is observed. In contrast, DiC₈, which transiently activates (as indicated by transient membrane translocation of the enzyme, *lower panel*) but does not downregulate

PKC α in IEC-18 cells, promotes downregulation but not hyperinduction of cyclin D1 in this system. Data are representative of four independent experiments.

Consistent with this notion, pharmacological inhibition of PKC activity with the broad spectrum inhibitor BIM I or the PKC α -selective inhibitor Gö6976 also resulted in hyperinduction of cyclin D1 protein, confirming a repressive effect of PKC α signaling on cyclin D1 accumulation even in cycling cells (Figure 54).

Figure 54

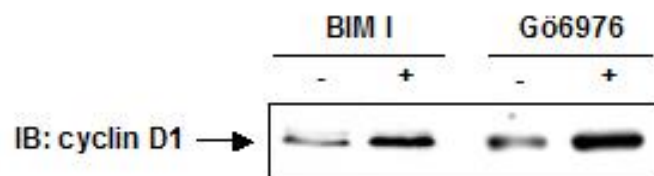


Figure 54 Both a general PKC-inhibitor and a PKC α -selective inhibitor result in hyperinduction of cyclin D1 in IEC-18 cells Subconfluent cells were treated with 5 μ M BIM I or 1 μ M Gö6976 for 2 h and WCL were subjected to immunoblot analysis for cyclin D1. Data are representative of at least four experiments.

To dissect further the effect of depletion of individual PKC isozymes on cyclin D1 accumulation in IEC-18 cells, we used short interfering RNA (**siRNA**) sequences to silence the expression of these molecules. The selected sequences promoted specific silencing of the target isozyme, with minimal effects on the expression of the remaining two isozymes (Figure 55). Notably, silencing of PKC α resulted in marked induction of cyclin D1 protein in IEC-18 cells, while silencing of PKC δ or ϵ did not have any significant effect on the levels of the molecule (Figure 55). Based on these data, we concluded that PKC α -associated signaling negatively modulates the expression of cyclin D1 gene in IEC-18 cells and that loss of this enzyme is responsible for the accumulation of cyclin D1 in PKC-depleted cells. Studies were next initiated to explore the molecular pathways involved in induction and maintenance of unusually high cyclin D1 expression upon disappearance of PKC α in these cells.

Figure 55

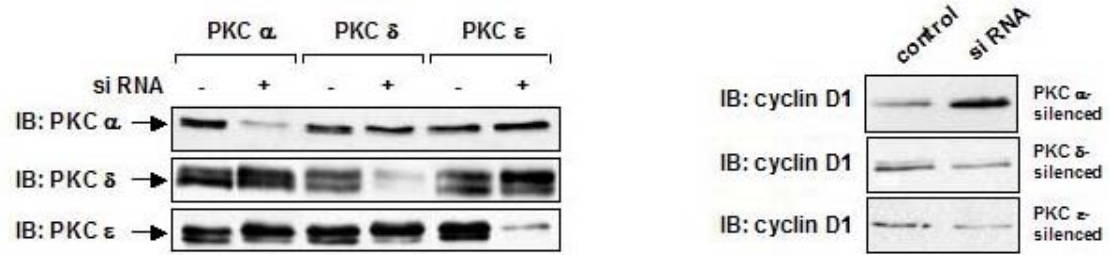


Figure 55 Silencing of PKC α , but not PKC δ or ϵ , results in hyperinduction of cyclin D1 in IEC-18 cells Cells grown in 6-well tissue culture plates were transfected with siRNA sequences interfering with the expression of PKC α , δ , or ϵ using Lipofectamine 2000 reagent. 4 h following transfection, cells were returned to fresh CGM. Cells were collected at 12, 24, 36, 48, or 72 h after media change. In this figure, data from the 48 h time point are presented. All conditions (10 μ g) were analyzed for the expression levels of PKC α , PKC δ , and PKC ϵ (*left panel*), and cyclin D1 (*right panel*) (studies by Pysz, M.A. and Black, J.D.). Data are representative of three independent experiments.

III.D.2 Depletion of PKC/PKC α is associated with recovery of cap-dependent translation initiation and translation elongation, increased cyclin D1 mRNA levels as well as enhanced synthesis of cyclin D1 protein

To determine the mechanism(s) underlying the striking increase in cyclin D1 protein expression in PKC/PKC α -depleted cells, we first examined the activation status of the translation machinery at the level of initiation and elongation by measuring 4E-BP1 phosphorylation and levels of phospho-Thr56-eEF2. As shown in Figure 56a, the activation of 4E-BP1 is significantly reversed between 2 and 6 h of PMA treatment, coincident with the disappearance of PKC/PKC α signaling. Furthermore, the reversal of hypophosphorylation of 4E-BP1 at 6 h of PMA treatment was accompanied by dissociation of 4E-BP1 from eIF4E-bound complexes, indicating the recovery of cap-dependent translation initiation, as demonstrated by the cap assay results shown in Figure 56a (lower panel). Recovery of eEF2 function in PKC/PKC α -depleted cells, appears to occur earlier (by 2-3 h) relative to that of the initiation machinery, as indicated by the decrease in phospho-Thr56-eEF2 levels (Figure 56b).

To explore further the mechanism(s) underlying the striking increase in cyclin D1 protein expression in PKC/PKC α -depleted cells, cyclin D1 mRNA levels were examined by Northern blot analysis. As shown in Figure 57b, a clear increase in levels of cyclin D1 message was detected in IEC-18 cells by 3 h of PMA treatment, paralleling the recovery and hyperinduction of cyclin D1 protein levels (Figure 57a). Interestingly, the hyperinduction of cyclin D1 mRNA observed at 3 h of PMA treatment is not coupled

with hyperinduction of protein, suggesting that recovery of both the translation initiation and elongation machinery may be required for the increase in cyclin D1 mRNA levels to be reflected in cyclin D1 protein levels. These data indicate that upregulation of cyclin D1 gene transcription or increased stability of the cyclin D1 mRNA are involved in the overproduction of cyclin D1 protein at these time points.

We next addressed whether the increased levels of cyclin D1 message are reflected in increased synthesis of the protein. [S^{35}]-Methionine biosynthetic labeling experiments were performed in cells treated with 100 nM PMA or vehicle for 4.75-6.25 h. Cyclin D1 was immunoprecipitated from cell lysates collected at 15-minute intervals and the immunoprecipitates were quantified by SDS-PAGE and autoradiography. A significant increase in radiolabeled cyclin D1 was detected in PMA-treated/PKC-depleted cells relative to cells treated with vehicle alone at each time-point examined (Figure 57c).

Figure 56

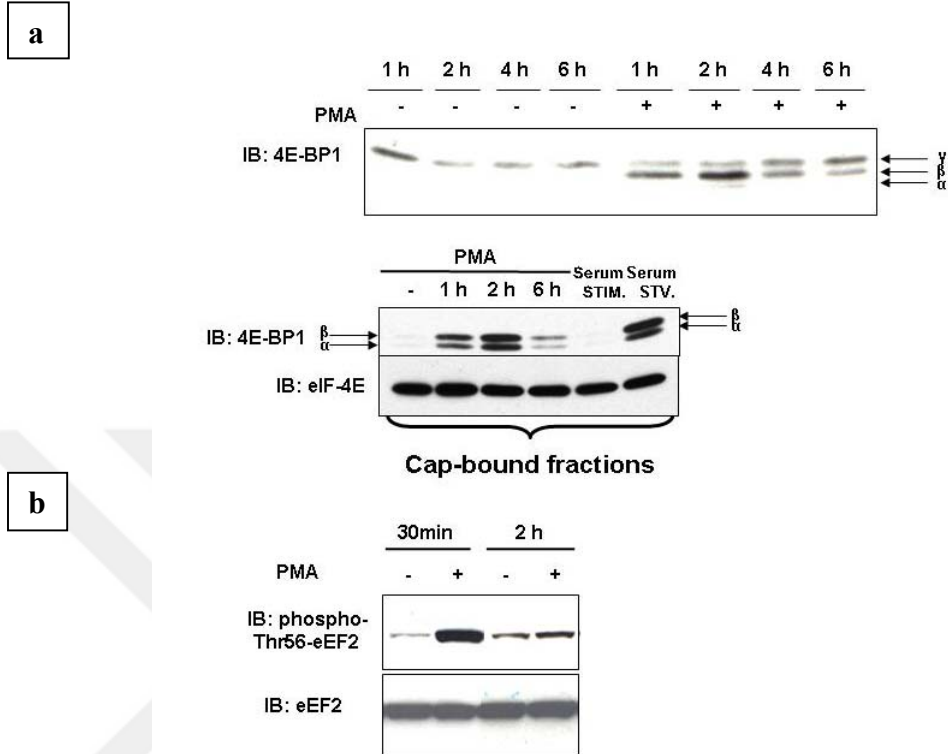
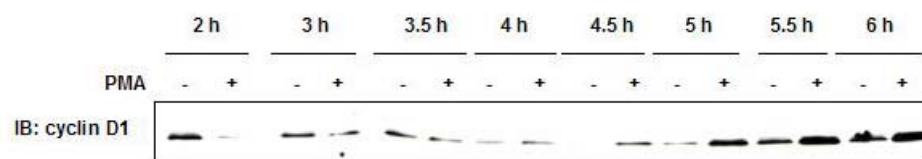


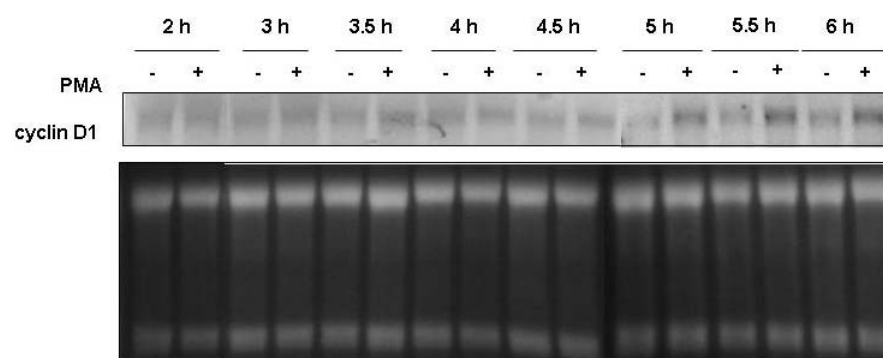
Figure 56 Enhanced cyclin D1 protein synthesis is associated with reversal of the inhibition by 4E-BP1 hypophosphorylation and loss of inhibitory phosphorylation of eEF2 on Thr56 (a) Upper panel: IEC-18 cells were treated with 100 nM PMA or vehicle for the indicated times and WCL were subjected to immunoblot analysis (IB) for 4E-BP1. *Lower panel:* Cells were treated with 100 nM PMA for 1, 2, or 6 h, serum-stimulated by an increase in serum from 5 to 10% for 10 min (Serum STIM.), or serum-starved for 22 h (Serum STV.). Cells were then harvested under cold lysis conditions and incubated with sepharose-conjugated m⁷-GTP cap analogue for 16 h. WCL and cap-bound fractions were subjected to immunoblot analysis (IB) for 4E-BP1 and eIF4E. **(b)** IEC-18 cells were treated with 100 nM PMA or vehicle for 2 h and subjected to immunoblot analysis (IB) for phospho-Thr56-eEF2. Data are representative of at least five independent experiments.

Figure 57

a



b



c

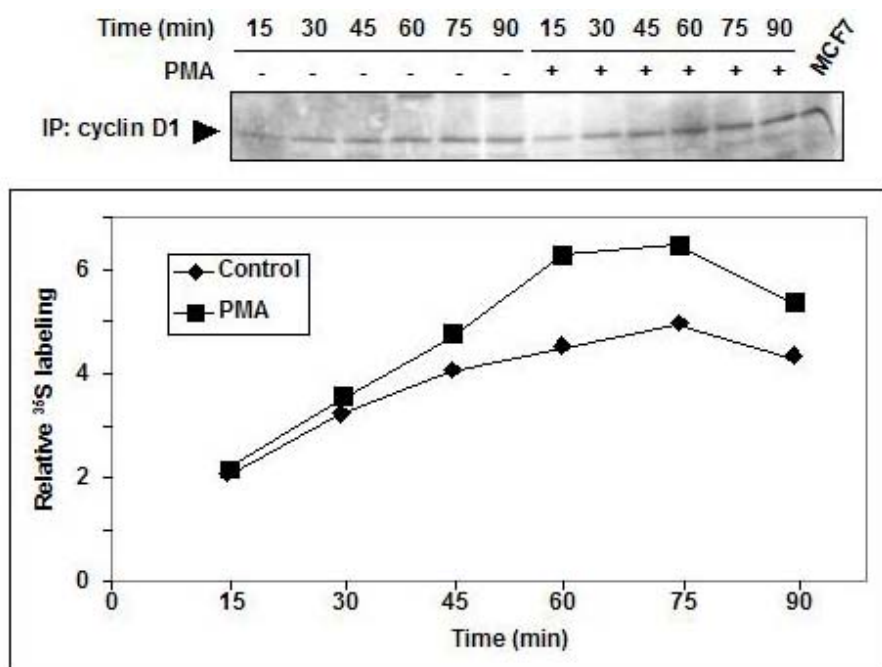


Figure 57 The hyperinduction of cyclin D1 that accompanies loss of PKC signaling in IEC-18 cells is associated with increased mRNA expression and enhanced synthesis of the protein (a) Following 100 nM PMA treatment, subconfluent cell populations were lysed in boiling 1% SDS lysis buffer for protein isolation at the indicated time points. 15 µg of WCL were immunoblotted for cyclin D1. **(b)** Cells treated with 100 nM PMA or ethanol for the indicated timepoints were harvested for RNA isolation. 7.5 µg of RNA were analyzed for cyclin D1 message by Northern blotting. **(c)** Subconfluent cell populations treated with 100 nM PMA or ethanol for 4 h 15 min were changed to methionine-starvation medium for 30 min and then returned to labeling medium for 15, 30, 45, 60, 75, and 90 min. Cell lysates were collected at (5-6.25 h) and cyclin D1 was immunoprecipitated from the samples using sc-450 antibody. Immunoprecipitates were resolved in a 20% SDS-PAGE gel and transferred onto PVDF membrane that was subsequently exposed to phosphorimager screens (upper panel). The signal from cyclin D1 protein in each condition was quantified and graphed using ImageQuant software (lower panel). Data are representative of two independent experiments.

Recovery of cyclin D1 synthesis was enabled by elevation of cyclin D1 mRNA expression in the context of reversal of PKC-induced hypophosphorylation/activation of 4E-BP1 and phosphorylation/inhibition of eEF2 following prolonged treatment with PMA (Figure 56). However, other mechanisms could also be involved in the *enhanced* synthesis and hyperinduction of cyclin D1 that accompanies loss of PKC signaling (e.g., alterations in other components of the translation machinery, altered nucleocytoplasmic transport of cyclin D1 mRNA). Note that alterations in cyclin D1 turnover have been excluded as a mechanism involved in this effect (Pysz, Black A.R., and Black J.D., data not shown).

III.D.3 Loss of PKC signaling and increased cyclin D1 expression are associated with accelerated growth of intestinal epithelial cells

To determine the effects of loss of PKC/PKC α signaling and increased levels of cyclin D1 protein on IEC-18 cell proliferation, phorbol ester-responsive isozymes were depleted from IEC-18 cells by overnight exposure to 1 μ M PMA. PKC-depleted cells were then washed, replated at equal densities, and counted at various times. As shown in Figure 58, PKC-depleted IEC-18 cells proliferated substantially faster than control cultures, consistent with an increase in cyclin D1 expression in the absence of PKC α . By 48 h, there were approximately 2-fold more cells in PKC-depleted cultures. Thus, the absence of PKC/PKC α signaling appears to confer a growth advantage to intestinal crypt cells, possibly by allowing the maintenance of high cyclin D1 expression.

Figure 58

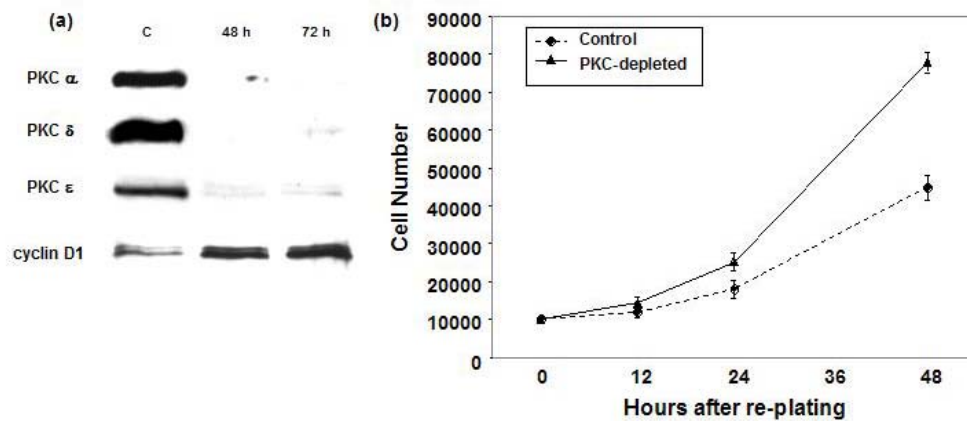


Figure 58 Loss of PKC/PKC α signaling in intestinal crypt cells is associated with prolonged hyperinduction of cyclin D1 protein and accelerated growth The sustained hyperinduction of cyclin D1 associated with long-term depletion of PKC/PKC α is associated with accelerated growth of IEC-18 cells. *Left panel:* Cyclin D1 levels remain elevated for at least 72 h in PKC/PKC α -depleted IEC-18 cells. *Right panel:* IEC-18 cells were incubated with PMA for 24 h to downregulate PKC/PKC α , re-plated at a lower density and cell number was determined at the indicated times (Studies by Frey, M.R. and Black, J.D.).

III.D.4. Intestinal neoplasms are characterized by decreased/abrogated PKC α expression and elevated levels of cyclin D1

Analysis of intestinal neoplasms revealed a similar relationship between the expression of PKC α and cyclin D1 (Figure 59). Immunostaining analysis of adenomas from Apc^{min} mice showed that, in contrast to Apc^(+/-) “normal” crypt and villus cells which express readily detectable levels of PKC α (albeit with different patterns of subcellular distribution) (Saxon et al., 1994 and Frey et al., 2000a), Apc^(-/-) adenoma cells exhibit dramatically reduced levels of the protein (Figure 59a). Conversely, while “normal” crypt cells express barely detectable levels of cyclin D1, adenoma cells markedly overexpress this molecule. Similarly, a majority of human colon adenocarcinoma cell lines express low levels of PKC α , in association with increased levels of cyclin D1 (Figure 59b). Interestingly, levels of cyclin D1 in PKC/PKC α -depleted IEC-18 cells (IEC-18/PMA) were comparable with those seen in neoplastic intestinal cell lines.

Figure 59

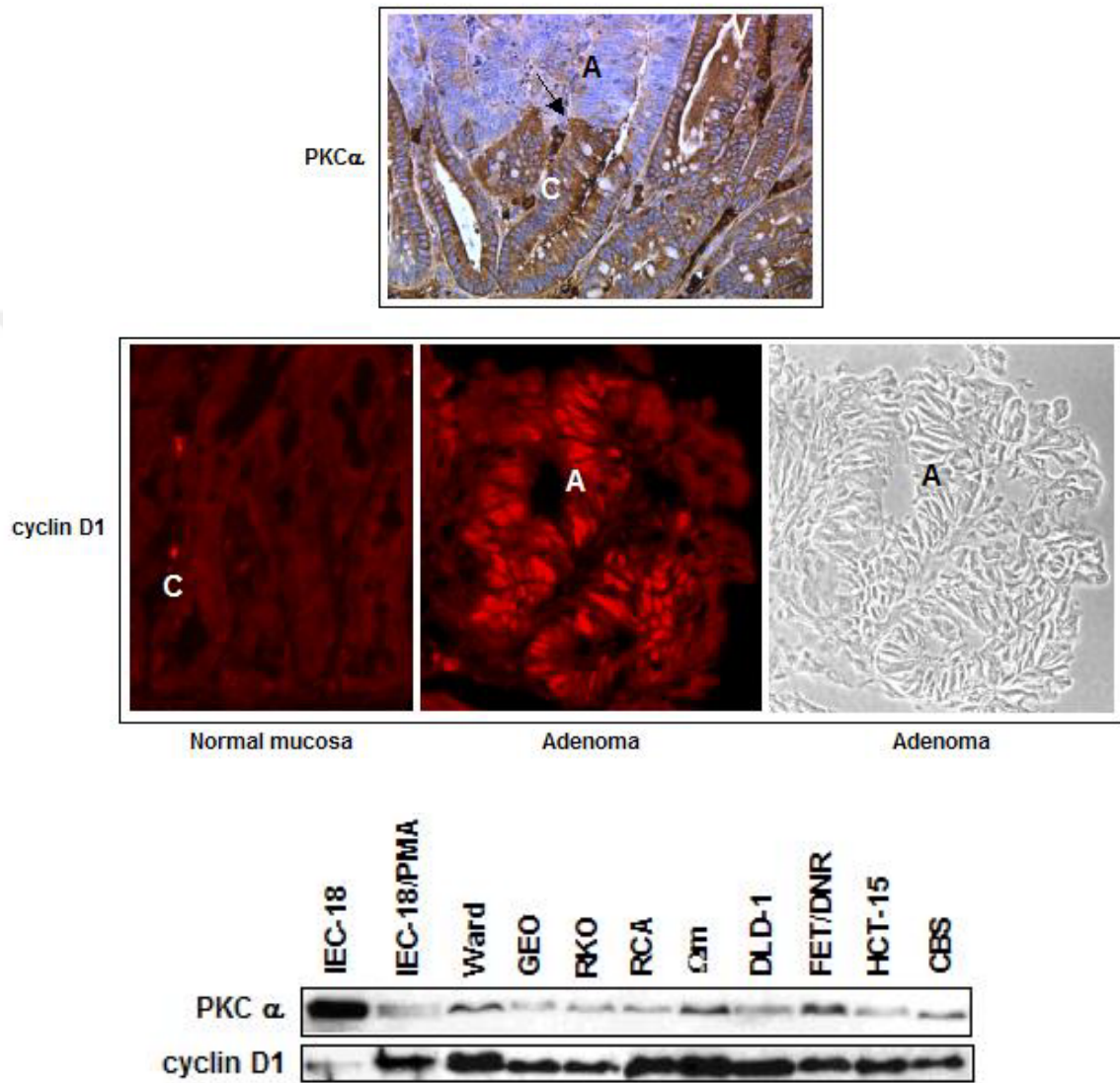


Figure 59 Intestinal neoplasms are characterized by decreased/abrogated PKC α expression and elevated levels of cyclin D1 (a) Expression of PKC α and cyclin D1 in Apc^{min} mouse adenoma tissue. Immunohistochemical analysis demonstrates that, while Apc^(+/-) “normal” crypt (C) and villus (V) cells express PKC α , Apc^(-/-) adenoma cells (A) exhibit dramatically reduced levels of the protein. Note the sharp delineation (arrow) between PKC α -positive normal crypt cells and PKC α -negative adenoma cells (Upper

panel). Comparison of cyclin D1 levels in $Apc^{(+/-)}$ “normal” crypt mucosa (C) and $Apc^{(-/-)}$ adenoma cells (A) by immunofluorescence analysis shows that cyclin D1 is barely detectable in normal crypt cells and is dramatically elevated in adenoma cells (Lower panel) (studies done by Esanullah, H. and Black, J.D.) **(b)** Protein samples from non-transformed IEC-18 cells (untreated and PMA-treated for 6 h), rat Ward colon adenocarcinoma cells and various human colon tumor cell lines (obtained from Drs. Michael Brattain and Ralph Bernacki) were immunoblotted for PKC α and cyclin D1 (data are provided by Pysz, M.A. Leontieva, O.V., and Hizli, A.A.).



IV. DISCUSSION

In this study, we demonstrated that activation of PKC α signaling promotes early loss of cyclin D1 protein by repressing its translation in the IEC-18 non-transformed intestinal cell line model (Figure 60a). Conversely, PKC α -depletion or inhibition of its activity results in hyperinduction of cyclin D1 (Figure 60b). In the next two sections, we will discuss our findings on the emerging molecular pathways involved in PKC α -regulated cyclin D1 downmodulation and hyperinduction in intestinal epithelial cells. We will compare and/or contrast PKC α -induced regulation of cyclin D1 expression with the findings of other studies describing cyclin D1 regulation in various systems. In the third section, we will address the significance of our findings in relation to observed alterations in the expression of these two genes in colorectal cancer. In the final section, future directions raised as a result of this study will be highlighted.

Figure 60

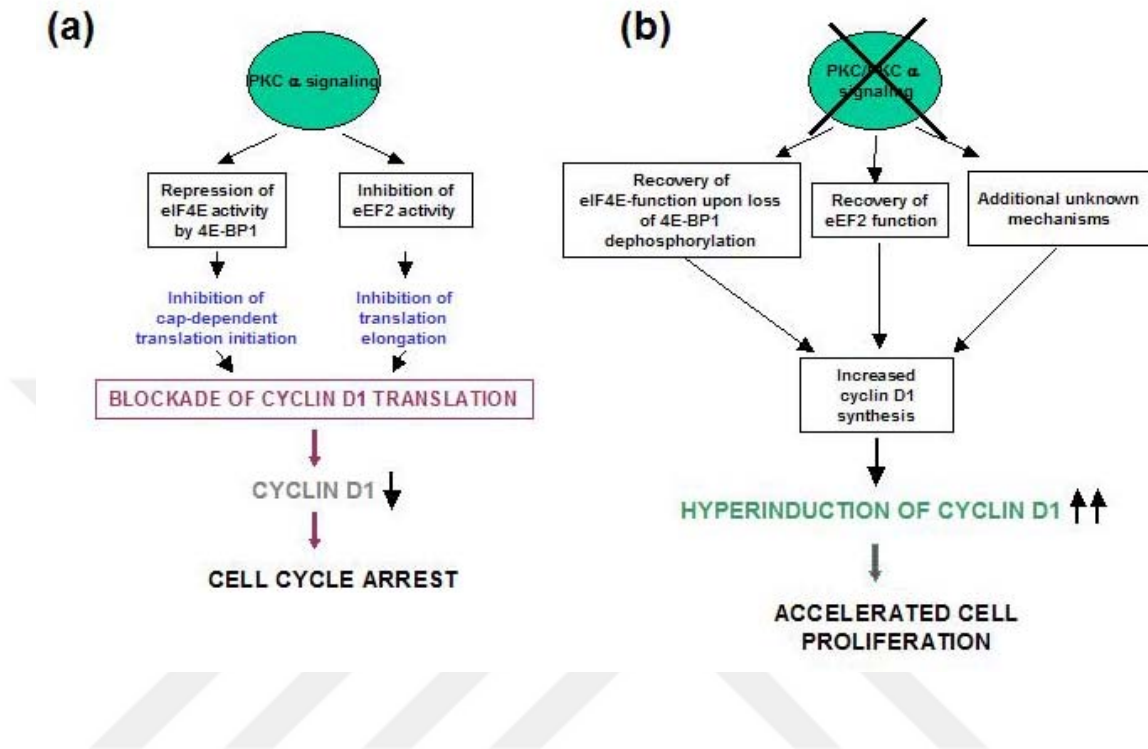


Figure 60 Model of the role of PKC α signaling in regulation of cyclin D1 levels in intestinal epithelial cells. (a) Activation of PKC α signaling in intestinal crypt cells leads to blockade of cyclin D1 translation through inhibition of cap-dependent translation initiation and translation elongation. Downregulation of cyclin D1 protein is associated with cell cycle arrest. **(b)** Loss of PKC α signaling leads to hyperinduction of cyclin D1 protein and accelerated cell proliferation.

IV.A PKC α signaling inhibits translation of cyclin D1 in intestinal epithelial cells undergoing G0 withdrawal

Activation of PKC α , δ , and ϵ by PMA leads to rapid downregulation of cyclin D1 in IEC-18 cells. PMA-induced downregulation of cyclin D1 is a PKC-dependent event because depletion of phorbol ester-responsive-PKC isozymes or inhibition of their activity by a general PKC inhibitor (BIM I) prevents loss of cyclin D1 upon PMA treatment. Several lines of evidence support a role for PKC α signaling, in particular, in mediating PKC agonist-induced loss of cyclin D1 in this system. First, previous studies from our laboratory demonstrated that PKC α activation promotes PKC agonist-induced downregulation of cyclin D1 in PKC δ and ϵ -depleted IEC-18 cells (Frey et al., 2000a). Second, a PKC α -selective inhibitor, Gö6976, blocks PMA-induced downregulation of cyclin D1. Third, the kinetics of downmodulation of cyclin D1 by different PKC agonists is closely correlated with the ability of these compounds to sustain activation of PKC α .

Our findings indicate that the early loss of cyclin D1 protein in response to PKC-signaling is not due to changes in transcription of its gene or stability of its mRNA, mechanisms which have been shown to regulate cyclin D1 expression in response to diverse stimuli in other systems (Lavoie et al., 1996 and Altucci et al., 1996, Watanabe et al., 1996, Albanese et al., 1995, Weber et al. 1997, Miyakawa and Matsushime, 2001, Guo et al., 2004, and Hashemolhosseini et al., 1998, and others see Introduction). Although a minor decrease in cyclin D1 mRNA levels is seen following PKC activation (around 2 to 3 h of PMA treatment), this change is always preceded by a decrease in

protein levels. Furthermore, analysis of cyclin D1 mRNA in nuclear and cytoplasmic subcellular fractions ruled out a possible blockade of cyclin D1 mRNA transport as the mechanism involved in the decrease in cyclin D1 expression.

While our findings point to a suppressive role of PKC α on cyclin D1 accumulation in intestinal cells and exclude the involvement of transcriptional control in the effect, previous studies in NIH-3T3 cells demonstrated that overexpression of the PKC α catalytic domain promotes transcriptional induction of the molecule (Soh and Weinstein, 2003). The discrepancy between these findings could be a reflection of differences in cellular responses to PKC α activation in the different cell types. However, differences in the approaches taken in each of the studies could also account for the conflicting findings. While our studies avoided interference with either the physiological levels or the biochemical properties of the enzyme (crucial parameters for the proper response of relevant signal transduction pathways to its activation), the study by Soh and Weinstein (2003) involved overexpression of *wt* and truncated PKC α constructs. As pointed out by the authors, the fact that overexpression of the truncated catalytic domain, but not the full size *wt* protein, induced cyclin D1 promoter activity in NIH-3T3 cells could reflect sensitivity of PKC α -mediated signaling to disturbances in the molecular structure of the enzyme.

PKC δ has also been implicated in regulation of cyclin D1 expression at the transcriptional level (Page et al., 2002). Overexpression of the active catalytic subunit of PKC δ in primary bovine airway smooth muscle cells attenuated PDGF-mediated

transcription from the cyclin D1 promoter, whereas overexpression of a dominant-negative PKC δ increased promoter activity. Similarly, inhibition of endogenous PKC δ activity by a PKC δ -specific pseudosubstrate led to an increase in cyclin D1 protein abundance. Although we have not addressed the mechanism(s) responsible for the moderate decline in cyclin D1 mRNA that follows the decline in protein in IEC-18 cells, it is possible that PKC δ activity may be involved in the effect. However, the absence of alterations in cyclin D1 levels in response to PKC δ silencing (Figure 55) suggests that PKC δ signaling does not play a role in cyclin D1 regulation in normal intestinal cells.

Pulse-chase analysis showed that the turnover of cyclin D1 is identical in PMA-treated and control cells, demonstrating that PKC signaling does not alter the stability of the protein in intestinal cells. The ability of PMA treatment to downregulate cyclin D1 proteolysis mutants confirmed that PKC activation does not lead to increased turnover of cyclin D1 protein via GSK-3 β or “destruction-box”-dependent mechanisms (Diehl et al., 1998 and Agami and Bernards, 2000). Thus, our findings differ from those of others (e.g., serum-starved NIH-3T3 cells, retinoic acid-treated human embryonic carcinoma cells, differentiating myoblasts, IR-treated MCF7 cells, and others) where loss of cyclin D1 protein in association with G1 arrest or cell cycle withdrawal is mediated by decreased cyclin D1 stability (Diehl et al., 1997 and 1998, Spinella et al., 1999, Zou et al., 2004, and Agami and Bernards 2000). However, the ability of both Li⁺ and proteasome inhibitors to promote accumulation of cyclin D1 in IEC-18 cells (Figure 28) suggests involvement of GSK-3 β and the proteasome in normal turnover of cyclin D1 in this system.

On the other hand, analysis of cyclin D1 protein synthesis revealed, for the first time, that PKC activation leads to suppression of cyclin D1 translation in IEC-18 cells withdrawing from the cell cycle. This observation parallels findings in several other systems where repression of cyclin D1 synthesis mediates inhibition of cyclin D/cdk4-associated kinase activity in response to G1 arrest-inducing signals. For example, decreased cyclin D1 synthesis has been observed in IEC-6 and RIE rat non-transformed intestinal cells in response to TGF- β treatment, in MCF-7 cells treated with prostaglandins, in NIH-3T3 cells treated with UPR-inducing agents (tunicamycin), and in herbimycin A-treated MCF7 and MCF7A cells (Ko et al., 1998, Campo et al. 2002, Brewer et al. 1999, and Muise-Helmericks et al., 1998). It is noteworthy that suppression of cyclin D1 expression can be induced by different mechanisms in the same cell line upon challenge by different stimuli. For example, in NIH-3T3 cells, downregulation of cyclin D1 protein occurs via increased turnover upon serum-withdrawal and inhibition of protein synthesis in response to tunicamycin-induced UPR (Diehl et al., 1997 and 1998, Choi et al., 1997 and Brewer and Diehl, 2000).

In the next section, we will discuss the results of our analysis of the effects of PKC signaling on key regulatory components of the translation machinery. Our findings point to inhibition of translation initiation and translation elongation as the underlying mechanisms mediating the decrease in cyclin D1 synthetic rates observed in IEC-18 cells.

IV.A.1 PKC/PKC α activation inhibits cap-dependent translation initiation of cyclin D1 in IEC-18 cells

We examined PMA-induced changes in translation initiation at its two crucial points of regulation: (a) abundance of eIF4F complexes, and (b) ability to recycle eIF2 α -GDP complexes back to active eIF2 α -GTP complexes which is required for uninterrupted loading of Met-tRNA_i to the 40S small ribosomal subunit. As discussed in the Introduction section, the impact of limited availability of eIF4F complexes on cyclin D1 protein synthesis is well-documented (Jiang et al., 2002 and De Benedetti and Graff, 2004). With regard to eIF2 α regulation, two studies have linked inhibition of eIF2 α function to suppressed translation of cyclin D1 (Brewer and Diehl 2000 and Campo et al., 2002). Brewer et al. (1999) demonstrated that the rapid block in translation of cyclin D1 induced by UPR in NIH-3T3 cells is associated with increased activity of PERK on eIF2 α . In the second study, increased Ser51-phosphorylation of eIF2 α by PKR was associated with a shift of polysomal cyclin D1 mRNA to nonpolysomal fractions in MCF7 cells treated with 15d-PGJ(2) (Campo et al., 2002). Although we did not detect an inhibition of eIF2 α processing (as indicated by the absence of alterations in the phosphorylation state of Ser51), we observed marked changes in the phosphorylation/activation of the eIF4E inhibitor 4E-BP1 in response to PKC activation in intestinal epithelial cells.

The PMA-induced dephosphorylation of 4E-BP1 was shown to be a PKC-dependent event as PMA does not lead to hypophosphorylation of 4E-BP1 in cells

depleted of phorbol ester-responsive PKC isozymes. Two additional lines of evidence indicate that dephosphorylation of 4E-BP1 is a PKC α -dependent event. First, PMA can induce hypophosphorylation of 4E-BP1 in PKC δ/ϵ -depleted cells. Second, the PKC α -selective inhibitor, Gö6976, blocks PMA-induced upregulation of both β and α -phosphoforms of 4E-BP1.

It should be noted that PKC δ has also been implicated in regulation of 4E-BP1 activity (Kumar et al., 2000). However, in contrast to our findings with PKC α , PKC δ was shown to promote phosphorylation of 4E-BP1 and its dissociation from eIF4E (Kumar et al., 2000). Although the specific function of PKC δ in regulation of 4E-BP1 phosphorylation in IEC-18 cells remains to be determined, our data indicate that any positive effects of activated PKC δ signaling on phosphorylation of this factor are dominated by the negative effects of PKC α in this system.

The increase in the levels of the α and β phosphoforms of 4E-BP1 was paralleled by an increased association of these 4E-BP1-species with the mRNA cap-binding protein eIF4E, which would result in limited abundance of translation initiation-competent eIF4F complexes. The degree of 4E-BP1 association with eIF4E in cells treated with PMA for 1-2 h was similar to that seen in serum-starved control cells; thus, signals promoting G0-exit in this system produce comparable effects on translation initiation. The hypophosphorylation of 4E-BP1 seen in response to activation of PKC α provides additional support for the previously defined role of PKC α -signaling in cell cycle withdrawal in the intestinal epithelium *in vivo* (Saxon et al., 1994 and Frey et al., 2000a).

In this respect, our data parallel those of others showing that induction of HL-60 and U-937 cell differentiation into monocytes/macrophages by IFN-gamma or PMA also results in hypophosphorylation and consequent activation of 4E-BP1 (Grolleau et al, 1999).

Increased stoichiometry of 4E-BP1 in eIF4E-associated complexes is a hallmark indication of shutdown of cap-dependent translation initiation, an event that is known to selectively impair translation of weak mRNAs (De Benedetti and Graff, 2004). Cyclin D1 message is an example of a weak mRNA; like the mRNAs of other potent mitogenic genes, cyclin D1 message has a 5' UTR which is long and complex in its secondary structure (Shi et al., 2004 and De Benedetti and Graff 2004). Translation of weak mRNAs is known to be highly susceptible to scarce abundance of eIF4F complexes, because the individual activity of eIF4F components is crucial for the progression of these transcripts through the multistage process of translation initiation. Translation of cyclin D1 message and of other weak mRNAs is highly dependent on the unwinding activity of the eIF4A component of eIF4F, which enables scanning for the initiation codon, as well as on the functions of the cap-binding eIF4E and the docking factor eIF4G for engagement to the holo-ribosomal complex (De Benedetti and Graff 2004). On the other hand, strong mRNAs, which have short 5' UTRs with relatively simple structures, have the advantage of being processed promptly under conditions of low eIF4F abundance. Our data confirm other findings demonstrating that inhibition of cap-dependent translation, either by overexpressing 4E-BP1 or by activating endogenous 4E-BP1 through rapamycin-induced hypophosphorylation, is associated with decreased cyclin D1 expression (Jiang et al., 2002 and Grewe et al., 1999). Importantly, our studies

provide a more direct link between increased activation of 4E-BP1 and PKC-induced suppression of cyclin D1 synthesis through IP-RT-PCR assays. Analysis of cyclin D1 mRNA levels associated with 4E-BP1 early during PMA treatment demonstrated acute sequestration of cyclin D1 mRNA in 4E-BP1-associated complexes. This piece of evidence implies that PMA-induced hypophosphorylation of 4E-BP1 can acutely inhibit the processing of cyclin D1 transcript at early stages of translation initiation by blocking the primary function of eIF4E which is to form eIF4F complexes.

PMA-induced sequestration of eIF4E by 4E-BP1 does not appear to impact the secondary function of eIF4E in regulation of cyclin D1 message nucleocytoplasmic transport (Proud 2002 and Scheper and Proud 2002). Since the nuclear shuttling protein 4E-T binds to eIF4E at the same site that is bound by 4E-BP1, one would predict an acute disruption of eIF4E subcellular distribution upon PMA treatment, resulting in a decrease in the nuclear eIF4E population. Although changes in subcellular localization of eIF4E in response to PMA were not addressed in this study, the absence of alterations in the levels of nuclear and cytoplasmic cyclin D1 mRNA suggests that PMA-induced sequestration of eIF4E by 4E-BP1 may not be imposing an immediate impact on the subcellular distribution of eIF4E in this system.

Strong and sustained phosphorylation of eIF4E at Ser209 in a MAPK-dependent manner was another observation made in IEC-18 cells arrested in G1/G0 as a result of PKC activation/PMA treatment. As mentioned earlier, the role of this phosphorylation on translation initiation is unclear, although recent biophysical and biochemical data argue

that phospho-Ser209-eIF4E has reduced affinity for 5' capped oligonucleotides (Scheper and Proud, 2002). The strong and sustained nature of the induction of Ser209 phosphorylation becomes noteworthy in light of data from studies using another differentiation-committed cell line. In this study, the differentiation-inducing cytokine thrombopoietin (TPO) was shown to promote a high degree of Ser209-eIF4E phosphorylation in haematopoietic stem cells undergoing megakaryocyte differentiation (Caron et al., 2004). However, the physiological implications of the strong and sustained phosphorylation of eIF4E at Ser209 in IEC-18 cells arrested in G0 remain to be addressed in future studies.

The mechanisms mediating PKC-induced hypophosphorylation of 4E-BP1 in intestinal epithelial cells remain to be defined. Our preliminary data and published studies point to two possible pathways: (1) PKC-induced inhibition of Akt activity and of downstream signaling molecules regulating translation initiation, and (2) activation of protein phosphatase 2A (PP2A). Akt-signaling has been shown to be a major regulatory pathway responsible for the hierarchical phosphorylation of 4E-BP1 (Gingras et al., 1998, 1999, and 2001, Mothe-Satney et al., 2000; Herbert et al., 2002). Phosphorylation of 4E-BP1 is sensitive to rapamycin treatment in several systems, suggesting dependence of 4E-BP1 phosphorylation on mTOR signaling which is thought to lie downstream of Akt (Mamane et al., 2004, Richardson et al., 2004, and see Figure A.1). Activation of Akt and its downstream effector mTOR has been proposed to be responsible for the phosphorylation and inactivation of 4E-BP1 by various mitogenic stimuli (Richardson et al., 2004 and Browne and Proud, 2002). Conversely, loss of Akt signaling in response to

stress signals or mitogen-deprivation has been implicated in inactivation of mTOR kinase activity, which can also be lost upon nutrient- or energy-deprivation, leading to hypophosphorylation of 4E-BP1 (Richardson et al., 2004 and Browne and Proud, 2002). However, it should be noted that whether 4E-BP1 is an mTOR substrate is still subject to ongoing debate, because a large number of factors are co-precipitated in mTOR-associated complexes displaying kinase activity on 4E-BP1 (Proud 2002).

The rapid and transient loss of Akt signaling observed in IEC-18 cells in response to PMA treatment parallels observations in PC12 and LnCap cells (Zeng et al., 2000 and Tanaka et al., 2003). Although neither of these studies addressed whether PMA-induced loss of Akt activity led to hypophosphorylation of 4E-BP1 or changes in expression of cyclin D1, the effect in LnCap cells was shown to be dependent on PKC α . Whether hypophosphorylation of 4E-BP1 is a direct consequence of inactivation of Akt-regulated signaling in IEC-18 cells will be addressed using Akt constructs and by analysis of downstream intermediates in future studies (see Future Directions section below).

Another possible mediator of PKC α -induced 4E-BP1 hypophosphorylation is **PP2A**. As suggested by Tanaka and colleagues in LNCap cells (2003), studies in IEC-18 cells also indicate that PKC signaling may stimulate PP2A activity. Activation of PKC by PMA or Bryo was shown to result in increased expression and perinuclear accumulation of PP2A in these cells, suggesting changes in activity of the enzyme (Leontieva and Black 2004). Notably, PP2A has been shown to dephosphorylate 4E-BP1 in Jurkat cells (Peterson et al., 1999) and in neonatal cardiac myocytes challenged by oxidative stress

(Pham et al., 2000), as well as Akt in skeletal muscle myotubes (Cazzolli et al., 2001). Whether PKC α -activation promotes simultaneous dephosphorylation of both Akt and 4E-BP1 via activation of PP2A or PKC α -induced inactivation of Akt becomes reflected in loss of downstream signaling that is required for 4E-BP1 phosphorylation, has yet to be addressed. However, preliminary data showing recovery of Akt activity at a time (1-2 h) when 4E-BP1 is still heavily hypophosphorylated favors the possibility that PP2A only targets 4E-BP1 in IEC-18 cells (Guan, L.J., Frey, M.F., Hizli, A.A., and Black, J.D. unpublished data).

Our observations in IEC-18 cells parallel those in herbimycin A-treated MCF-7 cells (Muise-Helmericks et al., 1998), where decreased cyclin D1 synthesis is associated with loss of Akt signaling but not with changes in GSK-3 β activity or with cyclin D1 destabilization. Instead, as discussed extensively above, loss of Akt in IEC-18 cells is associated with hypophosphorylation of 4E-BP1, inhibition of cap-dependent translation initiation, and decreased synthesis of cyclin D1 protein. Notably, the changes observed in IEC-18 cells in response to PKC α signaling are similar to the reported effects of mTOR inhibition by rapamycin in pancreatic cancer cells (Grewe et al., 1998). However, other reported inhibitory consequences of rapamycin on cyclin D1 expression including decreased stability of cyclin D1 mRNA and protein were not observed in IEC-18 cells undergoing G1/G0 arrest in response to PKC activation (Hashemolhosseini et al., 1998).

p70S6K, another effector of Akt/mTOR signaling, has been implicated in regulation of cyclin D1 translation in MDA-MB-453 breast cancer cells (Koziczak and

Hynes 2004). p70S6K activity has been shown to be crucial for the translation of a subset of mRNAs which have a 5' terminal oligopyrimidine tract (**5' TOP**) sequence in their leader (Kawasome et al., 1998). Due to the presence of a putative 5' TOP sequence in the cyclin D1 mRNA leader, expression of cyclin D1 has been proposed to be dependent on the activity of p70S6K. Alterations in p70S6K activity in response to PKC activation will be addressed in future studies (see below).

V.A.2 PKC signaling inhibits translation elongation in IEC-18 cells

The mechanisms leading to inhibitory Thr56-phosphorylation of eEF2 following PKC/PKC α activation have yet to be delineated. We observed that PKC α activation in PKC δ/ϵ -depleted IEC-18 cells promoted moderate phosphorylation of eEF2 at Thr56, suggesting that inhibition of translation elongation is at least partially dependent on PKC α signaling. The decrease in magnitude of Thr56-eEF2 phosphorylation in PKC δ/ϵ -depleted IEC-18 cells may reflect (a) the involvement of PKC δ and/or ϵ in induction of this effect, or (b) the slightly reduced levels of PKC α in PKC δ/ϵ -depleted cells (Figure 40 and Frey et al., 2000a); these possibilities will be investigated in future studies. Although our preliminary analysis did not detect PKC-induced alterations in the status of inhibitory phosphorylation on Ser366 of eEF2K, the only kinase activity so far identified to be active on the Thr56-eEF2 substrate, we cannot exclude eEF2K as a mediator of PKC-induced inhibition of eEF2 because regulation of this kinase is complex, with its multiple phosphorylation sites under the control of various signaling pathways (Browne and Proud

2002 and see Figure A.1). Thus, additional studies are required to address the role of PKC signaling in control of the activity of eEF2K in IEC-18 cells.

IV.A.3 Evidence for the role of inhibition of cap-dependent translation initiation and translation elongation in PKC-induced downregulation of cyclin D1

In summary, both PKC-induced activation of 4E-BP1 and inhibition of eEF2 were kinetically consistent with the downregulation in cyclin D1 levels in IEC-18 cells. Polyribosome analysis, in turn, provided strong support for the impact of alterations in the function of both of these molecules on translation of cyclin D1 message. The polysome profile of cyclin D1 mRNA in untreated IEC-18 cells is consistent with the relatively poor translation of this message reported in other systems (Shi et al., 2004 and Graff and De Benedetti 2004). Consistent with its inhibitory effects on translation initiation and elongation, PKC signaling did not alter the polyribosomal profile of cyclin D1 mRNA in these cells.

IV.A.4 Tight regulation of cyclin D1 expression and of the translation machinery is crucial for maintenance of cell growth control

Regulation of cyclin D1 levels, confirmed to be a key control point for G1 phase progression in numerous cell systems, will likely remain an important focus of future studies, especially after the recent demonstration that cyclin D1/cdk4 may be a better target than cyclin E/cdk2 activity for inhibiting the proliferation of malignant cells (Tetsu and McCormick 2003). Studies in a number of systems have clearly shown that malignant transformation can involve deregulation of the expression of cyclin D1 at different levels, including transcription, mRNA stability, translation, or protein turnover, emphasizing the importance of cyclin D overexpression in malignant transformation. Indeed, overexpression of cyclin D1 is one of the most frequent abnormalities seen in a wide variety of human cancers including colon cancer (Weinstein, 2000).

Increasing attention is being focused on the important role of protein translation in the process of cellular transformation. In an exciting recent study, Rajasekhar et al., (2003) introduced the novel concept that alterations in polysome loading of oncogenic factors may be a primary event in tumor development, preceding transcriptional effects and alterations in mRNA levels. Analysis of global effects of oncogenic Ras and Akt signaling pathways highlighted that these pathways cooperate to alter the translation of a subset of mRNAs that are key regulators of cancer cell biology (Rajasekhar et al., 2003). Alterations in polysome loading of these mRNAs by oncogenic Ras or Akt signaling were shown to be far more rapid and extensive than changes in total mRNA levels,

suggesting that differential polysome loading of a subset of mitogenic mRNAs may be sufficient to initiate and maintain tumor growth (Rajasekhar et al., 2003). The authors further propose that transcriptional aberrations in genes involved in tumor initiation/maintenance may be generated as secondary effects by transcription factors whose mRNAs were more efficiently processed due to positive translational alterations. Another recent report demonstrating that persistent expression of cyclin D1 upon hormonal stimulus in breast cells is maintained by the recruitment of cyclin D1/cdk4 complexes to the cyclin D1 promoter (Cicatiello et al., 2004), allows one to contemplate how an initial blockade of translation of cyclin D1 message could lead to ultimate control of its transcription.

From this perspective, translational control of cyclin D1 gene expression emerges as a critical checkpoint for maintenance of normal tissue homeostasis. The importance of tight control of cyclin D1 expression is further highlighted by evidence from our laboratory and others that levels of the protein in normal proliferating intestinal crypt cells are extremely low relative to those in neoplastic intestinal tissue. Multiple mechanisms, including both transcriptional and translational control, may be responsible for maintaining cyclin D1 levels in check in normal cycling cells. The following section will discuss our findings indicating that PKC α signaling not only regulates acute downregulation of cyclin D1 upon induction of cell cycle exit, but also appears to act as a critical mechanism to control the accumulation of this molecule in cycling cells.

IV.B Keeping cyclin D1 expression in check by the presence of PKC α

Our results show that as PKC agonists deplete PKC protein from IEC-18 cells, thus diminishing PKC α -associated signaling, the inhibitory impact on translation initiation (i.e., hypophosphorylation of 4E-BP1) and translation elongation, (i.e. phosphorylation of eEF2 at Thr56), is gradually reversed. This gradual recovery of function in the protein synthetic machinery is closely accompanied by an increase in cyclin D1 expression, from minimal back to control levels (around 3-4 h). However, continued exposure to PKC agonists results in further increases in cyclin D1 protein abundance, with levels ultimately surpassing those normally seen in control cells by several fold. Notably, silencing of individual PKC isozymes using RNA interference technology indicated that loss of PKC α , but not of PKC δ or ϵ , is responsible for this effect. Biosynthetic labeling analysis of cyclin D1 between 4 h 15 min and 6 h of PMA treatment demonstrated enhanced incorporation of label into cyclin D1 in PMA-treated cells relative to vehicle-treated controls, in the presence of an increase in cyclin D1 mRNA levels. These data point to changes in cyclin D1 transcription or mRNA stability as the mechanism(s) responsible for maintaining overproduction of cyclin D1 protein upon loss of PKC/PKC α signaling. Consistent with this notion, recent findings (Pysz, M.A., Hizli, A.A., Black, A.R., and Black, J.D.) demonstrated that specific silencing of PKC α expression in IEC-18 cells also results in elevated cyclin D1 mRNA levels, suggesting a role for PKC α signaling in regulation of cyclin D1 transcription or mRNA stability. Since preliminary data (Pysz, M.A., Black, A.R., and Black, J.D.) failed to show alterations in cyclin D1 protein stability at late times of PMA treatment, it is possible that

other post-transcriptional mechanisms, such as cap-independent translation or mRNA transport, may cooperate with the increase in cyclin D1 message levels to sustain hyperinduced levels of cyclin D1 in the absence of PKC α signaling.

In this study, candidate pathways that may be involved in hyperinduction of cyclin D1 have not been extensively investigated. A mechanism that may account for increased cyclin D1 synthesis in PKC α -depleted cells is enhanced function of an IRES element in the 5' UTR of cyclin D1 mRNA which was recently shown to be capable of mediating cap-independent translation of the protein (Shi et al., 2005). The activity of this IRES is negatively regulated by Akt signaling; thus, its function is stimulated when levels of active Akt are relatively low. Since our recent data show that PMA has a biphasic effect on Akt activity in IEC-18 cells and levels of phospho-Ser473-Akt are particularly low following ~ 4 h of treatment (Guan, L.J. and Black, J.D., data not shown), it is possible that an IRES-dependent mechanism becomes operative to enhance synthesis of cyclin D1 at later times of PMA treatment. It would be interesting to test whether the bicistronic constructs used in the studies by Shi et al. (2005), in which the 5' cyclin D1 leader is flanked by Renilla luciferase and Firefly luciferase, would show differential activity in control and PKC-depleted cells (Shi et al., 2005).

A second possible mechanism that could account for enhanced cyclin D1 synthesis in PKC α -depleted cells is increased expression of eIF4E, a phenomenon that has been reported to occur in colon tumors (Rosenwald et al., 1999). If changes in eIF4E levels are confirmed to occur following prolonged treatment of IEC-18 cells with PKC

agonists, the enhanced synthesis of cyclin D1 in the absence of changes in mRNA levels could be explained by increased primary (formation of competent eIF4F complexes) and secondary (enhanced nucleocytoplasmic transport of cyclin D1 mRNA) functions of eIF4E, which is liberated from the control of 4E-BP1 as a result of loss of PKC α signaling. Interestingly, both loss of PKC α and increased levels of eIF4E are recognized early events in colon carcinogenesis (Black 2001 and Rosenwald et al., 1999), and both appear to serve the purpose of sustaining elevated levels of cyclin D1.

IV.C Maintenance of high levels of cyclin D1 may provide the basis for frequent loss of PKC α expression in colon tumorigenesis

In this study, we show that sustained elevation of cyclin D1 protein in PKC-depleted IEC-18 cells is associated with a shortened cell doubling-time. Although the cells lacked expression of all of the phorbol ester-responsive PKC isozymes, our evidence that specific knock-down of PKC α , but not of PKC δ or PKC ϵ , results in increased cyclin D1 levels indicates that the ability of these cells to maintain aberrant cyclin D1 expression is due to the absence of PKC α signaling. The ability of the PKC α -selective inhibitor, Gö6976, to promote induction of cyclin D1 further supports the notion that PKC α is the isozyme in control of cyclin D1 expression in intestinal cells. Analysis of *Apc*^{Min} mouse adenomas and a panel of rodent and human colon adenocarcinoma cell lines for PKC α and cyclin D1 expression also revealed a reciprocal relationship between the expression of these protein, strengthening the view that PKC α exerts negative control on cyclin D1 expression in intestinal cells.

As was summarized in the Introduction section, loss of PKC α expression is seen frequently in colon tumors from patients. Furthermore, manipulation of PKC α levels/activity in cell lines derived from these tumors revealed that inhibition of PKC α signaling increases the tumorigenic potential of these cells, while enhanced PKC α activity has the opposite effect (Suga et al., 1998 and Scaglione-Sewell et al. 1998). We believe that findings presented in this study offer a molecular basis for the loss of PKC α during intestinal carcinogenesis. Among the characterized G0-related molecular events induced by PKC α (Frey et al., 2000a), the importance of loss of cyclin D1 is highlighted by several findings regarding the role of cyclin D1 in colon tumorigenesis (Arber et al., 1997 and Hult et al., 2004). For example, expression of cyclin D1 antisense cDNA constructs in SW480 human colon adenocarcinoma cells inhibited their growth and tumorigenicity (Arber et al., 1997). Furthermore, by crossing *Apc*^{Min} mice to cyclin D1 *nulls*, Pestell and colleagues showed that cyclin D1 heterozygosity confers tumor-resistance in this model, as reflected by a decrease in the number of adenomas and in the rate of adenoma formation induced by mutation of the *Apc* gene. Moreover, cyclin D1 deficiency overcame the inhibition of colonic epithelial cell differentiation that is induced by activation of the Apc/ β -catenin pathway, demonstrating that cyclin D1 functions to block differentiation and to promote DNA synthesis in the transformed intestinal epithelium (Hult et al., 2004). Thus, by establishing the importance of elevating cyclin D1 levels for the process of intestinal tumorigenesis, these results can also offer a perspective on the frequent loss of PKC α expression, whose presence would otherwise

exert a negative effect on cyclin D1 translation/accumulation (Hulit et. al., 2004 and Hizli et. al., in preparation).

IV.D Future Directions

IV.D.1 Addressing the role of loss of Akt signaling in mediating PKC-induced inhibitory effects on translation and cell cycle progression of intestinal epithelial cells

Findings in this study have brought up several questions regarding PKC α -mediated negative regulation of cyclin D1 expression in the intestinal epithelium. As we have already pointed out, the translational repressive effects of PKC α signaling on cyclin D1 expression may involve its modulatory effects on Akt signaling. Therefore, the role of loss of Akt activity in inhibition of translation initiation (hypophosphorylation of 4E-BP1) and translation elongation (induction of phospho-Thr56-eEF2), will be investigated using an inducible constitutively active Akt expression system in IEC-18 cells.

Whether PKC α -induced loss of Akt signaling is reflected in changes in the activity of downstream players such as mTOR and p70S6K also remains to be determined. If p70S6K is shown to be inactivated in response to PMA, it will be worth addressing whether this decrease is a consequence of decreased mTOR activity or increased PP2A activity, which has been shown to promote dephosphorylation of p70S6K in other systems (Peterson et al., 1999). A recent report on regulation of eEF2K

demonstrated that the inhibitory phosphorylation at Ser366 can be mediated by p70S6K (Wang et al., 2001).

As pointed out earlier, the effects of PKC activation on eEF2K activity remain to be determined. If activation of eEF2K in response to PMA treatment correlates with increased phospho-Thr56-eEF2 accumulation, it will be worth examining whether activation of eEF2K is possibly linked to a PMA-induced inactivation of p70S6K. Since both loss of p70S6K (in MDA-MB-453 breast cancer cells, Koziczak and Hynes, 2004) and loss of eEF2 activity (T-lymphocytes, Gutzkow et al., 2003 and IEC-18 cells, Hizli et al., in preparation) are implicated in negative control of cyclin D1 and cyclin D3 synthesis, respectively, it would be interesting to address whether attenuation of p70S6K activity particularly impacts elongation of cyclin D1 in IEC-18 cells.

Next, it would be interesting to determine whether inducible constitutively active Akt can abrogate PKC-induced growth arrest by rescuing the expression of cyclin D1. This analysis could throw some light on the question of whether the ability of PKC/PKC α to reduce cyclin D1 levels or to induce p21^{CIP1/WAF1} is the pivotal event mediating the G0/G1 arrest in IEC-18 cells. If cyclin D1 levels are protected under sustained Akt signaling, would p21^{CIP1/WAF1} be sequestered in cdk4 complexes to allow continued cyclin E/cdk2 activity, or would the surplus of p21^{CIP1/WAF1} induced by PKC activation be sufficient for sequestration by the cyclin D1/cdk4/6 complexes and inhibition of cyclin E/cdk2 activity?

IV.D.2 Addressing other consequences of loss of PKC α signaling that may contribute to cellular transformation

We have shown that elevation of cyclin D1 steady state levels upon sustained depletion of PKC/PKC α correlates with enhanced proliferation of IEC-18 cells. It would be worthwhile to address whether cells under these conditions acquire other transformation-related properties such as anchorage-independent growth, failure to contact inhibit, or resistance to mitogen withdrawal-induced cell cycle arrest.

IV.D.3 Addressing the anti-tumorigenic effects of PKC α overexpression in intestinal tissue using animal models

Based on the information on the suppressive effects of PKC α on tumorigenicity of colon tumor cell lines (Scaglione-Sewell et al., 1998 and Batlle et al., 1998) and the tumor-resistant properties conferred by cyclin D1 deficiency in *Apc^{Min}* mouse intestinal cells, it would be interesting to determine whether targeted overexpression of PKC α in the intestine can elicit the same effects as loss of cyclin D1 in a background of activated Apc/ β -catenin signaling. Therefore, crossing of *Apc^{Min}* mice and mice overexpressing PKC α in the intestine (*Apc^{Min}/PKC α .O.E.*) could not only offer *in vivo* confirmation of the findings in this study, but may also prove the anti-tumorigenic effects of PKC α -signaling on intestinal carcinogenesis. We would expect to find similar phenotypic changes, including decreased size/number of intestinal adenomas and increased intestinal epithelial cell differentiation in the *Apc^{Min}/PKC α .O.E.* model to those observed in

Apc^{Min}/cyclin D1^(+/-) mice. Based on the repressive function of PKC α signaling on translation of cyclin D1 expression, we propose that ectopic PKC α expression would antagonize the effects of β -catenin-induced cyclin D1 transcription resulting in insufficient levels of cyclin D1 expression for intestinal transformation. These studies, together with further dissection of the molecular circuitry downstream of PKC α -signaling, would not only improve our understanding of intestinal carcinogenesis but may also reveal novel targets for intervention in the prevention or therapy of intestinal cancer.

Appendix A PI3K/Akt and mTOR-signaling as major pathways in regulation of the translation machinery

In the current model, binding of mitogens to their target receptor tyrosine kinases can initiate two major signaling cascades of cellular growth, the Ras/Erk and PI-3K signaling pathways (Holland et. al., 2004). Activation of Ras leads to the stimulation of Erk 1/2, a member of the MAP kinase pathway, which positively regulates cyclin D1 transcription.

We now know that the PI3K and downstream mTOR-signaling pathways can modulate translation of cyclin D1, likely due to their effects on 4E-BP1 and p70S6 kinase (Jirmanova et. al. 2002, Kawasome et. al. 1998, Muis-Helmericks et. al. 1998). PI3K-signaling plays a key role in cell biology because it can influence cell survival and death, cell growth, motility, differentiation and insulin action. Dysregulation of this pathway has been associated with various types of human cancer and with diabetes (Toker and Newton, 2000).

Figure A.1

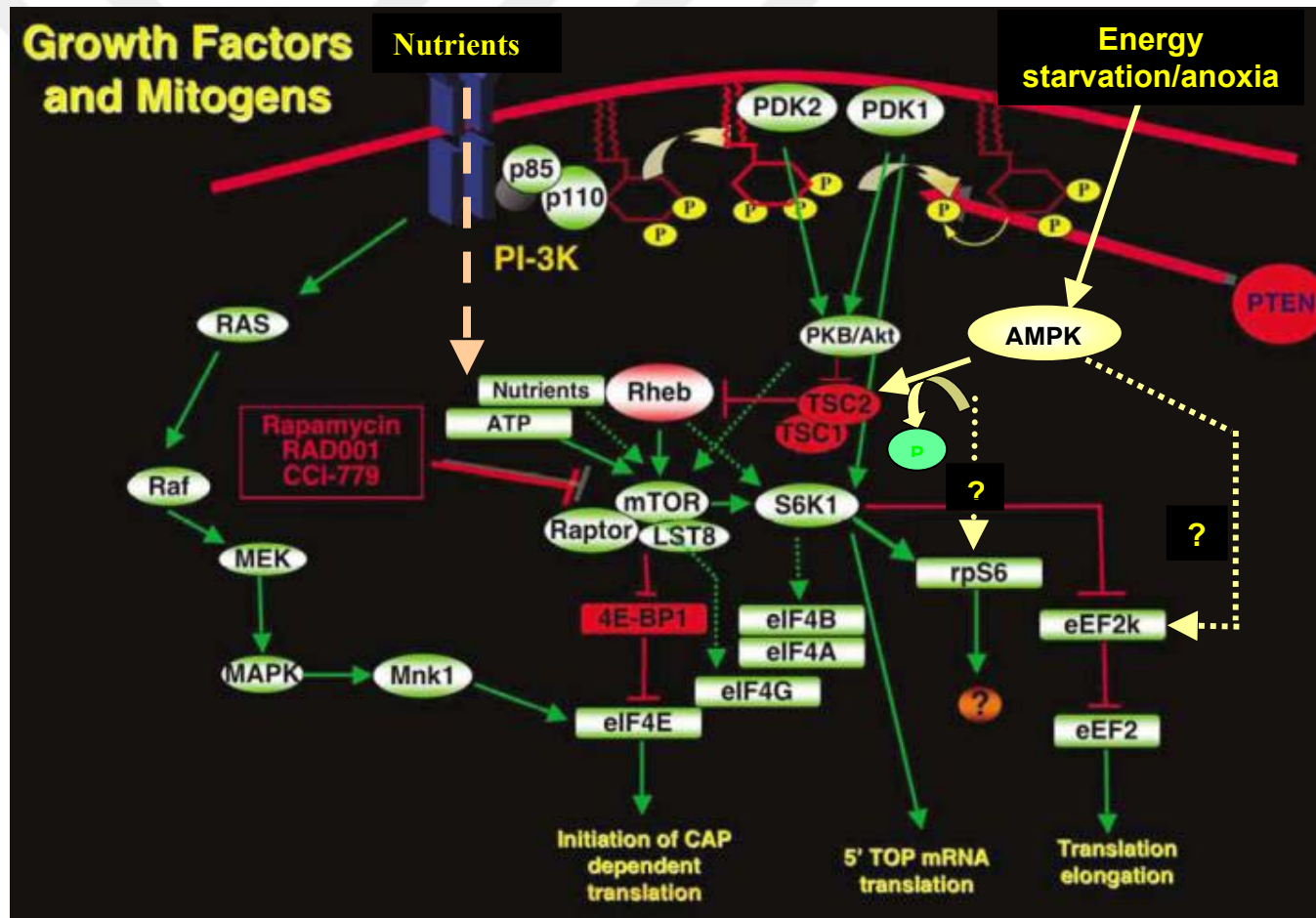


Figure A.1 PI3K and mTOR signaling pathways modulate components of the translation machinery Ras, PI3K, and signaling pathways regulated by nutrient and energy status converging on mTOR are depicted in terms of their ultimate effects on components of the translation machinery. The signal transduction events in each pathway are described in detail in text. (The figure is adapted from Holland et. al., 2004 and Mamane et. al., 2004)

As illustrated in Figures 7 (in the Introduction) and A.1, in response to various growth-promoting cellular signals, PI-3K catalyzes the accumulation of secondary messenger lipids by phosphorylating the 3'-OH position of phosphatidylinositols. The modified lipids, particularly PtdIns-3,4,5-P₃ and PtdIns-3,4-P₂, bind with high affinity to the PH domain of Akt/PKB, leading to engagement of Akt to the membrane through this PH domain. As a result of membrane tethering, Akt undergoes a conformational change which results in relief of active site autoinhibition. This allows phosphorylation of the active site (Thr308) and of the hydrophobic site in the C-terminus (Ser473). Phosphorylation of these sites is both mitogen- and PI3K-dependent.

Modification of Thr308 is catalyzed by 3-phosphatidylinositide-dependent kinase (PDK-1), which appears to be constitutively active and does not depend directly on PI3K for activity (Toker and Newton, 2000, Newton 2003). The name PDK-1 reflects the dependence of its *first*-identified substrate, Akt, on PI3K for activation (Newton 2003). Other known substrates for PDK-1, including PKC, PAK, SGK, p70S6K, RSK, and PRK, also undergo conformational changes, provided through interactions with other factors, in order to become phosphorylated by the enzyme. The lipid phosphatase, phosphatase and tensin homologue deleted from chromosome 10 (PTEN), which dephosphorylates (PtdIns-3,4,5-P₃) and (PtdIns-3,4-P₂), opposes signaling mediated by PI3K (Richardson et. al. 2004 and Figure A.1).

Active Akt phosphorylates TSC2 in the tuberous sclerosis complex (TSC) tumor suppressor; this phosphorylation destabilizes and inactivates the complex due to

disruption of the association of the TSC complex members TSC1(hamartin) and TSC2 (tuberin) (Mamane et. al. 2004 and Richardson et. al., 2004). TSC2-mediated inhibition of the small G protein Rheb is abrogated due to termination of the GTPase Activating Protein (GAP) activity of TSC2, leading to Rheb-mediated activation of mTOR through an as yet unknown mechanism (Mamane et. al. 2004). mTOR is a large protein (280-300 kDa) containing a C-terminal kinase domain, several protein-protein interaction surfaces in a large N-terminal domain, FAT/FATC domains which are characteristic of PI3-kinase-related protein kinase (PIKK) family members and thought to serve a scaffolding function, and an FRB domain where the inhibitory complex of FKBP12 and rapamycin binds.

mTOR, a phosphatidylinositol kinase-related kinase, is also regulated by nutrients (amino acids and glucose) and AMPK (Mamane et. al., 2004). Although the C-terminus of mTOR has sequence homology to lipid kinases, (e.g. PI3K), it phosphorylates Ser/Thr residues in proteins rather than lipids (Mamane et. al., 2004 and Richardson et. al. 2004). mTOR is part of a gigantic protein complex (approximately 1 megadaltons) including two other known components, raptor and GβL, which promotes the phosphorylation of p70S6K and the hierarchical phosphorylation of 4E-BP1 (Mamane et. al., 2004 and Holland et. al., 2004). Activated p70S6K phosphorylates the ribosomal protein S6 and eIF4B. These events correlate with the increased translation of a subset of so-called 5' TOP mRNAs, which encode components of the translation machinery such as ribosomal proteins, elongation factors, and poly(A)-binding protein (PABP) (Mamane et. al., 2004).

Since TOR was initially cloned in yeast and later in fungi, plants, worms, flies, and mammals, it has also been given the following names; FKBP12-rapamycin-associated protein (**FRAP**), rapamycin and FKBP12 target (**RAFT**), or rapamycin target (**RAPT**) (Mamane et. al., 2004). Rapamycin, and its derivatives such as CCI-779 (Wyeth-Ayerst) and RAD001 (Novartis) specifically interact with the immunophilin FK506-binding protein-12 (**FKBP12**). This complex directly binds and inhibits mTOR signaling. These compounds are currently in clinical trials due to their potent anti-tumor activity in several cancer types.

mTOR is not responsive to as wide a range of mitogenic signals as PI3K. It is primarily recognized as a sensor of nutrients and energy fluctuations. Changes in amino acid levels -particularly leucine, isoleucine, and valine- are immediately reflected in alterations in the activity of mTOR, and thereby of the protein synthetic machinery. Similarly, reduction of cellular ATP levels by 2-deoxyglucose (**2-DG**) leads to inhibition of mTOR-dependent phosphorylation of p70S6K and 4E-BP1. The sensor of cellular energy levels AMPK has recently been shown to inhibit mTOR upon energy shortage. Increases in the ratio of AMP/ATP are sensed by AMPK which, in turn, reduces ATP expenditure by inhibiting key enzymes of the biosynthetic pathways, activates enzymes that produce ATP, and stimulates glucose uptake (Richardson et. al. 2004). Thus, interplay between mTOR and PI3K signaling has been proposed to synchronize inputs from cellular growth pathways with proliferative mitogenic signaling, allowing growth only under conditions of adequate nutrients and energy.

The interplay between the so-called nutritional checkpoint, mTOR, and growth factor and hormone regulated-PI3K pathways has been addressed in numerous studies. The plethora of information coming from these studies suggests that connection between the two crucial pathways is rather complex and not linear. Currently, three major points of convergence between the PI3K and mTOR signaling pathways have been proposed (Richardson et. al., 2004): 1) The TSC complex, which negatively modulates mTOR and its downstream signaling events, is linked to the PI3K pathway by Akt. 2) Ser2448 near the C-terminus of mTOR, which seems to act as a repressor domain, is a direct phosphorylation target for Akt. 3) Phosphorylation of both 4E-BP1 and p70S6K as indicated by the evidence that inhibitors of both pathways block these modifications (Richardson et. al., 2004, and references therein).

Appendix B Optimization of the cyclin D1 antibodies raised in rat for Western blot and IP protocols

We confirmed that PMA induces a decrease in cyclin D1 protein by analyzing protein samples treated with vehicle or 100 nM PMA for 1 h using three additional anti-cyclin D1 antibodies. These antibodies were generated against peptides mapping to different parts of the mouse cyclin D1 sequence, and were raised in rat: (1) D1 (10G319) is referred to as **Ab A**, (2) D1 17A6-4 is referred to as **Ab B**, and (3) D1 (18B625) is referred to as **Ab C**. Since all of the antibodies were sent as ammonium sulfate precipitates (therefore, stable at 4 °C indefinitely), we dialyzed 100 µL aliquots as needed because (a) following dialysis, there was always a 2-2.5 fold increase in the volume of the purified antibodies, and (b) none of the purified antibodies were stable at 4 °C for longer than 1-2 months.

Antibodies were dialyzed against cold sterile PBS using Slide-A-Lyzer cassettes from Pierce. It should be noted that the manufacturer's instructions for preparation of the cassettes for the dialysis procedure included a 45-second membrane-hydration step in PBS; however, submerging the cassettes in PBS for this long resulted in deformation of the membranes and leakage of the samples. Therefore, this step was performed by briefly dipping the membrane in PBS for 2-3 seconds. For the rest of the dialysis protocol, all instructions given by the manufacturer were followed. After injecting samples into the dialysis cassettes, they were placed in pre-baked 500 ml beakers filled with sterile PBS and transferred to the cold room where they were kept with constant stirring until dialysis

was complete. The PBS in the beaker was changed two times at 2 h intervals, and after the third change dialysis was allowed to continue overnight.

Although all three antibodies performed somewhat satisfactorily in Western blots, all of them performed very poorly in immunoprecipitation protocols because of high background (see Figure B.1). Since Ab B detected cyclin D1 very weakly in IEC-18 lysates even at a 1:200 dilution, immunoblotting conditions for this antibody were not optimized any further. Good quality detection was obtained within 10-second exposures using Ab A at a 1:500 dilution in combination with secondary antibody from Santa Cruz (sc-2006) [goat anti-rat IgG HRP at (1:2000) dilution]. Ab C detected cyclin D1 within 6 min (or more) exposures when used at a 1:400 dilution in combination with goat anti-rat secondary (sc-2006) at (1:2000). In summary, these antibodies were useful tools to confirm our observations on changes in cyclin D1 levels upon activation of PKC in IEC-18 cells. However, they were not used routinely in our studies due to their low concentrations, reduced stability, and short storage life compared with the commercially available alternative (sc-753), which produced essentially identical results.

Figure B.1

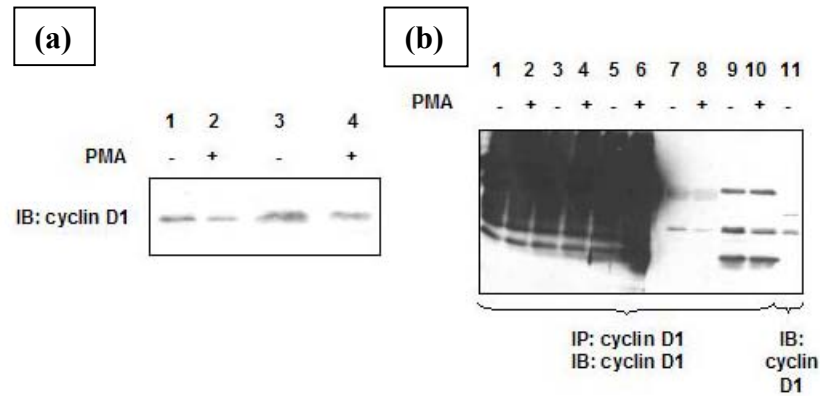


Figure B.1 Optimization of cyclin D1 antibodies raised in rat for Western blot and IP protocols (a) Subconfluent IEC-18 cells were treated in duplicates with 100 nM PMA for 1 h and harvested in boiling 1% SDS lysis buffer. Samples were immunoblotted for cyclin D1 using the monoclonal rat anti-cyclin D1 antibodies (Ab A lanes 1 and 2, Ab C lanes 3 and 4). Note that the signal for cyclin D1 using Ab A (in lane 1 and 2) was detected within 10 seconds whereas that obtained with Ab C (in lane 3 and 4) was detected in 6 min. (b) Subconfluent IEC-18 cells were treated in duplicates with 100 nM PMA for 1 h, and cells were harvested under cold lysis conditions for the IP procedure. Cyclin D1 was immunoprecipitated from control and 1 h PMA-treated samples using the following anti-cyclin D1 antibodies: Lane 1-2 Ab A, lane 3-4 Ab B, lane 5-6 Ab C, lane 7-8 sc-753, and lane 9-10 sc-450. Sample in lane 11 is a WCL included as a control for the immunoblotting of cyclin D1. Note that to avoid cross-reactivity of antibodies used in IPs with those used in immunoblotting, the membrane was cut between lanes 6 and 7, and lanes 1-6 were immunoblotted with rabbit polyclonal anti-cyclin D1 antibody (sc-753) while lanes 7-10 were immunoblotted with rat monoclonal anti-cyclin D1 (Ab A).

Appendix C Survey of GSK-3 β activity in response to PMA treatment with additional antibodies

As already presented in the Results section, we did not detect any changes in the phosphorylation of GSK-3 β at Ser9 in protein samples collected at early times of PMA treatment, despite evidence that Akt was inactivated by PKC activation at these times (indicated by a marked reduction in phospho-Ser473-Akt levels) (Figure B.1). These findings are in contrast to the effects of serum-starvation that led to a significant decrease in phospho-Ser9-GSK3 β in IEC-18 cells (Frey, M.F., data not shown). Furthermore, this observation did not reconcile with the characterized relationship between Akt signaling and GSK-3 β activity in other cell lines, where activated Akt phosphorylates GSK-3 β at Ser9 and inhibits its activity and, conversely, loss of Akt activity is linked to GSK-3 β activation (Cross et al., 1995 and Stambolic et al., 1996). To confirm the data obtained with phospho-Ser473-Akt and phospho-Ser9-GSK-3 β rabbit polyclonal antibodies (Cell Signaling), we used a phospho-(Ser21/9)-GSK-3 α/β rabbit polyclonal antibody that recognizes the inhibitory phosphorylations in both of the GSK3 isozymes (α and β), and is known to be more sensitive to phosphorylation differences at this site than phospho-Ser9-GSK-3 β (Dr. Livingstone, personal communication) (Figure C.1). Phospho-(Ser21/9)-GSK-3 α/β antibody detected two bands in lysates from IEC-18 cells, the slower migrating band representing the phospho-Ser21-GSK-3 α and the faster migrating band representing the phospho-Ser9-GSK-3 β . Like the phospho-Ser9-GSK-3 β antibody, the dual recognition antibody did not demonstrate any significant change in the activation status of GSK-3 β at early times of PMA treatment (lower band in Figure C.1c).

According to the manufacturers of phospho-Ser9-GSK-3 β and phospho-Ser473-Akt rabbit polyclonal antibodies, these reagents show decreased phospho-Ser-473-Akt accompanied by decreased phospho-Ser9-GSK-3 β -indicative of GSK-3 β activation- only in NIH-3T3 cells. Analysis of Jurkat cells or other cell lines gave similar results to those presented here for IEC-18 cells, i.e., inactivation of Akt did not coincide with activation of GSK-3 β (Dr. Livingstone, personal communication). One explanation for the lack of coupling of inactive Akt with activation of GSK-3 β could be that GSK-3 β activation requires additional events in these epithelial cells, such as activation of other phosphatases, which are not required in NIH-3T3 cells (Dr. Mark Livingstone, personal communication). We also confirmed the phospho-specificity of phospho-(Ser21/9)-GSK-3 α/β antibody by performing control CIP-treatment as described in Appendix D. We conclude that, despite its profound negative effects on Akt activity, PKC activation in IEC-18 cells does not induce changes in the activity of GSK-3 β , suggesting that activation of GSK-3 β in these cells requires additional events in the absence of Akt signaling.

Figure C.1

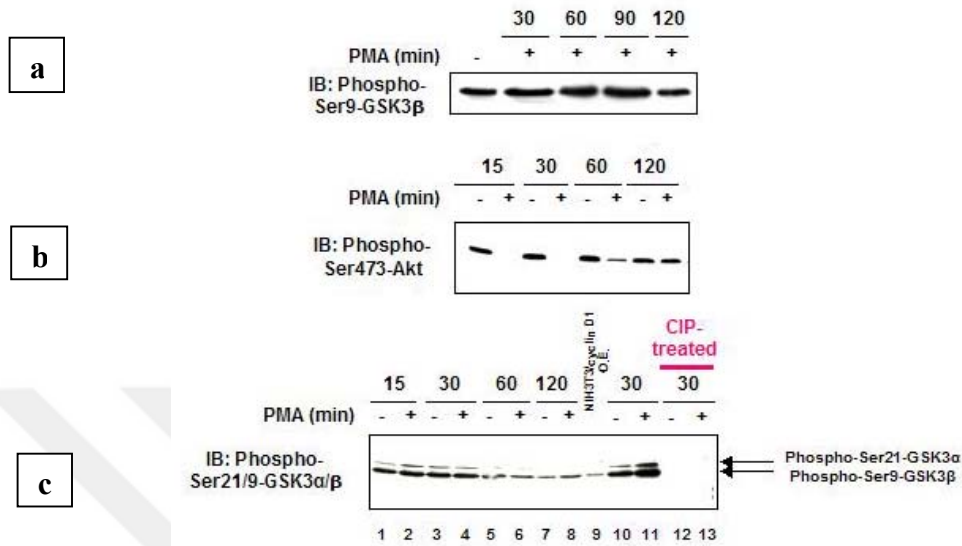


Figure C.1 PMA does not change the inhibitory phosphorylation on GSK-3 β (a)

Subconfluent IEC-18 cells were treated with 100 nM PMA for the indicated times.

Protein lysates were immunoblotted for phospho-Ser9-GSK-3 β . **(b)** Subconfluent cells were treated with 100 nM PMA for the indicated times. Protein lysates were

immunoblotted for phospho-Ser473-Akt. **(c)** Immunoblot analysis with the phospho-Ser21/9-GSK-3 α/β rabbit polyclonal antibody was performed on samples from cells

treated with 100 nM PMA for the indicated times (lanes 1-8), a protein lysate from NIH-3T3 cells overexpressing cyclin D1 (lane 9), cells treated with vehicle or 100 nM PMA

for 30 min from another experiment (lanes 10 and 11), and duplicate loading of the samples in lanes 10 and 11, which were treated with CIP (as described in Appendix B).

Arrows on the right point to the migration positions of phospho-Ser21-GSK-3 α and phospho-Ser9-GSK-3 β .

Appendix D Calf Intestinal Phosphatase (**CIP**) treatment as a control for phospho-Thr56-eEF2 and phospho-Ser366-eEF2K rabbit polyclonal antibodies

To confirm the phospho-specificity of phospho-Thr56-eEF2 and phospho-Ser366-eEF2K rabbit polyclonal antibodies, we performed a CIP treatment as a control reaction based on the instructions of the manufacturer (Cell Signaling). After resolving the samples in 7.5% SDS-PAGE gels, proteins were transferred onto 0.2 μ m nitrocellulose membrane. The segment of the membrane spanning from 75-250 kDa (both eEF2 and eEF2K are ~100 kDa) and lanes 5-8 and 8-11 were cut out and washed three times in 1X TBS, while the rest of the membrane pieces were saved in 1X TBS (Figure D.1). Following the three 1X TBS washes, the membrane pieces containing lanes 5-8 and 8-11 were incubated with CIP (5%; first 75% of the total amount of CIP recommended, New England Biolabs) in buffer #3 (NEB product M0290) at 37 °C for 24 h, and the remaining 25% of the CIP required was then added for an additional 3-4 h. After CIP treatment, the membrane pieces were washed in 1X TBS before being incubated in blocking buffer for 30 min at 37 °C. The membrane pieces covering lanes 1-8 were then immunoblotted for phospho-Thr56-eEF2 while the ones covering lanes 8-13 were immunoblotted for phospho-Ser366-eEF2K as described in Materials and Methods section, and the whole membrane was reconstructed before performing the chemiluminescence reaction (Figure D.1). Both the phospho-Thr56-eEF2 and the phospho-Ser366-eEF2K antibodies showed little or no reactivity in the portion of the membrane treated with the CIP (lanes 6 and 7, and 9 and 10), although they recognized the appropriate bands in the non-CIP-treated portion of the membrane (lanes 1-4 and 12-13). Thus, the CIP treatment confirmed the

phospho-specificity of both phospho-Thr56-eEF2 and phospho-Ser366-eEF2K rabbit polyclonal antibodies.

Figure D.1

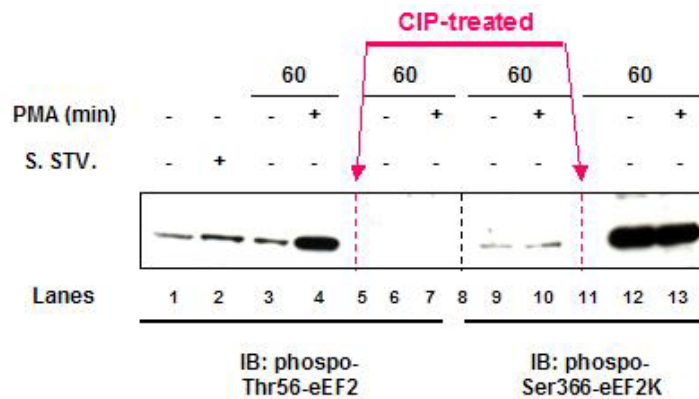


Figure D.1 Control CIP-reaction to confirm the phospho-specificity of phospho-Thr56-eEF2 and phospho-Ser366-eEF2K rabbit polyclonal antibodies The immunoblot for phospho-Thr56-eEF2 and phospho-Ser366-eEF2K presented in this figure includes the following samples: Lysates from cycling and serum-starved IEC-18 cells (Lane 1 and 2) (loaded once), and cells treated with vehicle or 100 nM PMA for 1 h (loaded four times) (lanes 3, 4, 6, 7, 9, 10, 12, and 13). The membrane was cut at the dashed lines and samples in lanes 6 and 7, and 9 and 10 were subjected to the CIP reaction before being immunoblotted with phospho-Thr56-eEF2 and phospho-Ser366-eEF2K antibodies, respectively. Note that lanes 5, 8, and 11 were loaded with 1X Laemmli Sample Buffer to avoid cutting of the lanes carrying a sample.

Appendix E Proteasome is involved both in regular turnover of cyclin D1 and PKC-induced downregulation of cyclin D1 in intestinal epithelial cells

To address whether PKC activation leads to enhanced turnover of cyclin D1 protein via activation of the proteasome pathway, we tested the ability of a panel of known proteasome inhibitors including lactacystin, MG132 (Figure E.1), and ALLN (data not shown) to block PKC-induced downregulation of cyclin D1. Each proteasome inhibitor appeared to block PMA-induced loss of cyclin D1 (compare lanes 1, 2, 4, and 6), while promoting accumulation of cyclin D1 by themselves (lanes 3 and 5). These results suggested that the proteasome degradation pathway is involved in PKC-mediated downregulation of cyclin D1 and, therefore, that there could be change in the stability of D1 protein as a result of activation of PKC-signaling. Furthermore, the ability of proteasome inhibitors to promote accumulation of cyclin D1 suggested a role for the proteasome degradation pathway in normal turnover of cyclin D1 in IEC-18 cells (compare lanes 1, 3 and 5 in Figure E.1). Since most specific proteasome inhibitor available, Lactacystin, blocked PKC-induced downregulation of cyclin D1, these results further ruled out the involvement of calpain protease, which was previously reported to mediate increased cyclin D1 proteolysis in actinomycin D and lovastatin-treated breast and prostate cancer cells and in serum starved NIH 3T3 cells (Choi et al., 1997).

Figure E.1

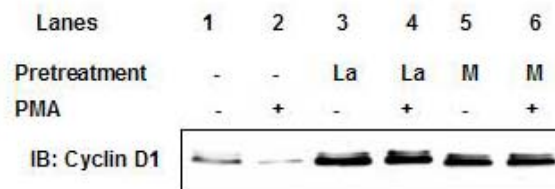


Figure E.1 Proteasome Inhibitors block PKC-induced downregulation of cyclin D1 protein Subconfluent cell populations were treated with 25 μ M Lactacystin or 25 μ M MG132 for 30 min prior to the addition of 100 nM PMA for 2 h. Cells were harvested in boiling 1% SDS lysis buffer and 30 μ g of each protein sample were immunoblotted for cyclin D1.

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