İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY AGAINST P60-KATANIN

Ph.D. Thesis by
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Programme : Molecular Biology-Genetics and Biotechnology

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P60-KATANİN'E KARŞI MONOKLONAL ANTİKOR ÜRETİLMESİ VE KARAKTERİZASYONU

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FOREWORD

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ABBREVIATIONS

µg : Microgram
µl : Microliter
aa : Amino acid
AAA : ATPases associated with diverse cellular activities
ADP : Adenosine diphosphate
AP : Alkaline phosphatase
APS : Ammonium persulfate
ATP : Adenosine triphosphate
ATPase : Adenosine triphosphatease
BCIP : 5-bromo-4-chloro-3-indolyl phosphate
BSA : Bovine serum albumine
C : Constant region
CBB : Coomassie brilliant blue
CH : Constant region of heavy chain
CL : Constant region of light chain
cm : Centimeter
cm^2 : Centimeter square
Da : Dalton
DMEM : Dulbecco's modified Eagle medium
DMSO : Dimethyl sulfoxide
DTT : Dithiothreitol
E.coli : *Escherichia coli*
EB : Elution buffer
ELISA : Enzyme-linked immunosorbent assay
FBS : Fetal bovine berum
FCA : Freund's complete adjuvant
g : Gram
GDP : Guanosine tri-phophate
GTP : Guanosine tri-phosphate
HAT : Hypoxanthine aminopterin thymidine
His : Histidine
HGPRT : Hypoxanthine-guanine phosphoribosyltransferase
HRP : Horseradish peroxidise
HT : Hypoxanthine thymidine
IFA : Incomplete freund's adjuvant
IgA : Immunoglobulin A
IgD : Immunoglobulin D
IgE : Immunoglobulin E
IgG : Immunoglobulin G
IgM : Immunoglobulin M
IPTG : Isopropyl β-D-1-thiogalactopyranoside
kDa : Kilo dalton
L : Liter
LB : Luria-bertani broth

M : Molar
mA : Miliampere
MAb : Monoclonal antibody
MAbs : Monoclonal antibodies
MAPs : Microtubule-associated proteins
mg : Miligram
min : Minute
ml : Mililiter
mM : Milimolar
mm : Milimeter
Ni-NTA : Nickel-nitriloacetic acid
ng : Nanogram
nm : Nanometer
nM : Nanomolar
RecP60 : Recombinant P60-katanin
nRecP60 : New recombinant P60-katanin
OD : Optical Density
PBS : Phosphate buffered saline
PBS-T : Phosphate buffered saline-tween 20
PEG : Polyethylene glycol
pH : Power of hydrogen
PNPP : Para-nitrophenylphosphate
PVDF : Polyvinylidine di-fluoride
rpm : Revolutions per minute
SDS : Sodium dodecyl sulphate
SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec : Second
SOC : Super optimal broth with catabolite repression
SPECT : Single photon emission computerized tomography
TBE : Tris-borate-EDTA
TBS : Tris-buffered saline
TCA : Trichloroacetic acid
TE : Tris-EDTA
TEMED : Tetramethylethylenediamine
TTBS : Tween 20-tris-buffered saline
V : Volt
γ-TuRC : γ -tubulin ring complex

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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY AGAINST P60-KATANIN

SUMMARY

Since microtubules serve as structural components within cells and maintain cellular processes, they are needed to be organized actively. Microtubule severing is an important microtubule organization mechanism for cellular activities. In mitosis and meiosis, microtubule severing is thought to contribute to spindle reorganization. In neurons, all neuronal microtubules are suggested to be severed to relocate to populate in growing processes such as axons and dendrites. Katanin is the predominant, heterodimeric microtubule severing enzyme that disrupts contacts within the microtubule lattice using energy derived from ATP hydrolysis. Two katanin subunits, P60 and P80, have been identified. P60 subunit has the microtubule severing activity and P80 is thought to target katanin to the centrosome and enhances the microtubule severing capacity of P60.

Katanin is one of the key protein for organizing microtubular structure in variety of cell types. Studies suggest that katanin is responsible for changes in microtubule dynamics during mitosis/meiosis, concentrated at mitotic spindle poles, may severe microtubules from centrosome. Inhibition of katanin suppresses spindle-shortening processes was shown in fibroblast mitotic spindles and *Caenorhabditis elegans* meiotic spindles. In neurons, the level of P60-katanin was found to be very high in axons which actively grow towards their targets, at the tips of growing neuronal processes at certain developmental stages and in dendritogenesis. When the axon reached its target and stopped growing, the level of P60-katanin was decreased. Blocking function studies of P60-katanin indicate that katanin-mediated microtubule severing is responsible for the release of microtubules from the neuronal centrosome and decrease microtubule length throughout the neuronal cell body.

Hybridoma technology has been developed by utilizing the immune system. The immune system is specifically responsible for targeting and removing the foreign macromolecules or antigens. In a humoral immune response, B lymphocytes are activated to secrete antibodies. Binding of an antibody inactivates viruses, microbial toxins and other foreign substances. Monoclonal antibodies are produced by hybridoma cell lines by tissue culturing. Each hybridoma is descended from a single B cell clone, hence the antibody it expresses is called monoclonal antibody. A monoclonal antibody belongs to one type of immunoglobulin class and has a single specificity so it recognizes only one epitope of the antigen and will rarely produce cross-reactions with others. This property brings the monoclonal antibodies the advantage as usage in larger areas than polyclonal antibodies.

In this study, 1G6, the first monoclonal antibody against P60-katanin was produced. Firstly, RecP60 (Recombinat P60-katanin) protein which was produced from a specific part of rat P60-Katanin was expressed in *Escherichia coli* and used as the

antigen. Monoclonal antibodies which were produced by hybridoma technology were screened with ELISA, examined in Western-blotting and immunocytochemistry. As a result of the study 1G6 was found to be the only antibody that recognized rat, mouse and human P60-katanin in Western-blotting and immunocytochemistry. 1G6 is the first monoclonal antibody, showing the specific cellular localization of P60-katanin of different species. Monoclonal antibodies are specific and produced permanently so they can be used in wide range of applications. Polyclonal antibodies have been used in studies with P60-katanin until now. Obtaining the hybridoma cell, the unlimited source, of the antibody against P60 results in a great advantage to investigate P60-katanin's functions.

P60-KATANİN'E KARŞI MONOKLONAL ANTİKOR ÜRETİLMESİ VE KARAKTERİZASYONU

ÖZET

Hücrelerin önemli yapısal bileşeni olan mikrotübüller hücresel süreçleri sürdürdükleri için aktif olarak organize edilmeleri gerekir. Mikrotübüllerin kesilmesi hücresel faaliyetler için önemli bir mikrotubul organizasyon mekanizmasıdır. Mitoz ve mayoz bölünmede iğ ipliklerinin reorganizasyonuna mikrotübüllerin kesilmesinin katkı sağladığı düşünülmektedir. Nöronlarda, mikrotübüllerin akson ve dentrit gibi gelişmiş hücre uzantılarına taşınması ve yerleşmesi için kesilmesi gerektiği önerilmiştir. Katanin, başlıca mikrotübül ağını kesen heterodimerik bir proteindir ve bunun için gereken enerjiyi ATP hidroliziyle sağlar. İki katanin altbirimi, P60 ve P80 olarak tanımlanmıştır. P60 alt ünitesi mikrotübül kesme aktivitesine sahiptir, P80 alt ünitesi ise mikrotübül kesilme kapasitesini arttırmakta ve katanini sentrozoma yönlendirdiği düşünülmektedir.

Katanin çeşitli hücre tiplerinde mikrotübül yapısını düzenleyen önemli proteinlerden biridir. Çalışmalar, kataninin mitoz/mayoz bölünme sırasında mikrotübül dinamiklerini değiştirmekten sorumlu olduğunu, mitotik iğ ipliği kutuplarında konsantre olması, mikrotübülü sentrozomdan kestiğini düşündürmektedir. Katanin inhibe edildiğinde, fibroblastta mitotik iğ ipliklerinin, *Caenorhabditis elegans* embriyosunda ise mayotik iğ ipliklerinin kısalma süreçlerinin de inhibe olduğu gözlenmiştir. Nöronlarda, hedefine doğru aktif olarak büyüyen aksonlarda, belirli gelişim evrelerinde büyüyen nöronal uzantıların uçlarında ve dendritogenezde P60-katanin düzeyi çok yüksek bulunmuştur. Akson hedefine ulaştığında ve büyümeyi durdurduğunda ise katanin seviyesi düşmüştür. P60-kataninin işlevi bloklanarak yapılan çalışmalarda ise katanin aracılı mikrotubul kesilmesinin mikrotübülleri nöronal sentrozomdan serbestleştirmeden ve nöronal hücre gövdesinde mikrotubul boyunu kısaltmadan sorumlu olduğunu göstermiştir.

Hibridoma teknolojisi bağışıklık sisteminden yararlanılarak geliştirilmiştir. Bağışıklık sistemi organizmaya yabancı olan makromoleküllerin veya antijenlerin spesifik olarak tanınması ve ortadan kaldırılmasından sorumludur. Antikor yanıtında, B lenfositler aktive olurlar ve antijene spesifik antikorları salgılarlar. Antikorun bağlanmasıyla virüs, mikrobiyal toksinler ve diğer yabancı maddeler inaktive edilir. Monoklonal antikorlar hibridoma hücre hatlarından doku kültürü yapılarak üretilirler. Her hibridoma tek bir B hücre klonu soyundan gelir, bu nedenle onun exprese ettiği antikor monoklonal antikor olarak adlandırılır. Monoklonal antikorlar bir immünglobulin sınıfına aittir ve tek tip spesifisite gösterirler. Bu yüzden antijenin tek bir epitopunu tanırlar ve nadiren diğer antijenlerle kross reaktivite verirler. Bu özelliğiyle polyklonal antikorlardan daha geniş alanlarda kullanım avantajına sahiptirler.

Bu çalışmada, P60-katanin'e karşı ilk monoklonal antikor olan 1G6, üretildi. Öncelikle, sıçan P60-kataninin belirli bir bölgesinden üretilen RecP60 (Recombinat

P60-katanin) proteini, *Escherichia coli* de exprese edildi ve antijen olarak kullanıldı. Hibridoma teknolojisi ile elde edilen monoklonal antikorlar, ELISA ile tarandı, Western-blot ve immünsitokimya yöntemlerinde denendi. Çalışmanın sonucunda, 1G6'nın Western-blot ve immunositokimya yöntemlerinde sıçan, fare ve insana ait P60-katanini tanıyan tek antikor olduğu belirlendi. 1G6, farklı türlere ait P60-katanini spesifik hücresel lokalizasyonunda gösteren ilk monoklonal antikordur. Monoklonal antikorların spesifik oluşu ve üretiminin sürekliliği sayesinde geniş uygulama alanlarında kullanılabilirler. Şimdiye kadar P60-katanin ile yapılan çalışmalarda poliklonal antikorlar kullanılmaktaydı. P60'a karşı monoklonal antikorun, yani onu sınırsız üreten hibridoma hücre kaynağının elde elde edilmesi, P60-kataninin fonksiyonlarını araştırmak için büyük bir avantaj sağlayacaktır.

1. INTRODUCTION

1.1 Cytoskeleton

Eukaryotic cells have protein fibers serving as structural components like a “skeletal system” in the cytoplasm which is called the “cytoskeleton”. For cells to function properly, they must rearrange their internal components as they grow, divide, and adapt to changing circumstances. They have to be correctly shaped, physically robust, and properly structured internally. Many have to change their shape and move from place to place. Eukaryotic cells have developed all these spatial and mechanical functions to a very high degree, and they depend on a remarkable system of the cytoskeletal filaments’ network.

Below mentioned are the basic functions of the cytoskeleton:

1) Pulls the chromosomes apart at mitosis and splits the dividing cell into two.

2) Drives and guides the intracellular transport of organelles and vesicles from one part of the cell to another.

3) Supports the fragile plasma membrane by providing the mechanical linkages that let the cells bear stresses.

4) Enables several types of cell motility such as sperm to swim, fibroblasts and white blood cells to crawl across surfaces.

5) Provides the machinery in the muscle cell to contract and in the nerve cell to extend an axon and dendrites.

6) Guides the growth of the plant cell wall and controls the diversity of eukaryotic cell shapes.

The different functions of the cytoskeleton depend on the behavior of three protein families, assemble to form three main types of filaments: microtubules, microfilaments, and intermediate filaments which together form an elaborate interactive network. Each protein fibers of the cytoskeleton has distinct mechanical properties, dynamics, and biological roles, but all three cytoskeletal filament systems must normally function collectively (Alberts et al., 2008; Karp, 2010).

Most eukaryotic cells contain all three types of cytoskeletal filaments, frequently concentrated in distinct locations. In the absorptive epithelial cells that line the lumen of the intestine, actin microfilaments are abundant in the apical region, where they are associated with cell-cell junctions and support a dense carpet of microvilli. Keratin intermediate filaments, forming a meshwork, connect microvilli and are tethered to junctions between cells. Lamin intermediate filaments support the inner nuclear membrane. Finally microtubules, aligned with the long axis of the cell, are in close proximity to major cell organelles (Figure 1.1) (Kreis and Vale, 1999).

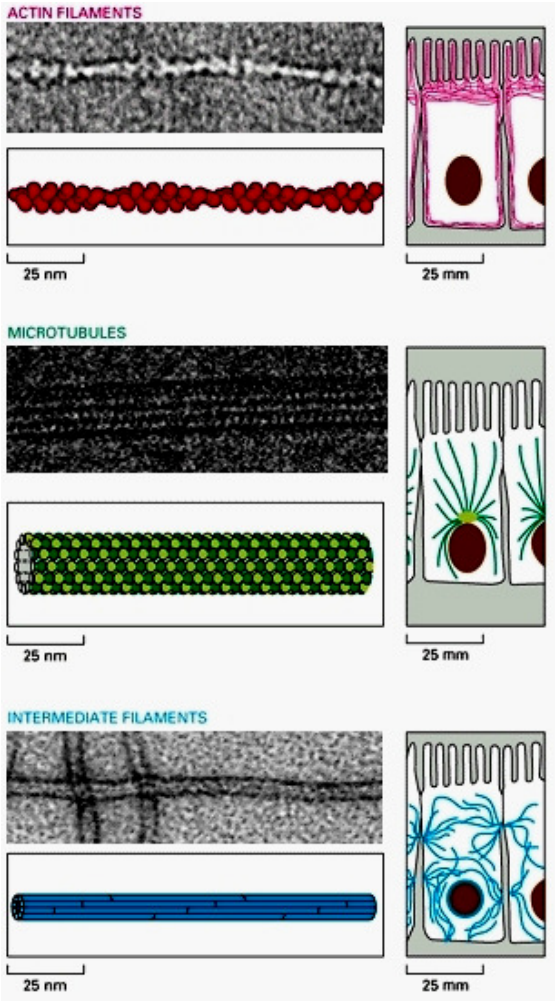

Figure 1.1 : Cytoskeletal filaments (Alberts et al., 2008).

1.1.1 Microfilaments

Microfilaments are also known as actin filaments are flexible tubes, 5-9 nm in diameter, composed of two-stranded helical polymers of the protein actin. Each filament is a twisted chain of identical globular actin molecules, all of which "point"

in the same direction along the axis of the chain. An actin filament possess a structural polarity, with a plus end and a minus end. Actin filaments able to grow by addition of actin monomers at either ends, but the rate of growth is faster at the plus end than at the minus end. Each free actin monomer carries a tightly bound ATP which is hydrolyzed to ADP soon after the incorporation of the actin monomer into the filament. Hydrolysis of bound ATP to ADP in an actin filament decreases the stability of the polymer, promoting depolymerization that helps the cell to disassemble filaments after they are formed. The function of actin filaments depends on a dynamic equilibrium between the actin filaments and the pool of actin monomers (Alberts et al., 2008).

Actin filaments are dispersed throughout the cell and concentrated at the narrow layer of cytoplasm beneath the plasma membrane called the cortex. In this region, most actin filaments are arranged in a network that excludes most organelles from the cortical cytoplasm (Lodish et al., 2004).

Actin filaments determine the shape of the cell surface and are necessary for whole-cell locomotion, for cells to engulf a large particle by phagocytosis and to divide. The distinctive shape of a cell depends on the organization of actin filaments and membrane-microfilament binding proteins. By association with actin binding proteins, actin filaments can form stiff and relatively permanent structures, such as the microvilli on the brush-border cells lining the intestine or small contractile bundles that can contact and act like the “muscles” of the cell. They can also form temporary structures, such as the protrusions formed at the leading edge of a crawling fibroblast or a contractile ring that pinches the cytoplasm in two during cell division (Lodish et al., 2004; Garner et al., 2004).

1.1.2 Intermediate filaments

Intermediate filaments are ropelike fibers with many long strands twisted together to provide great tensile strength. The subunits of intermediate filaments are elongated fibrous proteins composed of an N-terminal globular head, a C-terminal globular tail, and a central elongated rod domain. The rod domain consists of an extended α-helical region. Intermadiate filament proteins form stable dimer by wrapping around each other in a coiled-coil configuration. Two of the coiled-coil dimers associate by noncovalent bonding to form a tetramer and the tetramers bind to one another end-to-

end and side-by-side by non-covalent bonding and generate the final intermediate filament (Helfand et al., 2003).

Intermediate filaments' main function is to provide cells mechanical strength and resistance to shear stress when cells are stretched. They are tough fibers assembled from a large diverse family of proteins. The components of the cytoskeleton are highly dynamic structures capable of rapid and dramatic reorganization. The most common intermediate filaments, found in the nucleus, are composed of lamins called nuclear lamina, underlie and strengthen the nuclear envelope in all eukaryotic cells. Other proteins that construct intermediate filaments are expressed preferentially in certain tissues: for example *keratin*-containing filaments in epithelial cells, *desmin*-containing filaments in muscle cells, and *vimentin*-containing filaments in mesenchymal cells. (Lodish et al., 2004).

Intermediate filaments are found in the cytoplasm of most animal cells. Typically crisscross the cytoplasm, forming an internal framework that surrounds the nuclear envelope and stretches to the plasma membrane. They are attached by adapter proteins to the plasma membrane at specialized cell-cell junctions called *desmosomes* and *hemidesmosomes*, which mediate cell-cell adhesion and cell-matrix adhesion, respectively, particularly in epithelial tissues (Helfand et al., 2003).

1.1.3 Microtubules

In all eukaryotic cells, microtubules bear important organizing roles. They are involved in many cellular processes. Cell division, cytokinesis, cell motility, outgrowth of processes, cellular trafficking and differentiation are included. They are in charge of attaching membrane-enclosed organelles and leading intracellular transport serving as system of tracks along which vesicles, organelles and other cell components carried with motor proteins.

1.1.3.1 Microtubule structure

Microtubules are rigid tubes composed of subunits of the protein tubulin, a heterodimer of α-tubulin and β-tubulin, bound tightly together by noncovalent bonding.

Microtubules are arranged as a cylindrical tube, diameter of ~ 24 nm varying in length from hundred microns to a single micron in axons of nerve cells (Figure 1.2).

In a microtubule, lateral and longitudinal interactions between the tubulin subunits are in charge of maintaining the tubular form. Longitudinal interactions between heterodimers link the subunits head to tail at 8 nm intervals into a linear protofilament. Through lateral interactions, protofilaments associate side by side into a sheet and finally a cylinder to make microtubule. A single microtubule is comprised of 10–15 protofilaments, usually 13 in mammalian cells. The subunits in each protofilament in a microtubule all point in the same direction. Therefore, the microtubule itself has a distinct structural polarity, with α-tubulins exposed at one end called minus end and β-tubulins exposed at the other end, the plus end (Alberts et al., 2008; Lodish et al., 2004; Conde and Cáceres**,** 2009). The dynamic properties of the two ends of a microtubule are different. In vitro, in a concentrated solution of pure tubulin, tubulin dimers will add to either end of a growing microtubule, although they add more rapidly to the plus end than the minus end (Wittmann et al., 2001).

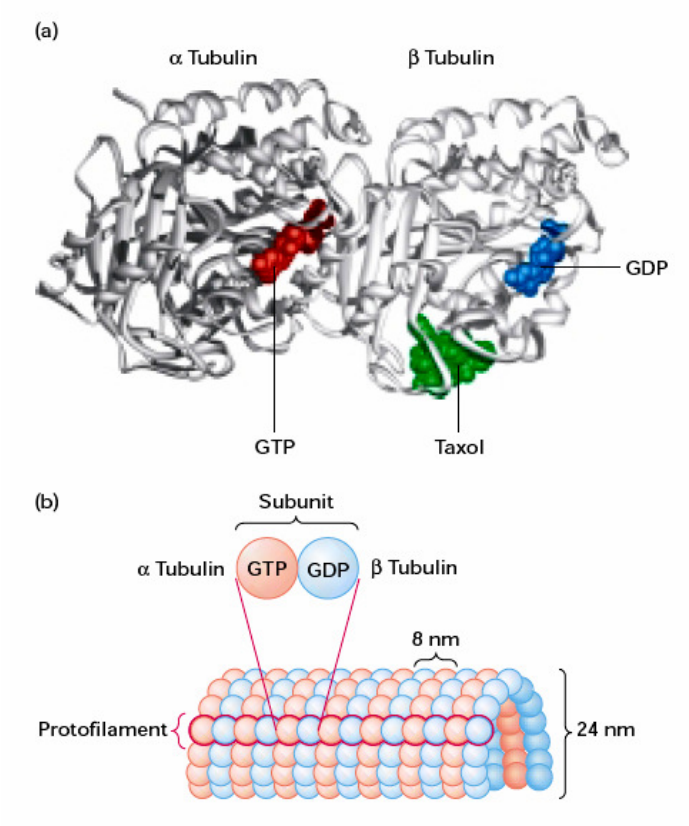

Figure 1.2 : Microtubule structure (Lodish et al., 2004).

α- and β- tubulin monomers are proteins of about 450 amino acids each and are about 50 % identical at the amino acid level. Each monomer has a molecular mass of about 50,000 Da. On each α or β monomer GTP binding sites are existed. Those sites are:

- Non-hydrolyzable site when binding GTP to α-tubulin is irreversible and the hydrolization incident of GTP does not occur.
- Exchangeable site on β-tubulin binds GTP reversibly and hydrolyzes it to GDP.

In the atomic structure of the tubulin subunit, the GTP bound to α-tubulin is trapped at the interface between the monomers and is thus nonexchangeable and never hydrolized. The GTP which lies at the surface of the β-tubulin monomer is freely exchangeable with GDP. The guanine bound to β-tubulin modulates the addition of tubulin subunits at the ends of a microtubule (Lodish et al., 2004).

1.1.3.2 Microtubule dynamics and organization

Like microfilaments and intermediate filaments, microtubules are not randomly distributed in cells. In animal cells microtubules radiate from the centrosome and this structure is called the primary microtubule-organizing center (MTOC). Microtubules are formed by outgrowth from MTOCs, which control the number of microtubules formed, their location, and their orientation in the cytoplasm. MTOC is a collection of microtubule-associated proteins that sometimes but not always contains a pair of centrioles. Because microtubules assemble from the MTOC, microtubule polarity becomes fixed in a characteristic orientation. In many nondividing animal cells, the MTOC is located at the center of the cell near the nucleus, and the radiating microtubules are all oriented with their (+) ends directed toward the cell periphery. In most interphase animal cells, the (-) ends of microtubules are proximal to the MTOC. In the nerve cells, all microtubules in the axon point their plus ends toward the axon terminal (Lodish et al., 2004).

The MTOC organizes cytosolic microtubules by nucleating the microtubule assembly and then anchoring and releasing microtubules. γ- Tubulin is the third member of the tubulin superfamily, is not a part of the tubulin subunit, but it is one of the protein that MTOC contains, for the first assembling a ring of αβ-tubulin dimers, than to add such dimers to a preexisting microtubule structure. γ- tubulin is also found as a part of a γ-tubulin ring complex (γ-TuRC). γ-TuRC can directly nucleate microtubule assembly at subcritical tubulin concentrations that is, at concentrations

below which polymerization would not take place in the absence of the γ-TuRC. By providing organizing centers containing nucleation sites, and keeping the concentration of free αβ-tubulin dimers low, cells can thus control where microtubules form (Lodish et al., 2004).

During the cellular processes, microtubules undergo dramatic alterations. One is called "dynamic instability" where microtubules' length oscillate between growth and shortening phases by reconfiguration through assembly and disassembly (Figure 1.3) (Kosik and Baas, 1999; Lodish et al., 2004). This ossilation stems from the intrinsic capacity of tubulin molecules to hydrolyse GTP. Each free tubulin dimer contains one tightly bound GTP molecule on β-tubulin site. The GTP is hydrolyzed to GDP, shortly after the subunit is added to a growing microtubule. Thus, this leads to its elongation and formation of the protofilament (Singh et al., 2008).

The GTP-associated tubulin molecules are packed efficiently together in the wall of the microtubule, but tubulin molecules carrying GDP have a different conformation and bind less strongly to each other. The majority of β-tubulin in the microtubule fiber is in the GDP-bound form but ''capped" with GTP-bound tubulin dimers at the plus end. When polymerization is proceeding rapidly, tubulin molecules add to the end of the microtubule faster than the GTP hydrolysis on the microtubule structure forming the GTP cap. When tubulin at the free end of the microtubule hydrolyzes its GTP before the next tubulin has been added, conformational change results and rapid depolymerization of the microtubule starts.

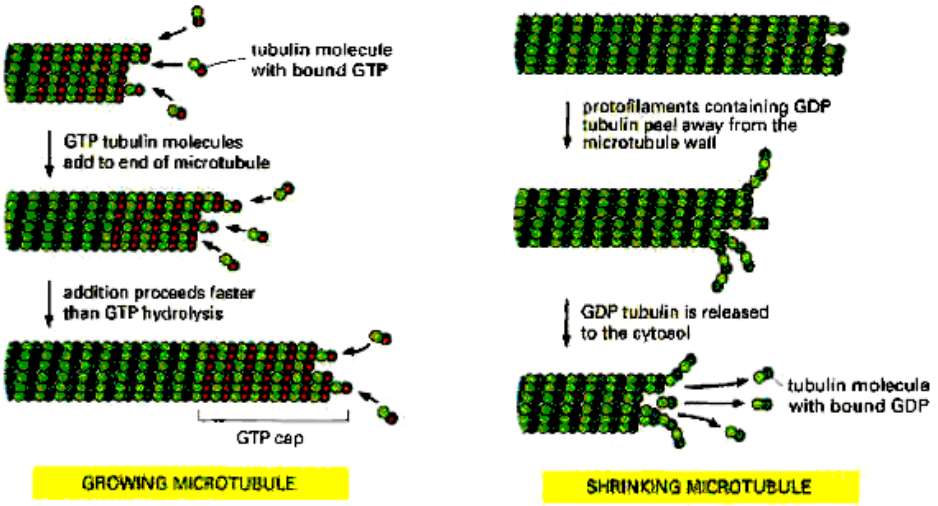

Figure 1.3 : Dynamic instability (Alberts et al., 2008).

Cells are able to modify the dynamic instability of their microtubules. As cells enter mitosis, switching between growing and shrinking much more frequently than interphase cytoplasmic microtubules.When a cell has differentiated into a specialized cell type and taken on a definite fixed structure, the dynamic instability of microtubules is often suppressed by proteins that bind to the ends of microtubules.

Most differentiated animal cells are polarized. The cells polarity is a reflection of the polarized systems of microtubules, which help to position organelles in their required location and to guide the streams of traffic moving between one part of the cell and another. Transport occurs along microtubules when the appropriate motor binds to a cargo and simultaneously binds to the rail. The motor then moves along the rail in such a "walking" manner powered by energy derived from hydrolysis of ATP (Mallik and Gross, 2004). Impaired axonal transport of molecules by motor proteins is linked to the pathogenesis of Alzheimer's disease (Stokin and Goldstein, 2006)

1.1.3.3 Microtubules' regulation and severing

Microtubule assembly and stability and their association with other cell structures are regulated by a large variety of accessory proteins called microtubule associated proteins (MAPs) (Table 1.1). MAPs are classified into two groups. One group stabilizes microtubules: MAP1A and MAP1B are found in neurons as well as in nonneuronal cells. MAP4, is found in most of the cells. In mitosis, MAP4 regulates microtubule stability, and CLIP170 cross-links microtubules to chromosomes. MAP2 and Tau is found only in neurons (Lodish et al., 2004).

Table 1.1: Properties of MAPs (Lodish et al., 2004).

Protein	MW	Location	Function
Microtubule-Stabilizing Proteins			
MAP1	250,000–300,000 (heavy chain)	Dendrites and axons; non-neuronal cells	Assembles and stabilizes MTs
MAP2	42,000 and 200,000	Dendrites	Assembles and cross-links MTs to one another and to intermediate filaments
MAP4	210,000	Most cell types	Stabilizes MTs
Tau	55,000–62,000	Dendrites and axons	Assembles, stabilizes, and cross-links MTs
CLIP170	170,000	Most cell types	Cross-links MTs to endosomes and chromosomes
Microtubule-Destabilizing Proteins			
Katanin	84,000	Most cell types	Microtubule severing
Op18 (stathmin)	18,000	Most cell types	Binds tubulin dimers

The second group of MAPs destabilizes microtubules: Katanin, severs intact cytosolic microtubules. Op18 (stathmin) increases the frequency of rapid disassembly of microtubules in the mitotic spindle (Lodish et al., 2004).

Binding to the tubulin subunits, MAPs can act on a microtubule directly, or they can restrict access to the microtubules to other MAPs or motor proteins by binding to microtubule (Baas and Qiang, 2005). A broad range of MAPs functions suggest that coordinated action of MAPs leads to the proper microtubule functioning (Maiato et al., 2004). Modulating the binding of MAPs is accomplished by the reversible phosphorylation of the MAP. Phosphorylated MAPs are unable to bind to microtubules; thus, they can control the length of microtubules, promoting microtubule disassembly. MAP kinase, a key enzyme for phosphorylating MAPs, is a participant in many signal-transduction pathways, indicating that MAPs are targets of many extracellular signals. MAPs, especially MAP4, are also phosphorylated by a cyclin-dependent kinase (CDK) that plays a major role in controlling the activities of various proteins in the course of the cell cycle (Lodish et al., 2004).

Microtubules must be actively organized and transported in the cell to serve as structural components and maintain cellular processes. Dynamic instability is not sufficient to explain the entirety of microtubule behaviors for these cellular activities. Microtubules can be severed along their length, and this affects microtubule dynamics (Baas et al., 2005; Quarmby, 2000). Microtubule severing activity is first identified and studied in mitotic extracts of Xenopus laevis eggs (Vale, 1991; McNally and Vale, 1993).

In mitosis and meiosis, microtubule severing on the spindles is thought to contribute to spindle reorganization (Ahmad and Baas, 1995). Severing near the centrosome could provide the opportunity for minus-end-directed flux of the mitotic spindle microtubules during metaphase (Mitchinison, 1989). Microtubule is released from minus end or severed by a severing enzyme and transport of these non-centrosomal microtubules are conveyed by motor proteins (Keating et al., 1997).

In neurons, microtubules were observed to extend from the cell body into the growth cone within the axon and dendrites. Important events for axonal differentiation such as elongation, branching, navigation, retraction, are achieved by changes in the configuration and behavior of microtubules (Baas and Buster, 2004). Suggesting that

all neuronal microtubules are nucleated at the centrosome, the tubulin proteins or microtubule arrays must be actively transported from the cell body into periphery, and then relocated to populate in axons and dendtires in order to maintain elongation and branching. Variety of cell types transform their microtubule array from one type of organization to another is explained by a model called “cut and run” (Baas et al., 2005). According to this model long immobile microtubules are severed into short pieces and rapidly transported into a new configuration, before the short pieces again elongate and lose their motility (Figure 1.4). Motor proteins and treadmilling have the main roles in transporting microtubules by carrying them and maintaining their dynamicity, recpectively. Due to the fact that long microtubules are stabilized by crosslinking with other components of the cytoplasm, longer microtubules can not be carried by motor proteins and tubulin subunits can not dissociate and then associate to longer microtubules by treadmilling mechanism. Consequently, the key step of the microtubule transport is the severing of long microtubules to be short enough. (Baas et al., 2005, 2006).

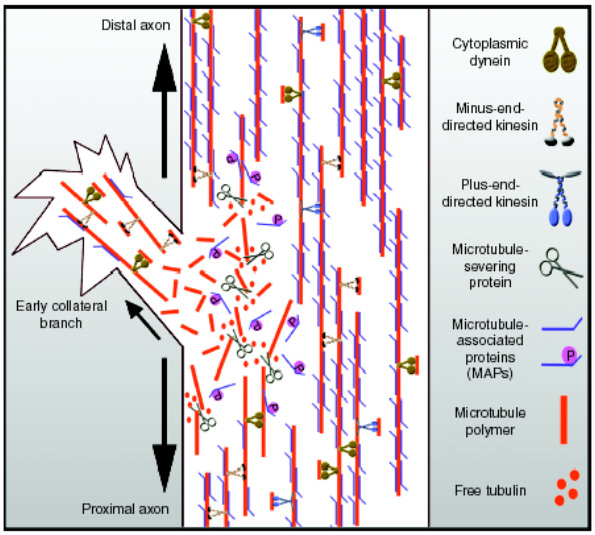

Figure 1.4 : The ‘cut’ and ‘run’ model (Baas et al., 2006).
Microtubule transport and axonal bra
ion regulation by microtubule-severing proteins.

Microtubule severing might be regulated by the stabilizing-MAP protection. It was observed that katanin-induced microtubule severing is much more active in interphase when frog homolog of MAP4 is depleted (McNally et al., 2002) and severing is more active in mitotic extracts based on phosphorylation of proteins, except katanin. Phosphorylated MAP4 lose its association with the microtubules. In

the model, fibrous MAPs protect the lattice of the microtubule from being accessed by katanin (Baas et al., 2005). Phosphorylation of the MAPs results in their detachment from the microtubule, thus enabling katanin to gain access. Signaling cascades can regulate microtubule severing by causing MAPs to dissociate from the microtubules at the site where an axonal branch is starting to form, thereby permitting katanin to break the microtubules into shorter mobile pieces where needed (Baas et al., 2006). Tau has been shown to be important for strong protection against severing by either katanin or spastin which is another microtubule severing protein. Hyperphosphorylation of tau causes it to dissociate from microtubules so microtubules become more accessible to katanin and this process is also observed in Alzheimer's disease pathology (Baas and Qiang, 2005). Tau that shields microtubules from severing has been found to have greater protection in the case of katanin (P60 subunit) than spastin (Yu et al., 2008).

1.2 Microtubule Severing Protein, Katanin

Katanin is the predominant, heterodimeric microtubule severing protein and has the ATPase activity which disrupts contacts within the microtubule lattice using energy derived from ATP hydrolysis. Katanin is characterized by purification from sea urchin eggs and its name is originated from the Japanese word for samurai sword "katana" (McNally and Vale, 1993). Two katanin subunits, P60 and P80, have been identified. P60 subunit has the microtubule severing activity and P80 is thought to target katanin to the centrosome and enhances the microtubule severing capacity of P60 (Hartman et al., 1998).

Katanin is responsible for changes in microtubule dynamics during mitosis. Katanin is concentrated at a microtubule-dependent structure at mitotic spindle poles, suggesting katanin may sever microtubules from their centrosomal attachments (McNally and Thomas, 1998) and mediates the disassembly of microtubule minus end during poleward flux (McNally et al., 1996; Quarmby 2000; Buster et al., 2002). In addition, *Drosophila melanogaster* katanin orthologues appear to function primarily on anaphase chromosomes, where it stimulates microtubule plus-end depolymerization and Pacman-based chromatid motility (Zhang et al., 2007). Inhibition of katanin inhibits spindle-shortening processes in fibroblast mitotic spindles and *Caenorhabditis elegans* meiotic spindles (McNally et. al., 2006).

Katanin may have other functions distinct from its role in cell division. In neurons, it was shown that katanin is present at the centrosome and widely distributed within the axon and all neuronal compartments (Baas et al., 2006). The level of P60-katanin was found very high in axons actively growing towards their targets, but then it decreased when the axon reached its target and stopped growing (Yu et al., 2005). High P60-katanin levels were also found at the tips of growing neuronal processes at certain developmental stages and in dendritogenesis. Blocking the function of P60-katanin prevents microtubule release from the centrosome and profoundly increases microtubule length throughout the neuronal cell body indicating that katanin-mediated microtubule severing is in charge of releasing microtubules from the neuronal centrosome (Ahmad et al., 1999; Karabay et al., 2004). Microtubule severing by katanin is also essential for regulating the length of the microtubules after their release (Ahmad et al., 1999). Katanin is likely to be the principal means for generating the short microtubules observed in axonal growth cones and interstitial branch points (Dent et al., 1999). Relatively short microtubules are mobile. In 'cut and run' model, katanin break the lattice of the microtubule polymer in order to generate mobilizable short microtubules which are transported to newly growing neuronal processes of neurons (Baas et al., 2005).

1.2.1 AAA superfamily and structure of Katanin

Katanin is a member of AAA (**A**TPases **A**ssociated with various cellular **A**ctivities) superfamily of ATPases (Confalonieri and Duguet, 1995). All members of the AAA family are Mg^{2+}dependent ATPases. The AAA motif is defined by a conserved ATP-ase domain of 230 to 250 amino acids that includes the Walker signature sequences of P-loop ATPases and other regions of similarity unique to AAA proteins. The classical AAA proteins are easily recognized by their strong sequence conservation of 30% identity in this domain (Patel and Latterich, 1998; Vale, 2000).

Although AAA proteins are monomers, they function as an oligomeric ring complex (Hartman et al., 1998, Patel and Latterich, 1998). Katanin is present in equilibrium between monomers and oligomers. In this case of the katanin, the oligometric state has been shown to be hexameric ring where ATP binding induces structural reorganizations at the interface region which increases interactions between adjacent AAA domains as well as between the AAA protein and its target. (Hartman and Vale, 1999). This creates a tense state of the AAA-target protein complex. The

tighter subunit-subunit interactions in turn accelerate ATPase reaction. Once the AAA modules are in ADP state, the complex reverts to a relaxed configuration in which interactions between AAA domains and the target protein weaken. Rings also provide a framework for binding target protein at multiple sites. If the ring-binding sites change their positions during the ATPase cycle, then tension could be applied to a bound protein (Vale, 2000).

P60, the 60 kDa subunit is 491 amino acid long polypeptide (Karabay et al., 2004) and composed of an N-terminal domain that binds microtubules (Hartman and Vale, 1999) and a conserved C-terminal domain sharing homology with a large family of ATPases. BLAST search revealed that ortholog of P60 exists in *C. elegans,* in *Arabidopsis thaliana* and there are vertebrate homologs of P60 (Hartman et al., 1998; Stoppin-Mellet et al., 2003; Bouquin et al., 2003; Yang et al., 2003). P60 alone displays a microtubule-stimulated ATPase activity. Although, when comparing the rates of microtubule disassembly, P60 is half as active as P60/P80 (Hartman et al., 1998). P80, the 80 kDa subunit is 658 amino acid long (Karabay et al., 2004) and composed of an N-terminal WD40 repeat domain, a central proline-rich domain and a C-terminal domain required for dimerization with the catalytic P60 subunit (Hartman et al., 1998).

It has been found that katanin's P60 subunit exhibits both microtubule-stimulated ATPase activity and microtubule- severing activity in the absence of the P80 subunit. P80 can enhance P60 mediated microtubule severing by increasing affinity of P60 to microtubules (McNally et al., 2000). P60/P80 was found to have a two-fold higher microtubule severing activity compared to P60 alone. The WD40 repeat domain of a human P80 homolog was shown to be sufficient to target the P80 homolog to interphase centrosomes (Hartman et al., 1998). WD40 domain and con80 domain of P80 katanin can enhance P60 mediated microtubule severing by increasing affinity of P60 to microtubules. (McNally et al., 2000). On the other hand, it was also found that WD40 domain of P80 acts as a negative regulator of microtubule severing by P60. (McNally and Thomas, 1998; Hartman et al., 1998; McNally et al., 2000).

In neurons, the levels of P60-katanin fluctuate at the developmental stage. In addition, katanin is typically viewed as a heterodimer, but it has been recently shown that the two subunits are not present within cells at equimolar levels. In fact, the ratio of the two subunits varies in different tissues and at different stages of development,

suggesting that the activity of the P60 subunit might be influenced by the levels of the P80 (Yu et al., 2005). In addition, a recent research also reported that the C terminal domain of a candidate tumor suppressor LAPSER1/LZTS2 (LAPSER1) inhibits katanin-mediated microtubule severing in vitro by binding to P80 katanin (Sudo and Maru, 2008).

1.2.2 Microtubule severing mechanism and regulation of Katanin

Katanin breaks microtubules along the length, removing the tubulin dimers from the wall of the microtubule. Released tubulin dimers are able to repolymerize again, so they are not phosphorylated, proteolyzed or otherwise changed. Katanin can disassemble microtubules under conditions that favor spontaneous assembly of tubulin into microtubules. This means that either katanin inhibits polymerization of tubulin dimers or dissociates tubulin dimers from microtubules faster than they re-associate (McNally and Vale, 1993).

Tubulin subunits dissociate very slowly from the microtubule wall. Severing of a microtubule at a specific spot requires the subunits to be dissociated from tightly bound neighbours above, below and on two sides. However, in the presence of ATP, katanin disrupts these tubulin–tubulin contacts and can sever and dismantle a taxol-stabilized microtubule within a couple of minutes (Vale, 2000). Microtubules act as a scaffold for katanin to oligomerize into a ring structure after it has exchanged its ADP for ATP. As a consequence of ATP hydrolysis and subsequent phosphate release, the katanin undergoes a conformational change leading to mechanical strain that destabilizes tubulin-tubulin contacts. The ADP-bound katanin dissolutes from the complex (Hartman and Vale 1999; Quarmby, 2000; McNally et al., 2000). This model suggests several possible points of regulation: a nucleotide-exchange factor could regulate loading of P60 with ATP; accessibility to microtubules could be regulated by removal of protective MAPs; oligomerization sites on P60 could be reversibly masked by regulatory factors; and other factors could stimulate or inhibit ATP hydrolysis and severing (Quarmby, 2000).

Although may katanin rich and is widely distributed in neurons, it should not sever microtubules everywhere. There should be a control mechanism which allows katanin to be locally activated/deactivated or being transferred to sites where microtubules need to be severed (Karabay et al., 2004). Activity of katanin might be

regulated by its synthesis and degradation levels. Tight regulation of the levels of katanin was observed in the neuron during axonal development (McNally et al., 2002), where katanin levels are high during their most active phases of growth but drop once axons have contacted their targets (Karabay et al., 2004). There were some factors (like cyclinB/cdc2, cyclinB/cdc1) discovered that changed microtubule-severing activity in M-phase *Xenopus* egg extract. However, experiments with isolated katanin showed that it is not directly activated or phosphorylated (McNally and Thomas, 1998; McNally et al. 2002). Activity of katanin might be also regulated indirectly by other MAP's which would restrict accession of katanin to microtubules (Baas and Qiang, 2005) as mentioned in detailed in the section 1.1.3.3.

1.3 The Immune System

Immunity is a defense mechanism against foreign substances. Microbes, macromolecules such as proteins and polysaccharides are included. The network of cells and molecules responsible for defending the body constitude the immune system. When an organism is threatened by microorganisms, viruses, the immune response acts to provide protection (Abbas and Lichtman, 2003). The first issue of the immune system is keeping out the foreign substances. If the system fails to keep them away, the foreigner will be seeked out and destroyed in the body. The key to a healthy immune system is its remarkable ability to distinguish the structure between the body's own and foreign molecules (Janeway et al., 2005).

The cells in the immune system specifically responsible for targeting and removing the foreign macromolecules or antigens are lymphocytes. They circulate in blood and lymph and populate areas of the body known as lymphoid tissues such as the spleen, lymph nodes, thymus, tonsils, adenoids, and Peyer's patches (Miller, 1996). There are two broad immune response classes that has specificity and memory: Antibody responses and cell-mediated immune responses.

The components of cell mediated immune responses are lymphocytes (T cells and B cells), plasma B cells, antigen-presenting cells (macrophages, dendritic cells) and memory B and T cells (Stites and Terr, 1991; Ruebush, 2007). In Antibody responses, B cells are activated to secrete antibodies, which circulate in the bloodstream and permeate the other body fluids. Binding of an antibody inactivates viruses, microbial toxins and other foreign substances (Brum et al., 1994).

Antibodies and T lymphocytes eliminate extracellular and intracellular microbes, respectively. These functions often require the participation of the nonlymphoid effector cells and other defense mechanism (Abbas and Lichtman, 2003). Activated T cells react directly against the antigen that is presented on the surface of a host cell, for example, a virus-infected host cell that has viral antigens on its surface is eliminated by T cell. In other cases, the T cell produces signal molecules that activate macrophages to destroy the microbes by phagocytosis (Cummings, 1996).

Immunological memory causes to "remember" each pathogen by a signature antigen. The specific immune response requires the recognition of antigens during "antigen presentation" process. Antigen specificity allows for the generation of responses that are fit to specific pathogens or pathogen-infected cells. The ability to mount these responses is maintained in the body by "memory cells" (Pancer and Cooper, 2006; Janeway et al., 2005). After the subsequent exposure to antigen the immune response' lag period is shorter and the response is greater than the primary response even the antigen is represented again after some weeks, months or even years (Alberts et al., 2002).

The specific immune response may be divided into five phases. The first phase starts by the specific recognition of antigens. This recognition guides to the activation of the lymphocytes that recognize the antigen and concludes in the development of effector mechanisms that mediate the physiologic function of the response which is called the elimination of the antigen. After the antigen is eradicated, the immune response subsides (returns to its basal resting state by apoptosis of antigen-stimulated lymphocytes) and homeostasis is restored (Abbas and Litchman, 2003).

According to "clonal selection hypothesis", antigen-specific clones of lymphocytes develop independently before exposuring to antigen. Cells of each clone arising from a single precursor are capable of recognizing and responding to a distinct antigenic determinant by their different antigen receptors exposed on their surface. The activation of lymphocytes requires firstly the antigen and secondly the microbial products or the non-specific immune components to microbes (Abbas and Litchman, 2003).

Activated lymphocytes synthesize and secrete cytokines which stimulate the growth and differentiation of the lymphocytes, cytokine receptors to make lymphocytes more responsive, many other proteins for gene transcription and cell division. In

response to these mechanisms, the antigen specific lymphocytes proliferate which is named clonal expansion. Some of the cells differentiate into effector cells, including helper T cells, Cytolytic T lymphocytes and antibody secreting B cells. Some of these B cells are identifiable as plasma cells. Plasma cells develop in lymphoid organs and at sites of immune responses and often migrate to bone marrow where some of them may survive even after the antigen is eliminated. These kind of plasma cells are the result of differentiation into memory cells. The same differentiation also accomplished for antigen-stimulated T cells. Memory B and T cells may survive in a functionally dormant or slowly cycling state for many years after the antigen is eliminated. Thus, the cells ensures rapid and enhanced responses to subsequent exposures to antigens (Abbas and Litchman, 2003).

1.4 Properties and Interactions of Antibodies and Antigens

Antibodies are serum immunoglobulins that specifically bind antigens in both the recognition phase of the antigen and the effector phase of the specific immune response. The first antibodies are produced in a membrane bound form by newly formed B lymphocytes, and these membrane molecules function as B cell receptors for recognition of the antigen. The interaction of antigen with membrane antibodies of immature B cells initiates B cell responses with the aid of a helper T cell and it differentiates into an antibody-secreting effector cell. These cells express and secrete large amounts of soluble antibody, which has the identical antigen binding site as its antigen receptor exposed earlier on the surface. All secreted antibody molecules from one B cell clone have the same specificity for their antigen binding site (Cummings 1996).

An antigen is any substance that may be specifically bound by an antibody molecule or T cell receptor. Any substance that activates immune response is called immunogen. All immunogens are antigens which react with the products of immune system, but all antigens are not immunogens. The immunogen antigens are in the structure of proteins, carbohydrates and phospholipids. The antigens that are not immunogens are smaller molecules may bind to antibodies, but can not activate B cells so they do not trigger immune response on their own. This kind of antigen is called hapten. Low molecular weight (1000 to 10000 Da) compounds including many drugs and antibiotics are haptens, hence they are conjugated to carrier proteins

in order to make them immunogen that cause to generate specific antibodies. Macromolecules are usually much bigger than the antigen-binding region of an antibody molecule. Antigens bind to antibodies with their small portions called antigenic determinants or epitopes. Antigens, typically contain multiple determinants, some of which may be repeated, and each of which, can be recognized by an antibody or receptors that belong to cells responsible for immunity (Stites and Terr 1991; Abbas and Litchman 2003; Ruebush 2007).

The two hallmarks of immunoglobulins are the diversity which meets the different antigenic structures and the specifity of each to select the one of the suited antigen in the environment.

The structure of immunoglobulins are glycoproteins composed of 82-96 % polypeptide and 4-18 % carbonhydrate (Stites and Terr, 1991). The simplest antibodies are Y-shaped tetrameric molecules consisting of two identical light (L) chains (about 220 amino acids/chain) and two identical heavy (H) chains (about 440 amino acids/chain). The four chains interact with each other by noncovalent and covalent (disulfide) bonds (Lewin, 2004; Alberts et al., 2002; Janeway et al., 2005). Light chains and heavy chains share the same organization consisting of two principal regions: the N-terminal variable region (V region); and the C- terminal constant region (C-region). The light chain has one variable (V_L) and one constant (C_L) domain, while the heavy chain has one variable (V_H) and three or four constant (C_H) domains (Figure 1.5).

The antibody has two identical antigen-binding sites in the variable domains. The variable domain (V) is generated by the interaction of V regions of light chain and heavy chain. Hundreds of V domains in different amino acid combinations create various antibodies to recognize diverse antigens. There are also three small hypervariable regions in the V domains of both chains. These regions are called complementarity determining regions (CDRs: CDR1, CDR2, CDR3) which protrude as loops at the end of V domain to form the three dimensional antigen-binding surface that is complementary to the antigen epitop (Figure 1.5 A-B). The effectiveness of antigen binding of the antibody molecule is increased by the hinge region, which allows flexibility to accomplish the required distance between the two antigen-binding sites to interact properly with the antigen (Abbas and Lichtman, 2003).

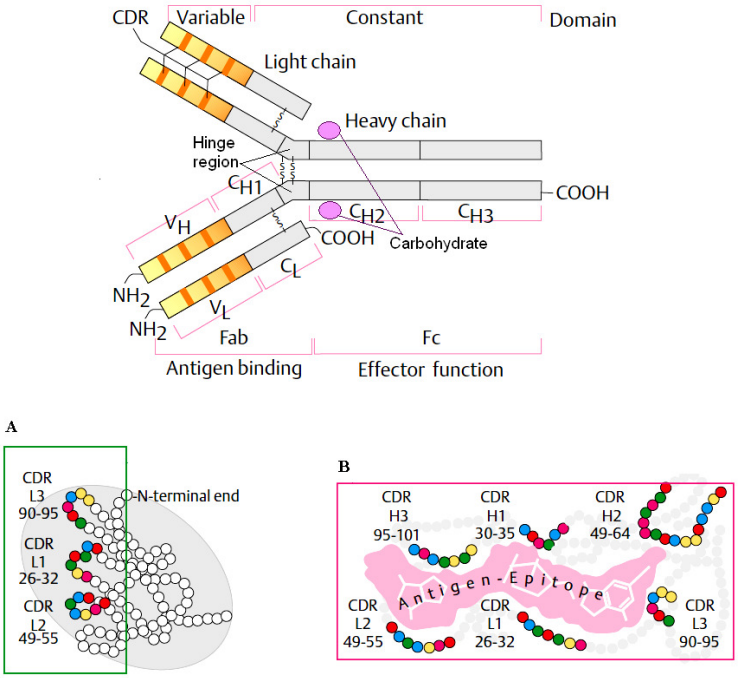

Figure 1.5 : Structure of a typical immunoglobulin (antibody) protein.
Hypervariable regions (CDR) of variable domains (A) determine the antigen specificity by the interaction of the antigen-epitope
(B) (Adapted from Burmester and Pezzutto 2003).

The protective properties of antibodies are due to their tails, the portion of heavy chain constant regions, which is called Fc region. This region acquire the antibody different effector functions, such as activating the complement system, attaching to phagocytic cells, transversing the placenta from mother to fetus. Antibody molecules can be divided into distinct classes and subclasses on the basis of differences in the amino acid sequence of their heavy constant regions including the Fc portions. In mammals, they are arranged as five classes, IgA, IgD, IgE, IgG, and IgM, which are named by the Greek letters- α, δ, ε, γ, and μ, respectively (Abbas and Lichtman, 2003; Alberts et al., 2008).

The isotype of IgG has four subclasses (IgG1, IgG2, IgG3 and IgG4), having $\gamma_1, \gamma_2, \gamma_3, \gamma_4$ heavy chains. IgG constitutes approximately 75 % of the total serum immunoglobulins, hence it is the dominant class in serum during a secondary immune response. IgG is the only isotype that crosses the placenta by its Fc region (passive immunity) (Minn and Quintans, 1999). IgA is the predominant immunoglobulin class in the mucosal immune system and appears to be an efficient antiviral antibody and also found in milk. IgA exists in two forms, IgA1 (90 %) and

IgA2 (10 %) that have α_1 and α_2 chains, respectively. IgM is a pentameric isotype which is synthesised in early immune responses. IgM has μ heavy chains and it constitutes 8-10% of the total serum immunoglobulins and it is also expressed on the surface of naive B cells. IgD has δ heavy chains and it is the predominant immunoglobulin which is localized on the surface of naive B lymphocytes, as IgM. IgE is responsible for many common allergies and may protect against parasitic infections. It binds to Fc receptors on mast cells and eosinophils and triggers mast cell degranulation and cell-mediated cytotoxicity involving eosinophils. (Abbas and Lichtman, 2003; Janeway, 2005).

1.5 Hybridoma Technology and Monoclonal Antibodies

Monoclonal antibodies are produced by hybridoma cell lines which can be produced in tissue culturing. In hybridoma technology, hybridomas are hybrid cells which are produced from the fusion of B cells and the myeloma cell line. In this technique, the idea is combining of two valuable cells together. B cells are derived from the lymphatic tissues of immunized animals and the myeloma cell line brings immortality to B cells when they are fused together (Figure 1.6).

Each hybridoma is descended from a single B cell clone, so the antibody it expresses is called monoclonal antibody. Monoclonal antibody has a single specificity and is in one type of immunoglobulin classes. Each monoclonal antibody is monospecific hence it recognizes only one epitope of the antigen (Burns, 2002).

In 1976, Georges Kohler and Cesar Milstein published the method for the production of monoclonal antibodies by hybridomas that come from mouse splenocytes and their myeloma fusion partner. Kohler's and Milstein's contribution, for which they were awarded together with Niels K. Jerne the 1984 Nobel Prize in Physiology (Mechetner, 2007). As described above, their technique was to immunize mice with a pathogen and then fuse their B-lymphocytes taken from the spleen with mutant non-secreted myeloma cells of mice. After limited dilution cloning and selection, hybridomas represent a pure and unlimited source of monoclonal antibodies with the desired antigen specificity (Chiarella and Fazio, 2008).

The steps of hybridoma technology are summarized as follows: Mice are immunized with desired antigen, in order to produce antibody against this antigen. Then, the

most actively immunized mouse is selected by Enzyme Linked Immunosorbent Assay (ELISA) which is an immunologic assay. The most actively immunized mouse's lymphoid organs are taken to isolate antibody producing B lymphocyte and myeloma cells are detached from their cultured flasks. Polyethyleneglycol (PEG) is used to fuse the two types of cells. Cells that are subjected to fusion will be cultured in Hypoxanthine Aminopterin Thymidine (HAT) selective medium. B lymphocytes can have a limited life time up to 4-5 days in cell culturing. Myeloma cells are the immortal cells that lose their reproduction control mechanism but sensitive to HAT. Therefore, the two types of unfused cells die and remained hybridomas continue to divide in culture plates. Hybridoma colonies that produced antibodies against desired antigen are detected by ELISA.

In the selected well which has antibody response, there can be more than one hybridoma colony which can result in polyclonal response. By "limited dilution" method, the cells in antibody responding wells are dispersed to new culture plates in order to get hybrid colony produced from single cell. Besides, hybridoma colonies that synthesize specific monoclonal antibody for desired antigen are selected by cross-reactivity ELISA method.After limited dilution and selection hybridomas are cultured in large scale and monoclonal antibodies are purified from their collected growth media.

HAT medium which is the most common selection medium, is used for the selection of hybridomas. Aminopterin inhibits de novo pathway which synthesize nucleotides from small-molecule precursors (Figure 1.7). Normal animal cells can also synthesize nucleotides from the partial breakdown products of nucleic acids such as hypoxanthine and thymidine (salvage pathway). The salvage pathway relies on the presence of the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). The mouse myeloma cell line is an HGPRT deficient (HGPRT−) mutant strain (Chung et al., 2000). Therefore, unfused myeloma cells die in HAT medium and unfused B cells die as they have a short life span in cell culture. In this way, only the B-cell-myeloma hybrids survive in the HAT medium culturing.

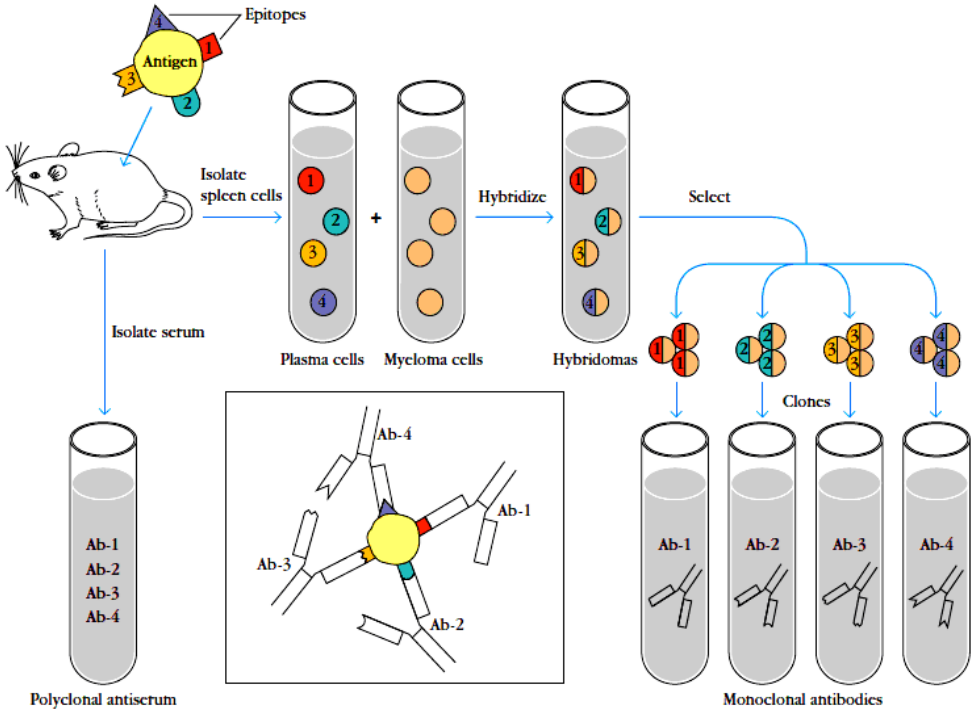

Figure 1.6 : General steps of hybridoma technology (Goldsby et al., 2003).

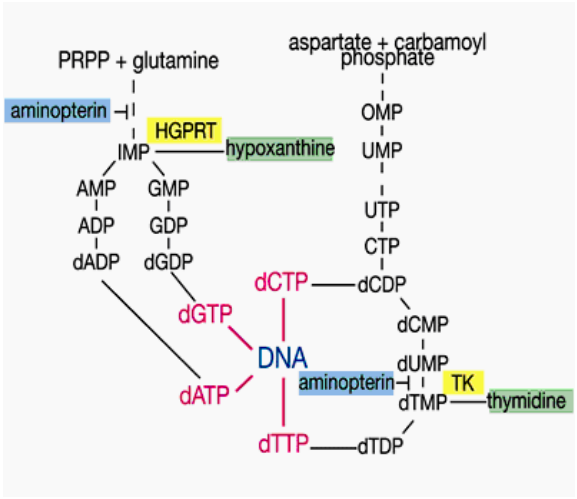

Figure 1.7 : Pathways for nucleotide synthesis (Adapted from Lodish et al., 2004).

1.6 Aim of the Study

Microtubules are structural cytoskeletal components maintaining cellular processes, must be actively organized in the cell. Microtubule severing is an important mechanism for microtubule dynamics for cellular activities. In mitosis and meiosis,

microtubule severing on the spindles is thought to contribute to spindle reorganization (Ahmad and Baas, 1995). In neurons, microtubules are suggested to be severed to generate mobilizable microtubules to be transported in growing processes such as axons and dendrites (Baas et al., 2005). Katanin is an important heterodimeric protein that severs microtubule network to regulate cellular functions using energy derived from ATP hydrolysis (McNally and Vale, 1993). Two katanin subunits, P60 and P80, have been identified. The subunit P60 of katanin which is responsible for microtubule severing, encourages shortening processes of mitotic-meiotic spindle poles and plays an important role in the developing of neuronal processes. P80 is thought to target katanin to the centrosome and enhances the microtubule severing capacity of P60 (Hartman et al., 1998).

This study is subjected to produce monoclonal antibody against P60-katanin, using rat sequence based RecP60 (Recombinat P60-katanin) protein as antigen. Monoclonal antibodies are produced from hybridoma cell lines which were descendant from a single B cell clone are monospecific. Monoclonal antibodies are produced unlimitedly hence they have more advantages then polyclonal antibodies. So far, P60-Katanin polyclonal antibodies were used in studies until now (McNally and Thomas, 1998; Karabay et al., 2004), development of a monoclonal antibody against P60, constitute an important resource to monitor the behavior of P60-katanin in cells and for the research of P60-katanin's functions.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Lab equipments

Centrifuge, Beckman Coulter Allegra 25R

Ultra centrifuge, Beckman Coulter Avanti™ J-30 I

Minicentrifuge, Beckman Coulter Microfuge®18

Magnetic stirrer, Labworld Standard Unit

pH Meter, Mettler Toledo MP220

Precision Weigher, Precisa 620C SCS

Weigher, Precisa BJ 610 C

Ice machine, Scotsman AF 10

Vortex, Heidolph, Reaxtop

Water Baths, Memmert, Elektro-mag M 96 KP

Thermomixer, Eppendorf Thermomixer Comfort

Shaker, Forma Orbital Shaker, Thermo Electron Corporation

Freezers:

-20°C, Uğur

+4 °C, Uğur

-80 °C, New Brunswick Scientific

UV-Visible Spectrophotometer, UV-1700 (Japan) PharmaSpec Shimadzu

Pure water system, TKA Wasseraufbereitungssysteme

Pipettes:

2.5µl, 10µl, 100µl, 200µl, Eppendorf

1000µl, Finnpipette Thermo

Electronic pipette Finnpipette Thermo

Multichannel (12 channel) pipette, 50-300µl, Genex beta

Microfuge tubes, 1.5-2ml, Axygen

Centrifuge tubes, 15-50ml, Avant Plus

Autoclave, Tomy SX-700E

Confocal microscope, TCS SP2 AOBS inverted laser scanning, Leica

Dialysis tubing cellulose membrane, Sigma

2.1.1.1 Equipments for Cell Culture

Laminar air flow cabinets, Faster BH-EN 2003

CO_2 Incubator, Biolab Shel Lab

Inverted light microscope, Olympus CK40

Tissue culture plates, 6-12-96 well, TPP

Tissue culture flasks, 25-75-150cm^2, TPP

Cell Scraper, 30cm length, 20 mm width, TPP

Hemacytometer, Fisher Scientific

Syringe filters, 0.22µm, TPP

Vacuum filtration- system, 150ml, TPP

Serological pipette, TPP

Electroporator, Nucleofector Amaxa Biosystems

Centrifuge, Thermo Electron Corporation IEC CL10

2.1.1.2 Equipments for ELISA

ELISA plate washer, Nunc-Immuno^{TM} Wash 8

Microplate reader, Bio-Rad Benchmark Plus 3550-UV

ELISA plates, 96 well, Nunc

2.1.1.3 Equipments for SDS-PAGE

SDS-PAGE gel electrophoresis system, Bio-Rad MiniProtean

Power supply, Thermo Electron Corporation EC250-90

2.1.1.4 Equipments for Western-blotting

Mini Blot Module, Thermo Electron Corporation EC140

Trans-Blot Semi-Dry Electrophoretic Transfer Cell, Bio-Rad

PVDF membrane, Roche

Filter Paper, 3MM Whatman

2.1.2 Chemicals

$NaH_2PO_4.2H_2O$, J.T. Baker

Na_2HPO_4, J.T. Baker

KH_2PO_4, J.T. Baker

K_2HPO_4, J.T. Baker

NaAc, J.T. Baker

Glycerol, Fluka

Acetic acid (glacial), Fluka

Bradford Reagent, Sigma

Tween 20 (P9416), Sigma

Albumine from bovine serum (BSA), Sigma

Freund's complete adjuvant, Sigma

Freund's incomplete adjuvant, Sigma

4-Nitrophenyl phosphate di (tris) salt, Sigma

50% Ni-NTA agarose suspension, Qiagen

Tris Hydroxymethil aminomethane, BDH Laboratory

$ZnCL_2$, Riedel- de Haën

Acetone, Riedel- de Haën

Absolute methanol, Riedel- de Haën

Absolute ethanol, Riedel- de Haën

Diethyl ether, Lachema

Glycine, Merck

Boric acid, Merck

Glucose, Merck

Pipes, Sigma

Hepes, Invitrogen

EGTA, Sigma

KCl, Merck

Imidazole, Merck

PEG 4000, Merck

Dithiothreitol (DTT), Merck

HCl, Merck

$MgCl_2.6H_2O$, Carlo Erba

NaCl, Carlo Erba

Non-fat dry milk, Pınar

Ammonium Sulphate, Merck

2.1.2.1 Chemicals for SDS-PAGE

SeeBlue Plus2 Prestained Protein Ladder, Invitrogen

Bencmark Prestained Protein Ladder, Fermentas

TEMED, AppliChem

2-propanol (Isopropanol), Fluka

Coomassie Brilliant Blue (CBB) R-2500, Fluka

N,N'- Dimethyl- bis- Acrylamide, Sigma

Acrylamide, BDH Laboratory

Ammonium peroxodisulphate (APS), Merck

SDS, Merck

Bromophenol blue, Merck

2.1.2.2 Chemicals for Bacterial Cell Culture

Isopropyl-β-D-1-thiogalactopyranosid (IPTG), AppliChem

Tryptone, Lab M ™

Kanamycin, Sigma

Yeast Extract, Merck

Agar, Merck

2.1.2.3 Chemicals for Cell Culture

DMEM- High Glucose- Liquid Media (SH30243), HyClone

DMEM-High Glucose Powder (D5648), Sigma

Fetal Bovine Serum (FBS), Biochrom

Gentamicin, Gibco

HT Supplement (100X), Gibco

HAT Supplement (50X), Gibco

DMSO, Riedel-de Haën

2.1.3 Antibodies

Anti-Mouse IgG (γ-chain specific)- Alkaline Phosphatase Conjugate antibody produced in goat, Sigma

Anti-Mouse Polyvalent Immunoglobulins (G,A,M)−Alkaline Phosphatase Conjugate antibody produced in goat, Sigma

Chicken anti-GFP Antibody, Aves

Rabbit anti-β tubulin Antibody, CST

Anti-mouse IgG Antibody-Alexa 488, Invitrogen

Anti-mouse IgG Antibody-Alexa 546, Invitrogen

Anti-mouse IgG Antibody-Cy5,

Anti-chicken IgY Antibody-FITC, Aves

Anti-Rabbit IgG Antibody Alexa 647, Invitrogen

Anti-mouse IgG Antibody-HRP (Horse Radish Peroxidase), Pierce

Anti-rabbit IgG Antibody-HRP, Pierce

2.1.4 Commercial kits

MAbTrap Kit, Sigma

Endofree Plasmid Maxi Kit, Qiagen

Nucleofactor Kit VCA-1001, Amaxa

Supersignal West Femto Maximum Sensitivity Substrate, Pierce

2.1.5 Buffers and solutions

2.1.5.1 Buffers for Ni-NTA Affinity Purification

Lysis Buffer (10 ml)

$NaH_2PO_4.2H_2O$	78 mg
NaCl	175.4 mg
Imidazole	6.8 mg

The pH was adjusted to 8.0 with NaOH.

Wash Buffer (10 ml)

$NaH_2PO_4.2H_2O$	78 mg
NaCl	175.4 mg
Imidazole	13.6 mg

The pH was adjusted to 8.0 with NaOH.

Elution Buffer (5 ml)

$NaH_2PO_4.2H_2O$	39 mg
NaCl	87.7 mg
Imidazole	2.72 g

The pH was adjusted to 8.0 with NaOH.

2.1.5.2 Buffers for SDS- PAGE

Acrylamide and N,N'methylene-bis-acrylamide (200 ml)

Acrylamide	58,4 g
Bis-acrylamide	1,6 g

Filter and store at 4°C in the dark.

10 % Sodium Dodecyl Sulfate (100 ml)

SDS	10 g

Filtered and stored at room temperature.

0,5 M Tris-Cl Solution (50 ml)

Tris	3 g

The pH was adjusted to 6,8 with HCl.

Filtered and stored at 4°C.

1,5 M Tris-Cl Solution (200 ml)

Tris	36,3 g

The pH was adjusted to 8,8 with HCl.

Filtered and stored at 4°C.

%10 APS (1ml)

APS	0,1 g

Stored for 1 week life at 4°C

2X Sample Buffer (10 ml)

Tris- HCl pH:6.8 (of 0.5 M)	2.5 ml
SDS (of 10%)	4 ml
Glycerol (of 100 %)	2 ml
Bromophenol blue	5 mg
DTT	231 mg

Tris-glycine Running Buffer (1 lt)

0.025 M Tris	3g
0.192 M Glycine	14.4 g
0.1 % SDS	10 ml (of 10%)

Staining Solution (500 ml)

CBB R-2500	5 g
Methanol	250 ml
Acetic acid	50 ml

Destaining Solution (1 lt)

Methanol	50 ml
Acetic acid	100 ml

2.1.5.3 Buffers for Western-blotting

Transfering Buffer (150 ml)

Tris	1,6 g
Glycine	0,9 g
Methanol	20 ml
%10 SDS	800 µl

Tris-Buffered Saline (TBS) (300ml)

NaCl	2.4 g
KCl	0.06 g
Tris	0.9 g

The pH was adjusted to 7.4.

Tris-Buffered Saline -Tween 20 (TTBS)

Tween 20 (0.05 %)	90 µl
TBS	180 ml

Blocking Solution

Non-fat dry milk (3 %)	0.9 mg
TBS	30 ml

Prepared just before the experiment.

Primary Antibody Solution I

Non-fat dry milk (3 %)	0.3 mg
Hybridoma supernatant	10 ml

Primary Antibody Solution II

Non-fat dry milk (3 %)	0.3 mg
Purified monoclonal antibody (1:1000)	10 µl
TTBS	10 ml

Secondary Antibody Solution I

Anti-mouse polyvalent immunoglobulins-AP (1:5.000)	2 µl
Non-fat dry milk (3 %)	0.3 mg
TTBS	10 ml

Secondary Antibody Solution II

Anti-mouse IgG-AP (1:10.000)	1 µl
Non-fat dry milk (3 %)	0.3 mg
TTBS	10 ml

BCIP/ NBT Substrate Buffer (250ml)

$MgCl_2.6H_2O$	2,665 g
Tris	3,03 g
NaCl	1,46 g

BCIP/NBT Substrate Solution

BCIP/NBT stock solution	200 µl
BCIP/ NBT substrate buffer	10 ml

The substrate buffer and the stock solution was mixed just before the application.

2.1.5.4 Buffers for Immunocytochemistry

PHEM (2X) (500 ml)

Pipes 18,14 g

Hepes 5,96 g

EGTA 3,8g

MgCl2 0,41 g

pH was adjusted to 6,9 with NaOH.

Made aliquots and stored at -20^{o}C.

Gluteraldehyde Fixation/Permeabilization Solution (40 ml)

Gluteraldehyde (25%) 320µl

Triton X100 (10%) 400 µl

Taxol (10 mM) 40µl

PHEM solution (2X) 20 ml

Fixative was warmed in 37 ^{0}C water bath before using.

Paraformaldehyde Fixation Solution (25 ml)

Paraformaldehyde (PFA) 1 gr

PBS 25 ml

The pH was adjusted to 7,3 by NaOH.

The solution was heated to 60^{o}C with stirring overnight.

Triton X100 Permeabilization Solution

10% TritonX100 was dilued with PBS into final concentration of 0,2 %.

Blocking solution (1 ml)

Goat serum 100 µl

BSA 10 mg

PBS 900 µl

Filtered with 0.8 µm Nalgene filter.

Stored for 15 days at 4 ^{0}C.

Primary Antibody Solution I (200 µl /dish)

Hybridoma supernatant

Centrifuged at 10.000 rpm for 10 minutes at 4^{0}C.

Primary Antibody Solution II (200 µl /dish)

Monoclonal Antibody (MAb) (1/1000)

Filled up to final volume with PBS.

Centrifuged at 10.000 rpm for 10 minutes at 4^0C.

Primary Antibody Solution III (200 µl /dish)

Chicken anti-GFP (1/1000)

MAb (1/1000)

Filled up to final volume with PBS.

Centrifuged at 10.000 rpm for 10 minutes at 4^0C.

Primary Antibody Solution IV (200 µl /dish)

Rabbit anti-β tubulin (1/500)

MAb (1/1000)

Filled up to final volume with PBS.

Centrifuged at 10.000 rpm for 10 minutes at 4^0C.

Secondary Antibody Solution I (200 µl /dish)

Anti-mouse IgG (Alexa 488/ Alexa 546/ Cy5) (1/1000)

Filled up to final volume with PBS.

Centrifuged at 10.000 rpm for 10 minutes at 4^0C.

Secondary Antibody Solution II (200 µl /dish)

Anti-chicken IgY FITC (1/1000)

Anti-mouse Cy5 (1/500)

Filled up to final volume with PBS.

Centrifuged at 10.000 rpm for 10 minutes at 4^0C.

Secondary Antibody Solution III (200 µl /dish)

Anti-Rabbit IgG Alexa 647 (1/1000)

Anti-mouse IgG Alexa 488 (1/1000)

Filled up to final volume with PBS.

Centrifuged at 10.000 rpm for 10 minutes at 4^0C.

2.1.5.5 Buffers for cell culture and ELISA

Polyethylene Glycol (PEG) solution (2 ml)

2 g PEG-4000

Solution was sterilized by autoclave and kept at 37^{o}C.

PBS

10 mM KH_2PO_4 solution: 186 mg/L KH_2PO_4

10 mM K_2HPO_4 solution: 174 mg/L K_2HPO_4

10 mM KH_2PO_4 solution of pH was adjusted to 7.2 with 10 mM K_2HPO_4.

0.15 M NaCl was added to final mixture solution.

PBS-Tween 20

0.05 % tween 20 was added to PBS buffer.

Primary Antibody Solution I

Serum of mouse (1/200)

Filled up to final volume with PBS.

Primary Antibody Solution II

Hybridoma Supernatant

Secondary Antibody Solution I

Anti-Mouse Polyvalent Immunoglobulins (G,A,M)-AP

Filled up to final volume with PBS.

Secondary Antibody Solution II

Anti-Mouse IgG-AP

Filled up to final volume with PBS.

Substrate Buffer

1 mM $ZnCl_2$

1 mM $MgCl_2$

0,1 M Glycine

The pH was adjusted to 10.4 with KOH.

1 mg/ml 4-paranitrophenylphosphate was added just before using.

2.1.6 Bacterial strains

BL21(DE3)pLysS *Escherichia coli* strain harbouring pET30a vector with cloned P60-katanin

Genotype: F^{-} *dcm ompT hsdS*(rB^{-} mB^{-}) *gal* λ(DE3)[pLysS Cam^{r}], Novagen

DH5α *Escherichia coli* strain

Genotype: F^{-}, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^{-}, mk^{+}), *phoA*, *supE*44, λ^{-}, *thi*-1, *gyrA*96, *relA*1, Invitrogen

2.1.7 Bacterial culture media

LB medium (1 lt)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Sterilized by autoclaving at 121°C for 15 minutes.

34 μg/ml chloramphenicol and 30 μg/ml kanamycin was added.

LB-agar

15 g/l of agar to LB medium and sterilized by autoclaving.

34 μg/ml chloramphenicol and 30 μg/ml kanamycin was added.

2.1.8 Cell lines

FO Myeloma, ATCC CRL-1646

NIH/3T3 Fibroblast, ATCC CRL-1658

HeLa, ATCC CCL-2

SHSY-5Y Neuroblastoma, ATCC CRL-2266

2.1.9 Cell culture media

Freezing medium

70 % DMEM

20 % FCS

10 % DMSO

2.1.9.1 Hybridoma cell culture

Growth medium

80 % DMEM (including 3.5 g/L glucose, 2 g/L $NaHCO_3$ and 25 mM Hepes)

20 % FCS

50 µg/ml Gentamicin

HAT medium

2 % (40ml/L) HAT (50x) was added in growth medium.

HT medium

2 % 20ml/L HT (50x) (100X HT) was added in growth medium.

2.1.9.2 HeLa growth medium

90 % DMEM (including 3.5 g/L glucose, 2 g/L $NaHCO_3$ and 25 mM Hepes)

10 % FCS

20 µg/ml Pen/Strep

2.1.9.3 Neuroblastoma and Fibroblast growth medium

90 % DMEM (including 1 g/L glucose, 2 g/L $NaHCO_3$ and 25 mM Hepes)

10 % FCS

20 µg/ml Pen/Strep

2.1.10 Experimental animals

The 6-8 week old female BALB/c mice were used for immunization and fusion. Rat embryos on the day 18 were used for preparing tissue samples for Western- blotting. All experimental animal procedures were approved by animal care and use committee (Appendix A).

2.1.11 P60-katanin construct

pEGFP-C1 (Invitrogen) vector with full length P60-Katanin.

2.2 Methods

2.2.1 Antigen preparation

2.2.1.1 Recombinant P60-katanin expression induction

- Frozen glycerol stock of BL21(DE3)pLysS colonies harbouring pET30a vector with cloned P60-katanin was spread on LB-agar petri plates.
- Single colony from the plate was inoculated into 5 ml LB and left for overnight at vigorous shaking at 37°C.
- Next day, 5 ml of overnight culture was diluted with 500 ml LB media and was incubated with shaking at 37°C.
- After OD_{600} reached 0.6-0.8, 2ml cell culture was collected for uninduced total and soluble-unsoluble protein analysis and total culture was induced with 0,5 mM IPTG and grown for 12 hours, taking 2 ml culture samples at 4th, 8th and 12th hours.
- Total cell culture was centrifuged for 5 minutes at 14000 *x g* at 12. hour after induction and Ni-NTA affinity chromatography was applied.

2.2.1.2 Total protein analysis of the cell

- After induction, collected 1 ml cell cultures at 4th, 8th and 12th hours were centrifuged for 5 minutes at 14000 *x g* .
- Pellet was mixed with 50 µl 2X SDS sample buffer and stored at 4°C until SDS-PAGE analyses.

2.2.1.3 Soluble-unsoluble total protein analysis of the cell

- After induction, collected 1 ml cell cultures at 4th, 8th and 12th hours were centrifuged for 5 minutes at 14000 *x g* .
- Pellet was resuspended in 1 ml of ice-cold 20 mM Tris-HCl pH 7.5.
- Suspension was incubated at -80°C until SDS-PAGE analyses.
- On the day of SDS-PAGE, they were thawed on ice and centrifuged at 14,000 × *g* for 10 minutes.
- The pellet which would give the unsoluble fraction was resuspended with 10 ml lysis buffer.

- The supernatant which would give the soluble fraction, was concentrated with TCA (trichloracetic acid) method:
    - 100 µl (1/10 volume) of 100% TCA (w/v) was added to 1 ml of supernatant, vortexed and placed on ice for 15 minutes.
    - Centrifuged at 14000 *x g* for 10 minutes.
    - Pellet was washed with 100 µl of acetone, vortexed and centrifuged at 14,000 × *g* for 5 minutes. This step was done twice.
    - Pellet was allowed to air dry.

2.2.1.4 Protein separation by SDS-polyacrylamide gel electrophoresis

To analyse total and soluble-unsoluble proteins and to separate proteins by size, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed.

- Firstly, 12.5 % separating gel was prepared:

2,08 ml	30 % acrylamide
1,25 ml	1,5M Tris, pH 8.8
1,56 ml	dH_2O
50 µl	10 % SDS
30 µl	10 % APS
10 µl	TEMED

  The solution was applied into the gel cassette and isopropanol was added drop by drop onto the gel. After polymerization isopropanol was carefully removed.

- 5% stacking gel was prepared.

0,33 ml	30 % acrylamide
0,25 ml	1 M Tris, pH 6.8
1,38 ml	dH_2O
20 µl	10 % SDS
10 µl	10 % APS
5 µl	TEMED

  The stacking gel solution was poured into the gel cassette, and the gel comb was placed.

- After polymerization the comb was removed and the gel cassette was set into the PAGE apparatus and filled with Tris-glycine running buffer.

- The samples containing proteins were prepared:
  - Samples were resuspended in 2X sample buffer and denaturated for 5 minutes at 95ºC
  - 7.5 µl of each sample was loaded on the SDS PAGE gel.
- The proteins were run in the stacking gel for 15 min at 150 V, 250 mA, 25 W
- When the proteins passed through the separating gel, they were separated for 105 min at 100 V, 250 mA, 25 W.
- SDS-PAGE was stained in staining solution for overnight at room temperature and destained in destain solution at 65^{o}C for 30 minutes with shaking at 50 rpm.

2.2.1.5 Metal affinity purification of 6xHis tagged RecP60

The NI-NTA (nickel-nitriloacetic acid) affinity purification uses the interaction between nickel (or other metal ion, e.g. copper, zinc, cobalt) and imidazole ring to purify His-tagged proteins. When the lysate of the cell culture is mixed with Ni-NTA agarose, nickel binds imidazole ring structure of histidine. To elute histidine tagged protein, highly concentrated imidazole which replace with histidine is used.
Purification of RecP60 was performed under native conditions.

- After induction with IPTG, 500 ml bacterial culture was centrifuged for 5 minutes at 14000 *x g*.
- The pellet was stored frozen at -80^{o}C until the next step was done.
- The frozen pellet was thawed on ice, resuspended in 5 ml lysis buffer and centrifuged for 10 minutes at 14000 *x g*.
- 500 µl of 50 % Ni-NTA agarose was added to the supernatant and gently mixed for 1 hour at 4^{o}C.
- Short spin was done to pellet the resin.
  - Supernatant (flow-through) was collected to analyse the unbound protein in SDS-PAGE.
- The resin was washed three times with 2,5 ml of wash buffer. Short spin was done after each wash step.
  - Supernatant was collected to analyse in the SDS-PAGE, if the unbound protein was removed totally.

- Finally, 250 µl elution buffer was added, short spin was done and supernatant containing purified protein was taken to a fresh tube and stored at -20ºC. This step is done for 4 times.

2.2.1.6 Bradford analysis for purified RecP60

The Bradford assay is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The protein-dye complex causes a shift in the absorption maximum at 595 nm. In order to determine the concentration of purified P60-katanin 96 well plate assay protocol was applied. This assay is performed in a 96 well plate. It is possible to quickly assay multiple protein samples with this assay, while using a small sample volume (5 µl of a 0.1-1.4 mg/ml protein sample is used).

- BSA standard ranging from 20 µg -1,25 µg/20µl was prepared in PBS and distributed to wells in the 96 well plate.
- P60 elution samples were prepared at different dilutions by PBS with an approximate concentration between 20 µg -1,25 µg/20µl and distributed to wells.
- 200 µl of the bradford reagent was added onto each well and mixed on a shaker and incubated at room temperature for 5 minutes.
- Absorbance was measured at 595 nm.

2.2.2 Immunizing mice with purified RecP60 and screening antibody sesponse by ELISA

BALB/c female mice were immunized two times intraperitoneally with 50 µg of RecP60/mouse. At first immunization, P60 was injected with Freund's complete adjuvant and at the second immunization, P60 was injected with Freund's incomplete adjuvant. After immunization anti-P60 antibody response was assayed by indirect ELISA which is described in the following steps:

- 120ng P60/well was coated on ELISA plate and incubated for overnight at $4^{o}C$.
- Washing: The plate was washed with PBS-Tween-20, 3 times by using microplate washer.
- Incubation: The plate was blocked with %1 milk powder and incubated at $37^{o}C$ for 1 hour.

- The plate was washed as explained at the above step
- Serum of P60 immunized mice at 1/200 dilution or hybridoma supernatants were treatened onto plates and incubated as the above step.
- The plate was washed.
- Polyclonal antibody response was screened by adding 1/1000 diluted alkaline phosphatase conjugated anti-mouse polyvalent immunoglobulins (conjugate) and IgG antibody response was screened by adding 1/2000 diluted alkaline phosphatase conjugated anti-mouse IgG and incubated.
- The plate was washed five times.
- Conjugate-antiserum reaction was monitored by detection of *p*-nitrophenyl phosphate hydrolysis reaction which could be understood by appearance of a yellow color. *p*-nitrophenyl phosphate was added onto wells and after 1 hour incubation at the dark, the optical density at 450 nm was read by microplate reader.

2.2.3 Cell culture studies

2.2.3.1 Thawing cells from -80^{o}C storage

Cells which had been stored in -80^{o}C for at most 6 months were thawed in 37^{o}C water bath and taken into 10 ml PBS buffer for centrifugation for 5 minutes at 900 *x g*. Cell pellet was dissolved with growth medium and transferred to the culture flasks.

2.2.3.2 Counting cells

Cell pellet was suspended with 20 ml PBS. Then 10 µl suspension was taken and dispersed on the hemacytometer, cells were counted as the number of cells per 25 square (1mm^2). Total cell number was calculated by the two formula, given below:

10^4 (constant number) x Amount of Counted Cell = Cell Number/ml **(2.1)**

Cell Number/ml x Volume of Cells (ml) = Total cell number **(2.2)**

2.2.3.3 Cell passage

Growth medium of cells was removed and 10-15 ml PBS was added in their growing 75 cm^2 flasks. FO and hybridoma cells that attach on the culture dish were lifted via cell scraper and the cells are suspended.

For HeLa, NIH/3T3 Fibroblast, SHSY-5Y Neuroblastoma cells to detach from the culture dish and make the cell suspension several steps were performed. PBS was removed from the flask and 1 ml Trypsin/EDTA was added. Flasks were placed in CO_2 free incubator for 2 minutes. Flask was smacked couple of times, to make cells flow freely.

Suspended cells were put into the centrifuge tubes and centrifuged at 900 *x g* for 2 minutes. Centrifugation step was repeated two times. The cell pellet was resuspended with fresh medium and divided into additional flasks.

2.2.3.4 Cell freezing

After the cell passage, cells were taken into the centrifuge tubes and they were washed with PBS. The cell pellet was transferred to freezing tubes with freezing medium for stocking them. Estimated cell number per freezing tube was $2x10^6$ cell/ml.

2.2.4 Hybridoma Technology

2.2.4.1 Preparation of feeder cells

Feeder cells were isolated from the unimmunized, normal mouse a day before the fusion. After the mouse was sacrificed by cervical dislocation method and disinfected in 70 % alcohol, was taken into laminar flow and placed on the dissecting board in the dorsal position. Abdomen skin of mouse was separated from the underlying peritoneum. To obtain feeding cell (macrophage) mixture from the peritoneum internal face, 5 ml DMEM was injected into the peritoneum cavity and taken back. The mixture was taken to the sterile tube and the cell number was counted. ~6000 feeder cell/100µl was transferred to each well of the cell culture plate which would be used for growing hybridomas after fusion.

2.2.4.2 Isolating spleen and lymph node cells from immunized mouse

Among the mice having the highest immune response was selected by indirect-ELISA and was sacrificed by cervical dislocation method. The mouse was sterilized with 70 % ethanol and taken into laminar flow. The animal was laid on its backside on the dissecting board. With the sterile scissors, incision was made started from genital region to inferior chin and the skin was flayed laterally on two sides. Four pairs of cervical superficial, one pair of axillary, brachial, inguinal and sciatic lymph nodes which are the superficial were collected. When the incision was made in the peritoneal wall, several nodes of mesenteric and two pairs of lumbar which were deep lymph nodes were collected (Figure 2.1). On the left side, spleen was pulled gently, any connective tissue was removed using the blunt end of the sterile forceps and removed from the body. As two groups spleen tissue and lymph nodes were put into petri dishes separately, containing 5 ml PBS in order to purify it from the fatty tissues.

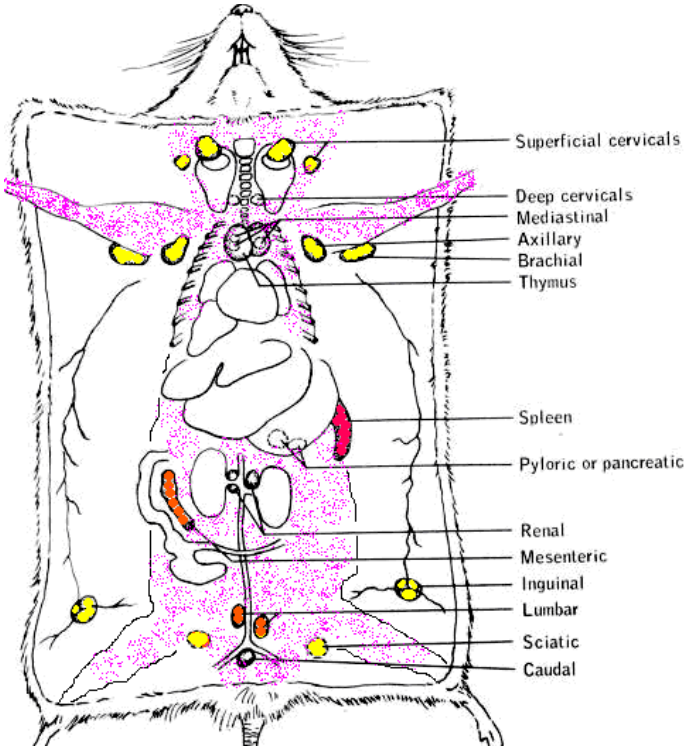

Figure 2.1 : Lymphatic system of mouse (Adapted from Reeves and Reeves, 2003).
Superficial lymph nodes (yellow) deep lymph nodes (orange) and spleen (pink)

The isolated tissues were teased in metal mesh by sequeezing with a glass tube to reveale cells. Cells were suspended in the petri and washed 2 times with 20 ml PBS

buffer by centrifugation at 900 *x g* for 5-10 minutes. Pellet was dissolved in 10 ml medium and cells were counted.

2.2.4.3 Preparation of myeloma cells

10 days before fusion, FO myeloma cells were started to be cultivated in growth medium. At the beginning of the myeloma cell culture studies, 20 µg/ml azoguanin was added to the medium to eliminate HGPRT (+) mutant cells. In the fusion day, myeloma cells were removed from the flasks by making passage and cells were washed 2 times with PBS buffer by centrifugation at 900 *x g* for 5 min. Pellet was taken into 20 ml medium and cells were counted. Cell mixture was held in 5 % CO_2 incubator while preparing spleen cells and lymph nodes for fusion.

2.2.4.4 Fusion

Myeloma/spleen and myeloma/lymph cells were combined in proportion to 1/10-1/2 and centrifuged at 900 *x g* for 10 minutes. After the centrifugation, supernatants were removed. 1 ml (for spleen cells) and 500 µl (for lymph cells) PEG-4000 which was heated at 37^{o}C was added to the pellet of cell mixture drop by drop within 1 min. and mixed gently for 1 minutes. 4 ml (for spleen cells) and 2 ml (for lymph cells) DMEM was added within 1 minutes. 20 ml (for spleen cells) and 10 ml (for lymph cells) DMEM was added within 2 minutes. Finally, 20 ml (for spleen cells) and 10 ml (for lymph cells) DMEM that contains 15 % FBS was added within 2 minutes. Mixture was held for one hour at 37^{o}C in 5 % CO_2 incubator and they were taken to the centrifuge at 800 *x g* for 5 min. After the centrifugation, pellet was suspended with HAT medium and dispersed as 150 µl/well to the cell culture plates which contained feeder cells, prepared one day before the fusion.

2.2.4.5 Following culture after fusion

6-10 days after the fusion, medium of 100 µl/well was removed from the cell culture plates and fresh HAT medium was added as 100 µl/well. 14 days after the fusion, the culture medium was changed with HT medium with the same method. Growing medium was applied according to the period of cells' growth. In 6-10 days period, cells have big and clear images and some small hybridoma colonies can be observed. Each cell culture well was screened by light microscope in order to find out wells including colonies that would be tested by indirect ELISA.

2.2.4.6 Subcloning and large scale production of hybrid cells

Subcloning is required to make the hybridoma cell line coming from the offspring of the single hybridoma cell which results monospecific monoclonal antibody secretion. The procedure involves distributing the hybridoma cell suspension with graded dilutions which results one cell per well in the cell culture plate and the method is called limited dilution (Liddell and Cryer, 1991). This method removes other kinds of antibody producing and nonproducing cells that avoids the suppression of the one kind of antibody producing cells when they overgrow in the culture.

The limiting dilution method was repeated at least 3 times and searched for antibody producing hybrid wells which grew in the last limiting dilution step. The cells from the selected hybrid well were cultured and stored frozen.

Hybrids were transferred to the different size culture flasks to have high amount of antibody yield. For that reason, hybrids were cultivated in 25 cm^2 and 75 cm^2 culture flasks. After cell growing, supernatants containing desired antibody were collected in tubes.

2.2.5 Characterization of anti-RecP60 monoclonal antibodies

Obtained monoclonal antibodies against RecP60 katanin (antigen) were screened if they recognize P60-katanin at tissues by Western-blotting and in mitotic cells by immunocytochemistry.

2.2.5.1 Western-blotting

Western-blotting procedures involve the transfering of proteins that have been separated by gel electrophoresis onto a membrane, followed by immunological detection. For immunoblotting analysis HeLa, NIH/3T3 Fibroblast, SHSY-5Y Neuroblastoma cells and brain, liver, lung and heart lysates of embryo and adult of rats were applied to 12,5 % SDS-PAGE.

Sample Preparation

Cells were detached from the flasks and tissues were isolated from rats , they were washed twice in ice cold PBS and homogenized in 2X Laemmli sample buffer and denaturated for 5 minutes at 95ºC.

SDS-PAGE

50 µg samples were loaded on the SDS-PAGE gel and run in the stacking gel for 15 minutes at 150 V, 250 mA, 25 W and when the sample passed through the separating gel, they were separated for 105 minutes at 100 V, 250 mA, 25 W until the dye of sample buffer migrate to the bottom end of the gel.

Transfer

In this step the separated proteins in the gel were electrophoretically transferred to PVDF membrane.

- The PVDF membrane was prepared for transferring, soaked in methanol, washed with dH_2O and kept in transfer buffer for 10 minutes.
- The gel was placed in the "sandwich" chamber with 6 filter papers and prepared PVDF membrane (3 filter paper/gel/PVDF membrane/3 filter paper) all soaked in transfer buffer.
- The trans-blot was run at 20 V for 30 minutes.

Immunologic detection

For immunological detection two types of antibodies are utilized. The primary antibody is directed against the target protein. The secondary antibody is specific for the constant region of the primary antibody and it is conjugated to an enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP). Anti-mouse IgG or Anti-mouse polyvalent was used as secondary antibody. Two kinds of detection method were used. In chromogenic detection, BCIP/NBT substrate was used to visualize AP cojugated antibody binding and in chemiluminescant detection, luminol substrate (super signal substrate kit) was used to visualize HRP cojugated antibody binding.

- The membrane was incubated with blocking solution by shaking for 2 hours at room temperature.
- It was washed for 10 minutes with TTBS solution, 3 times.
- The membrane was incubated with primary antibody solution by shaking overnight at 4^oC.
- The membrane was washed as explained at the above step.
- The membrane was incubated with secondary antibody solution for 1 hour at room temperature.

- The membrane was washed twice with TTBS for 10 minutes and once with TBS for 10 minutes.
- Finally, membrane was soaked into NBT/BCIP substrate solution and kept in the dark until the bands were observed for chromogenic detection.
- The reaction was stopped by washing the membrane with dH_2O and it was dried at room temperature in the dark.
- For chemiluminescent detection, immunoreactive bands were monitored by incubation of the membrane with supersignal west femto kit including luminol enhancer with peroxidase solution for 5 minutes in the dark and analyzed with CCD camera by UV exitation.

2.2.5.2 Purification of monoclonal antibodies

In order to store the obtained monoclonal antibody (1G6) in large amounts and to use for a long time, it is needed to be concentrated and purified. Therefore, purified antibody can be examined in large amounts that is needed in performing characterization methods and the antibody's affinity in different range of concentrations can also be monitored.

Protein precipitation of hybridoma supernatants by ammonium sulphate

45 % Ammonium sulphate was added to 100 ml of cultured 3H4 and 1G6 hybridoma cell supernatants, in 30 minutes and mixed overnight at 4^oC. The mixture was centrifuged at 20.000 *x g*, 1 hour, at 4^oC. Pellet was dissolved in PBS and dialised against PBS two times, overnight.

Protein-G Immuno-affinity Column Chromatography

Protein-G column was used to purify monoclonal antibodies from concentrated proteins of cell culture medium. Column was washed with 5 ml dH_2O and 4 ml of binding buffer. Ammonium sulfate precipitated and dialized medium was filtered and loaded on the column and binding buffer was added to the column. 1 ml of fractions were collected and absorbance was measured at 280 nm. Binding buffer was transfered from the column until the absorbance of fractions were zero and elution buffer was applied. Eluted fractions were collected as 0,5 ml in 50 µl neutrilizing buffer containing tubes. After absorbance at 280 nm became zero, 5 ml binding buffer and 2 ml ethanol was loaded to store the column. Collected column fractions were tested by ELISA to select the eluted antibody fractions by its activity.

2.2.5.3 Immunocytochemistry with monoclonal antibodies

HeLa (human), SHSY-5Y Neuroblastoma (human) and NIH/3T3 Fibroblast (mouse) cells were grown on coverslips in 33 mm cell culture petri plates for 48 hours in growth medium. The cells were fixed and permeabilized with methanol and they were treated with IgG type of monoclonal antibodies (MAbs), 1G6, 3H4, 3H6, 9H3. To visualize these MAbs' recognition of intrinsic P60-katanin, cells were incubated with flourescence labelled secondary anti-mouse IgG antibodies (Alexa 488; Alexa 546 or Cy5). Paraphormaldehyde, gluteraldehyde and methanol fixation methods were tried to find out the optimum method for our antibody stainings. MAbs were examined with their supernatant and also with their purified forms. Since 1G6 was found to interact with P60-katanin in all fixation methods, to further confirm the localizations, overlay of P60-katanin and tubulin was analyzed on methanol fixed HeLa cells. 1G6 and rabbit anti-β tubulin antibody were used as primary and Alexa-488 anti-mouse IgG and Alexa-647 anti-rabbit IgG antibody were used as secondary antibodies.

Fixation-Permeabilization of cells by Gluteraldehyde

- The fixation/extraction solution was warmed at 37^0C water bath.
- Petri dishes containing the cultured cells were filled with 2 ml of fixation/ permeabilization solution and incubated at room temperature for 15 minutes.
- 10 mg/ml sodium borohydrate solution was prepared in PBS.
- Cells were quenched 2 times for 15 minutes with sodium borohydrate solution then rinsed with PBS 3 times for 5 minutes.
- Dishes were kept in 4 ^{0}C until the day of immunostaining in PBS.

Fixation-Permeabilization of cells by Methanol

- MeOH was placed at -20 ^{0}C in beaker for 10 minutes to be chilled.
- The media from the dishes containing the cultured cells were removed and filled with ice-cold MeOH and kept for 15 minutes.
- MeOH was removed from the dishes.
- The dishes were rinsed with PBS 3 times and kept at 4^0C until the day of immunostaining in PBS.

Fixation of cells by Paraformaldehyde and Permeabilization by TritonX100

- The paraformaldehyde fixation solution was warmed at 37^0C and filtered.
- The cell were fixed with the solution for 15 minutes at room temperature.
- For permeabilization, the dishes were filled with 2 ml of TritonX100 solution and kept for 10 minutes at room temperature.
- Cells were rinsed with PBS 3 times for 5 minutes.
- 10 mg/ml sodium borohydrate solution was added to cells and quenched two times for 15 minutes.
- Cells were rinsed with PBS and kept at 4 ^{0}C until the day of immunostaining.

Immunostaining

- Fixed cells were blocked for 1 hour at room temperature with 150 µl blocking solution.
- Blocking solution was removed carefully and 150 µl primary (1^o) antibody (Ab) solution was put on.
- 1^o Ab containing cells were incubated at 4^0C, overnight. During this staining period, the dishes were placed in a "humidified chamber," a large petri dish containing moistened filter paper, which minimizes evaporation of the antibody.
- 1^o Ab was removed and dishes were washed by using PBS 3 times for 5 minutes.
- Cells were blocked again for 1 hour at room temperature.
- Secondary (2^o) Ab solution was prepared and centrifuged in the same way as 1^o Ab and 150 µl of 2^o Ab solution was applied to the dishes.
- Cells were incubated for 1 hour at 37 ^{0}C incubator in the dark.
- All the dishes were rinsed with PBS 3 times for 5 minutes each.
- 20 µl of mounting medium was added and cover slips were placed on the dishes.
- Coverslips were fixed from the edges by using nail polisher.
- Dishes were visualized and analyzed by using confocal microscopy.

2.2.5.4 Overexpression of P60-Katanin in mitotic cells

For overexpression of the katanin, HeLa cells were transfected with the pEGFP-C1 vector (Clontech, Mountain View, CA) carrying full P60-katanin (human). Firstly, the constructs were transformed into competent DH5α *E.Coli* bacterial strain for copying (replicating) them in large scale. It is aimed to concentrate the amount of the plasmid because it is prerequisite for eukaryotic transfection. The plasmids were purified from the transformed and large scale cultured cells so the concentrated amount would be obtained. The consruct that was produced and purified in large amounts were transfected into HeLa cells.

Transformation of P60-pEGFP-C1 into DH5α

- The competent DH5α cells were taken from –80^{o}C and thawed on ice.
- When cells were thawed on ice, 1 µl of P60-katanin construct was added immediately to 50 µl of competent cells and the mixture was incubated on ice for 30 minutes.
- Then, heat shock was done by putting the cells in the 42^{o}C waterbath for 45 seconds and they were then immediately put back on ice for 3 minutes.
- 100 µl of SOC medium was added to treated cell-plasmid mixture and short cultured at 37^{o}C for 1 hour by shaking at 200 rpm,.
- 100 µl of cells were then spreaded onto LB-agar plate containing 30µg/ml kanamycin. The plates were incubated at 37^{o}C overnight.

Large-Scale Purification of P60-pEGFP-C1

Endotoxin-free DNA will improve transfection into sensitive eukaryotic cells. Therefore, in this step EndoFree Plasmid Maxi Kit was used for obtaining endotoxin-free plasmid DNA.

- Starter culture: A single bacterial colony was picked from the cultured transformation plate and inoculated into 5 ml LB media with kanamycin and incubated for 6 hours with shaking at 200 rpm, at 37ºC.
- 200 µl of the starter culture was diluted into 100 ml LB media with kanamycin and incubated for overnight with shaking at 200 rpm, at 37ºC.
- The next day, 50 ml of the culture was distributed into 2 falcon tubes and centrifuged for 30 minutes at 6000 *x g*.

- The supernatants were discarded completely and the bacterial pellet was resuspended in 10 ml of suspension buffer (5ml/falcon) and collected to one of the falcon tubes.
- 10 ml lysis buffer was added and mixed by vigorously inverting the tube 6 times and incubated at room temperature for 5 minutes.
- Cell lysate was precipitated by addition of 10 ml chilled neutralization buffer and the tube was again mixed as told at the above step.
- The lysate poured into the barrel of the cartidge and incubated at room temperature for 10 minutes.
- The cap from the cartridge outlet nozzle was removed and the plunger was inserted into it and cell lysate was filtered and collected into a falcon tube.
- 2,5 ml endotoxin removal buffer was added to the lysate and mixed by inverting the tube 10 times and incubated on ice for 30 minutes.
- Qiagen column was equilibrated by adding 10 ml equilibration buffer, allowing the column to empty by gravity flow.
- The endotoxin removal buffer added lysate was applied to the equilibrated column and allowed to enter the resin by gravity flow.
- The filter was washed 2 times with 30 ml washing buffer.
- The plasmid was eluted by passing 15 ml elution buffer which was previously warmed at 40^{o}C, through the washed filter. The eluate was collected in a polystyrene or polypropylene falcon tube.
- The eluted DNA was precipitated by adding 10,5 ml (0,7 volumes) room-temperature isopropanol. The mixture was centrifuged immediately at 3600 *x g* for 60 minutes at 4°C. The supernatant was decanted.
- The DNA pellet was washed with 5 ml of endotoxin-free 70 % ethanol at room temperature and centrifuged at 3600 *x g* for 60 minutes at 4°C. The supernatant was decanted carefully, without disturbing the pellet.
- The pellet was air-dried for 15 minutes, and redissolved in 200 µl of endotoxin-free Buffer TE.
- Concentration of the purified plasmid was determined by UV spectrophotometry at 260 nm. Its migration was analysed in agarose gel electrophoresis. Absorbance at 260 nm was used to calculate the obtained amount of the plasmid by comparing and proportioning with the standard plasmid.

Transfection of purified P60-pEGFP-C1 into HeLa Cells

- 15 μg of P60-katanin pEGFP-C1 construct was put into tube.
- Cultured cells were detached and counted with hemacytometer.
- $1x10^6$ cells/transfection were taken from the cell suspension and centrifuged at 900 *x g* for 5 minutes.
- Supernatant was removed and the cell pellet was resuspended with 100 μl solution R (Amaxa nucleofactor kit).
- Cell-solution R suspension was put into each tube containing plasmids and mixed.
- The mixture was transferred into the electroporation cuvette (Amaxa) and placed into electroporator.
- The transfection was done in the nucleofector instrument by using the program I-013.
- Electroporated cells were taken out immediately by special pipettes provided in the nucleofactor kit and transferred into new tubes containing 900 μl fresh medium. As a result we had 10^6 cells/ml that means ~$1x10^6$ cells per transfection set.
- 10.000 cells/dish were plated into 33 mm plastic culture dishes. Hence, for 5 dishes 10.000 cells x 5 = 50.000 cells were needed which was in 50 μl. 50 μl of transfected cells were suspended in 700 μl medium and plated as 150 μl/dish.
- Cells were put in 37 ^{0}C 5% CO_2 incubator for 2-3 hours.
- 2 ml warm medium was added into the dishes and cells were cultured at 37^{0}C 5% CO_2 incubator.

After electroporation, cells were fixed-permeabilized with methanol at 4th, 8th, 12th, 24th, 48th and 72th hours to determine different expression levels of P60-GFP and then treated with 1G6 MAb and chicken anti-GFP antibody. Cy5 conjugated anti-mouse and FITC conjugated anti-chicken IgY antibodies were used as secondary antibodies, respectively. Images of the cells were obtained with a TCS SP2 AOBS inverted laser scanning confocal microscope (Leica, Wetzlar, Germany) using a 63x oil immersion objective.

3. RESULTS

Production of monoclonal antibody against P60-katanin studies have been performed in four steps. The first step was cloning the selected RecP60 sequence performed in Karabay's laboratory previous to the study. For the second step, the antigen which named recombinant P60-katanin (RecP60) has been expressed in large scale. For the third step monoclonal antibodies against RecP60 were produced by hybridoma technology. For the fourth step the monoclonal antibodies were characterized by Western-blotting and immunocytochemistry. When the study was concluded one antibody named 1G6 was selected as monoclonal antibody recognizing P60-Katanin.

3.1 Preparation of RecP60 Protein As The Antigen

3.1.1 Optimization of large scale RecP60 expression time

RecP60 which was inserted in pET30a vector was expressed in BL-21(DE3)pLysS cells under 0,5 mM IPTG induction. After induction, culture samples were collected at 4^{th} , 8^{th} and 12^{th} hours in order to determine the maximal soluble amount of RecP60 expression time for large scale production. In order to check whether over-expressed P60-katanin was in the soluble fraction, cells were disrupted by freezing and thawing. Soluble and insoluble fractions were separated by centrifugation after they were suspended in Tris buffer. The supernatant which was the soluble fraction, was concentrated by TCA method and these samples were compared with total cell protein samples by SDS-PAGE.

The SDS-PAGE results showed that RecP60 was expressed at 35 kDa and found in maximal amount at the soluble fraction of 12^{th} hour of IPTG induction. Beginning from the 4^{th} hour, the amount of soluble RecP60 seemed to increase proportionally to the amount of total cell protein and became distinguishable in a maximum level at 12^{th} hour after IPTG induction. In the uninduced control sample any distinguishable overexpressed protein was not found (Figure 3.1). Amount of soluble protein seems to increase proportionally to the amount of total protein. Studies of soluble and

insoluble fractions showed that most of the expressed protein is in soluble state means that the His tagged protein was purified in soluble state succesfully under native conditions.

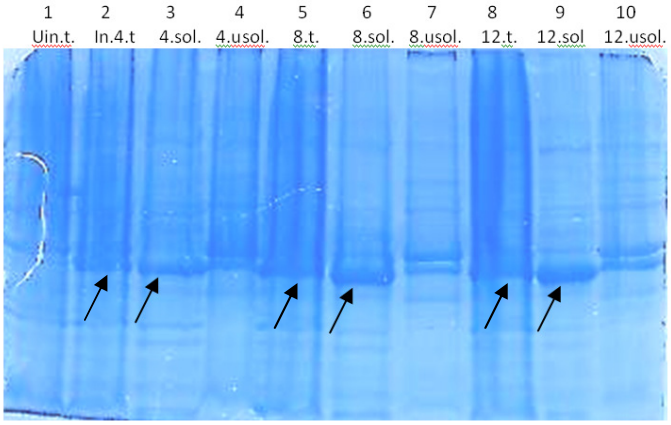

Figure 3.1 : SDS-PAGE analysis of the expression level and soluble proportion of RecP60.

P60 is seen in total and soluble protein fractions which is shown with arrows. Uninduced total protein (Uin.t.), Induction after 4h. total protein (In.4.t), Induction after 4h. soluble protein (4.sol), Induction after 4h. unsoluble protein (4.usol), Induction after 8h. total protein (8.t.), Induction after 8h. soluble protein (8.sol.), Induction after 8h. unsoluble protein (8.usol), Induction after 12h. total protein (12.t), Induction after 12h. soluble protein (12.sol), Induction after 12h. unsoluble protein (12.usol.)

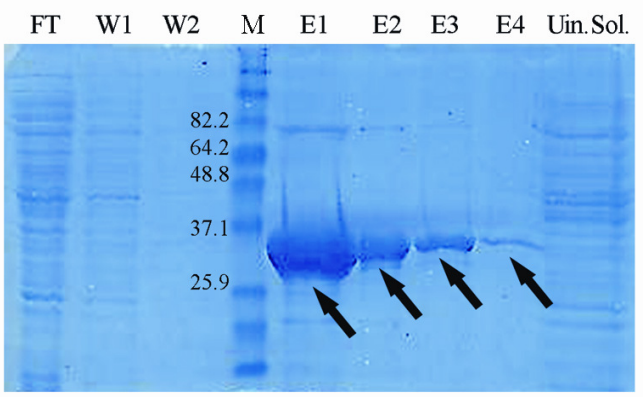

Figure 3.2 : SDS-PAGE analysis of RecP60 purification by Ni-NTA affinity.

Purified P60 is shown with arrows. Flow through (FT), First wash fraction (W1), Second wash fraction (W2), M: Benchmark prestained protein ladder, First elution fraction (E1), Second elution fraction (E2), Third elution fraction (E3), Forth elution fraction (E4), Uninduced soluble protein (Uin.Sol.)

3.1.2 Expression and purification of RecP60 under native conditions

As the RecP60 nucleotide sequence was cloned into pET30a vector, it was expressed as a fusion protein with His-Tag sequence. Since studies of soluble and insoluble fractions showed that most of the expressed RecP60 was in soluble form at 12^{th} hour of induction, the pellet of large scale culture induced for 12 hours was collected. RecP60 was purified by Ni-NTA agarose purification method under native conditions, using the affinity of the His-Tag sequence to Ni.

The lysate of 12^{th} hour culture was treated with Ni-NTA agarose. After washing step, RecP60 was eluted. The fractions of each purification step were analysed with SDS-PAGE (Figure 3.2). A very dense protein band was seen in the 1^{st} elution fraction clearly and as expectedly in the following elution fractions the protein amount decreased gradually. The absence of RecP60 in flow-through and wash fractions showed that RecP60 was captured by Ni-NTA agarose. Uninduced culture was also applied to the SDS-PAGE as the negative control. It was clearly seen that overexpressed band was only observed in purified, induced pET30a-P60 transformed fractions, missing in the negative sample.

3.1.3 Measurement of purified RecP60 concentration by Bradford Assay

The protein concentrations of each RecP60 purified fractions were measured by Bradford reagent. At the first stage of the application standard curve was drawn by using BSA prepared at different concentrations as standarts and the measurement of the absorbance at 595 nm of the standards with Bradford reagent (Figure 3.3). RecP60 elution fractions were prepared by serial dilutions and their absorbance at 595 nm was measured (Figure 3.4).

To calculate the protein concentration of RecP60 elution fractions, increasing micrograms of BSA standard absorbtions were compared with the absorbtions of elution fractions at increasing volumes. The absorbance of the certain volume of each fraction was spotted on the standard curve and the corresponding concentration was revealed on the same curve. If 4 µl of each fraction was considered, the concentrations were calculated as follows: E1, 20 µg/4µl (5 mg/ml); E2, 10µg/4µl (2,5 mg/ml); E3, 3.5 µg/4µl (875 µg/ml); E4, 1.5 µg/4µl (375 µg/ml).

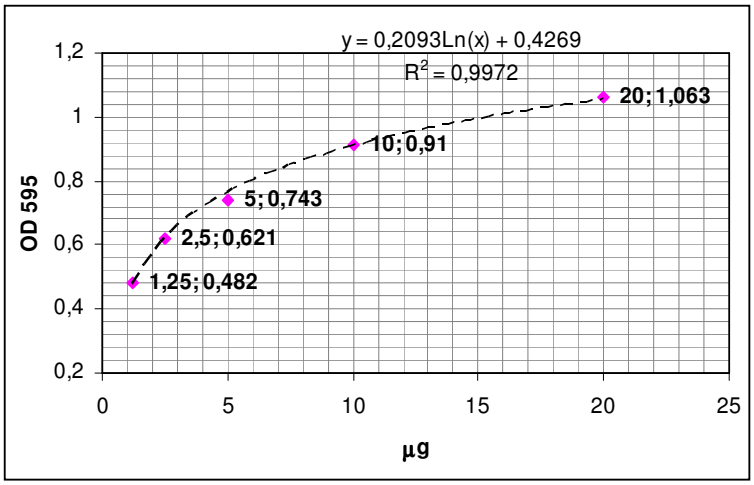

Figure 3.3 : BSA standart curve of the Bradford assay.

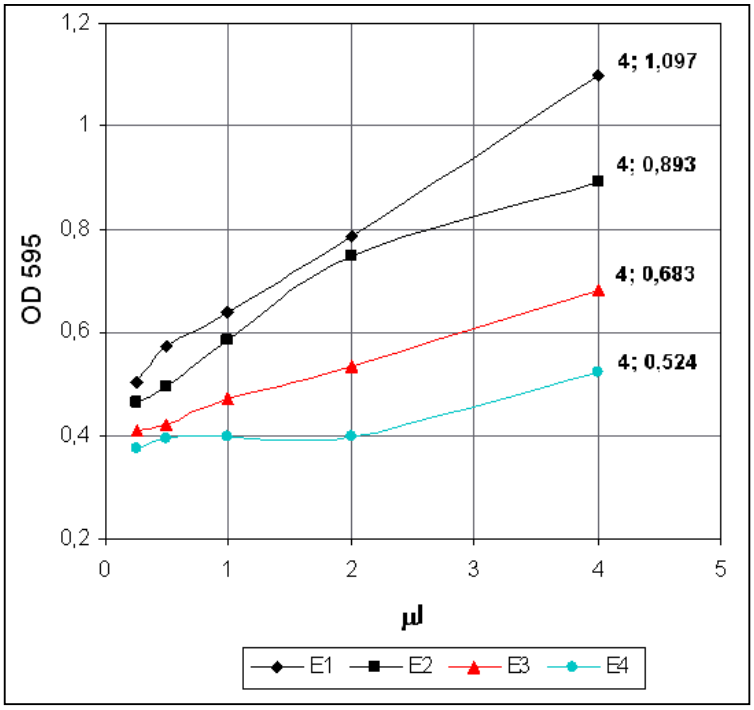

Figure 3.4 : Results of the bradford assay of RecP60 purified fractions.
The graph shows absorbtions at 595nm of purified RecP60 at increasing volumes. Purified fractions: First elution fraction; E1, Second elution fraction; E2, Third elution fraction; E3, Forth elution fraction;E4.

The fractions were collected as 250 µl in the purification step, so from the 1st fraction of elution (E1) to the 4th fraction (E4), 1250 µg/250µl, 625 µg/250µl, 219 µg/250µl, 94 µg/250µl RecP60 protein was obtained, respectively. As a result, 2,2 mg/ml RecP60 protein was in total purified with Ni-NTA purification method.

3.2 Monoclonal Antibody Production

3.2.1 Immune response control of mice and determining RecP60 coating amount for ELISA

Anti-RecP60 polyvalent and anti-RecP60 IgG antibody response of BALB/c mice immunized with RecP60, were assayed by ELISA. For the first ELISA test, it was necessary to find the optimum coating amount of RecP60 on ELISA plates. Therefore, the immune sera of mice were treated with the coated various amounts of (30, 60, 120 ng/well) RecP60 during the ELISA. Immune response against RecP60 was controlled by using two types of secondary antibody. Polyvalent response which would indicate the three types of antibody response was analysed by using alkaline phosphatase conjugated anti-mouse polyvalent immunoglobulins (IgA, IgM, IgG) as secondary antibody. To analyse one type, the IgG type antibody response alkaline phosphatase conjugated anti-mouse IgG antibody was used.

In this ELISA result, the highest immune response was found at 120 ng coated wells on test plates. 10 days after second immunization, all sera of mice showed anti-RecP60 polyvalent and IgG antibody response over 0,8 RecP60 (120ng) coated wells (Figure 3.5). According to the results, the most suitable amount for RecP60 coating was determined as 120ng for following ELISA tests. 1st mouse which developed the highest IgG response against RecP60 was decided to be used for the 1st fusion study. The second highest immune mouse, 4th mouse, was selected for the 2nd fusion and the others were selected the same way, respectively. One week before every fusion, a booster immunization was done to the selected mouse as the 3rd immunization which was called rapel. After rapel, all mice which were used for fusions showed high IgG response over 1,5 in ELISA (to 120ng RecP60). Generating IgG response in mice was the evidence that the secondary immunization was done successfully. Obtaining IgG antibody response was important because it enhanced our chance to find hybridomas, producing IgG type antibodies.

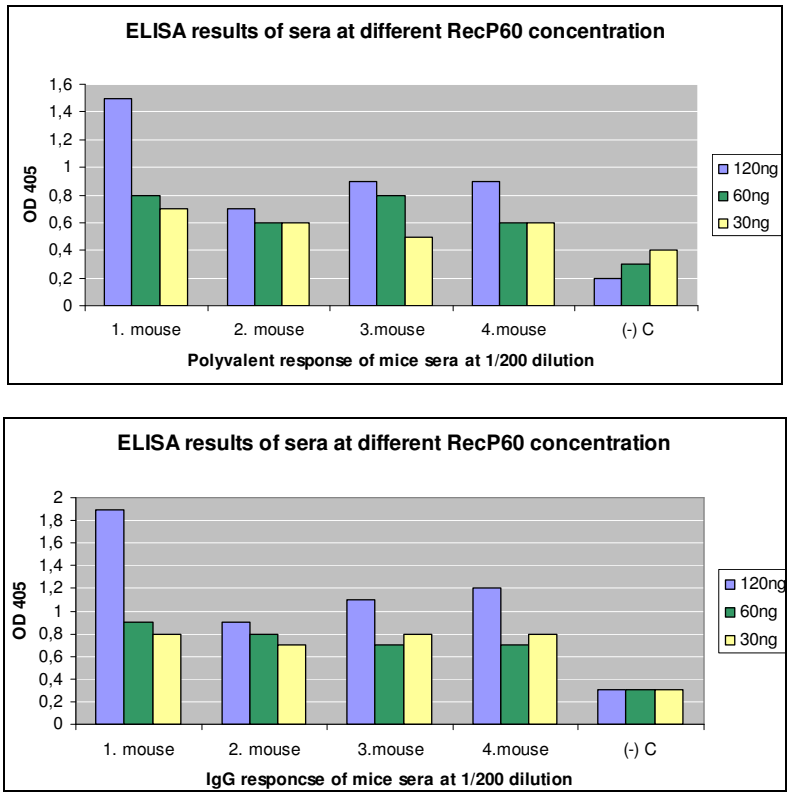

Figure 3.5 : Anti-RecP60 polyvalent and IgG antibody response of immunized BALB/c mice.

RecP60 was coated on ELISA test plates at different concentrations: 120ng, 60ng, 30ng. Sera of mice were diluted in proportion to 1/200 with PBS for measuring their immune response. (-) C : negative control, non-immunized mouse.

3.2.2 Obtaining hybridomas by cell fusion studies

Mice developed IgG response against RecP60 were selected to fuse their spleen and lymphoid cells with FO, myeloma cells. There were 7 fusions performed. For the first 6 fusions, the 2 fusions performed for one mouse: spleen and lymph node cells of the mouse fused with FO cells, separately. Only in the 7th fusion, cells of spleen and lymph node were mixed together and fused with FO cell line. In other words, only 1 fusion was performed with the mixture of two types of lymphocytes from the last mouse (Table 3.1). Following the fusion studies, at around day 10 hybrid colonies appeared. The culture medium (supernatant) was replaced with fresh medium in around every 10 days according to growing speed of the colonies and the supernatants were tested by ELISA.

Each antibody responding cell was passaged into a large scale gradually and tested for its antibody response with its collected supernatants to watch antibody producing

continuity. They were named with the well number of which they were first grown in the 96 well plate. The name contains 2 number and 1 letter, for example 1G6, the first number represents the plate number, the following letter stands for the row and the last number corresponds to the column of the plate.

As a result, from the 4th fusion only one, 3H4; from 7th fusion twelve, 1F8, 1G6, 2B11, 2C8, 3H6, 4G1, 6B6, 6E3, 6G3, 7F11, 9H3 and 11D3 antibody producing hybridoma cells were obtained (Table 3.1). In the 7th fusion, 4G1, 6E3 and 11D3 lost their anti-RecP60 activity, the reason can be chromosome loss in the hybrid line.

Table 3.1: Fusion conditions and results.

	1st fusion	**2nd fusion**	**3rd fusion**	**4th fusion**
Number of spleen cells	75 x 10^6	**-**	71 x 10^6	**-**
Number of lymphoid cells	**-**	10 x 10^6	**-**	11 x 10^6
Number of myeloma cells	7 x 10^6	3 x 10^6	56 x 10^6	18 x 10^6
Number of wells for fusion plates	576	480	576	480
Hybridoma cells	1	0	11	12
Hybridoma cells producing antibody response	0	0	0	1 **(3H4)**
Hybridoma cells producing a-RecP60 response	0	0	0	1 **(3H4)**

	5th fusion	**6th fusion**	**7th fusion**
Number of spleen cells	-	110 x 10^6	150 x10^{6*}
Number of lymphoid cells	130 x 10^6	-	
Number of myeloma cells	30 x 10^6	30 x 10^6	60 x 10^6
Number of wells for fusion plates	384	288	1056
Hybridoma cells	15	10	1056
Hybridoma cells producing antibody response	0	0	12 (**1F8, 1G6, 2B11, 2C8, 3H6, 4G1, 6B6, 6E3, 6G3, 7F11, 9H3, 11D3)**
Hybridoma cells producing a-RecP60 response	0	0	6 (**1G6, 3H6, 6B6, 6G3, 7F11, 9H3**)

*Spleen and lymphoid cells were mixed during 7th fusion.

The specificities of obtained antibodies against RecP60 were tested by their cross reactivity against some proteins, such as BL21 and skim milk powder. BL21 lysate which was prepared from the cell lysate of *E.Coli* BL21 strain without pET-30a-RecP60 vector, was used to detect whether an activity of a monoclonal antibody is observed against a bacterial protein that comes from RecP60 purification. Skim milk which was used in the blocking step of ELISA, was used to detect the activity of MAbs against the blocking agent.

In the cross-reactivity ELISA, the activity of monoclonal antibodies against RecP60, cellular lysate of BL21 and skim milk powder was compared. As a result, 3H4, 6B6 and 6G3 monoclonal antibodies reacted mostly with RecP60. 1G6, 3H6 and 9H3 recognized RecP60 higher than BL21 lysate and milk powder. 1F8, 2B11, 2C8 showed high cross reactivity with milk powder and BL21. 7F11 differently recognized BL21 lysate higher than RecP60 (Figure 3.6). Although 1G6, 3H4, 3H6, 6B6, 6G3, 9H3 seemed to recognize RecP60 specifically according to these results, all the 10 antibodies were tested for further characterization because there can be common epitops on these three proteins and on the native P60-Katanin.

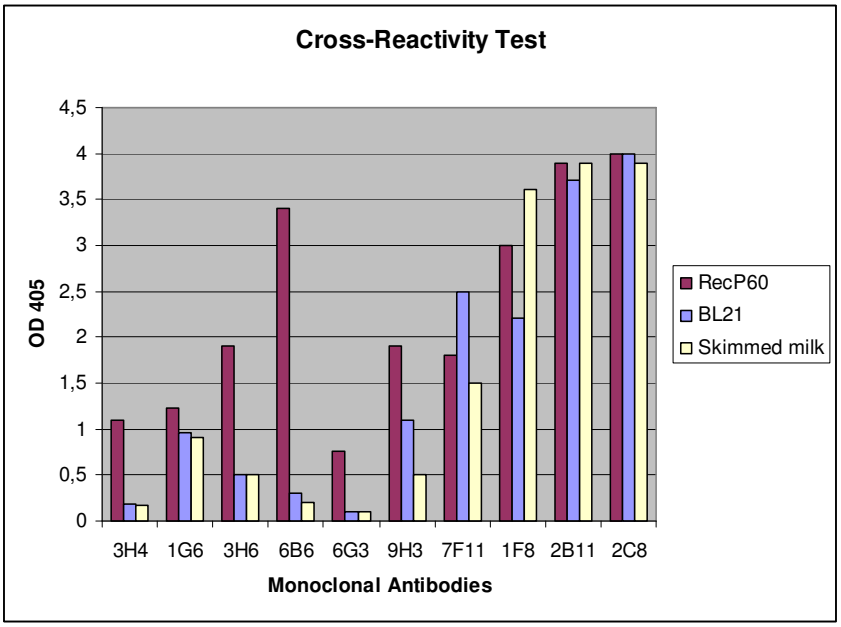

Figure 3.6 : Cross-reactivity of the monoclonal antibodies.
E.Coli BL21 lysate without pET30a and skim milk activity was compared with RecP60.

The immune response of screening and cross-reactivity ELISA tests were visualized by anti-mouse polyvalent immunoglobulins (IgA, IgM, IgG). It was also tested whether these antibodies were IgG type or not. To understand that, the activity against RecP60 was compared with anti-mouse polyvalent immunoglobulins and anti-mouse IgG immunoglobulin. According to the ELISA results 1G6, 3H4, 3H6 and 9H3 were found to be IgG type antibodies but 7F11 was not IgG, it could be either IgA or IgM type (Table 3.2).

Table 3.2: Isotype of anti-RecP60 monoclonal antibodies.
1G6, 3H4, 3H6 and 9H3 were IgG type antibodies. 7F11 could be either IgA or IgM.

	1G6	**3H4**	**3H6**	**7F11**	**9H3**
Anti-RecP60 polyvalent response	1	1,7	1,1	1,4	1,6
Anti-RecP60 IgG response	1,1	1,7	1,3	0,2	1

As a summary, IgG type 3H4 from the 4th fusion; 1F8, 2B11, 2C8, 6B6 and 6G3 and IgG type 1G6, 3H6, 7F11 and 9H3 from the 7th fusion, hence in total 10 hybridoma cells and their the same named monoclonal antibodies were obtained. All the hybridoma cells were subcloned by limiting dilution and cultured in large scale. The hybrid cells were also frozen in freezing medium at -80^{o}C to be stored for a long time.

3.3 Characterization of Anti-RecP60 Monoclonal Antibodies

Since the aim was producing monoclonal antibody that would recognize P60-Katanin, it was necessary to select the antibody by examining it in further studies. Their monoclonal antibody containing supernatanats were collected for further selection and characterization studies to test if they recognize P60-Katanin of cells by Western-blotting and immunocytochemistry.

3.3.1 Western-blotting with monoclonal antibodies

Western-blotting studies were performed to test whether obtained MAbs would recognize native P60-katanin in tissues. Rat embryonic brain, rat embryonic liver tissue and HeLa cell lysates were used to test for all MAbs obtained after fusion studies. Liver and Hela cell line were chosen to represent the mitotic tissue and brain was chosen as nervous tissue. 50 µg/well tissue lysates were loaded on % 12 polyacrylamide gel for SDS-PAGE. The six antibodies 3H4, 3H6, 6G3, 6B6, 7F11 and 9H3 recognized RecP60 at 36 kDa but there was no significant protein band at 55-65 kDa where native P60-katanin migrates. Only 1G6 recognized protein bands at 55 kDa on brain and 65 kDa on HeLa lysate. Anti-β Tubulin and KATNA1 antibody were used as positive kontrol. Anti-β Tubulin antibody which was used as general Western-blotting method control (internal control), recognized a band at 55 kDa in

all tissues. KATNA1 recognized a band at about 55 kDa in brain which was used to control visualizing P60-Katanin protein (Figure 3.7). β Tubulin staining showed us the steps of the method was performed succesfully. As KATNA1 was a commercial antibody produced against P60-Katanin, it showed us that 1G6 reacts with the same molecular weight band where P60-Katanin migrates on nervous tissues.

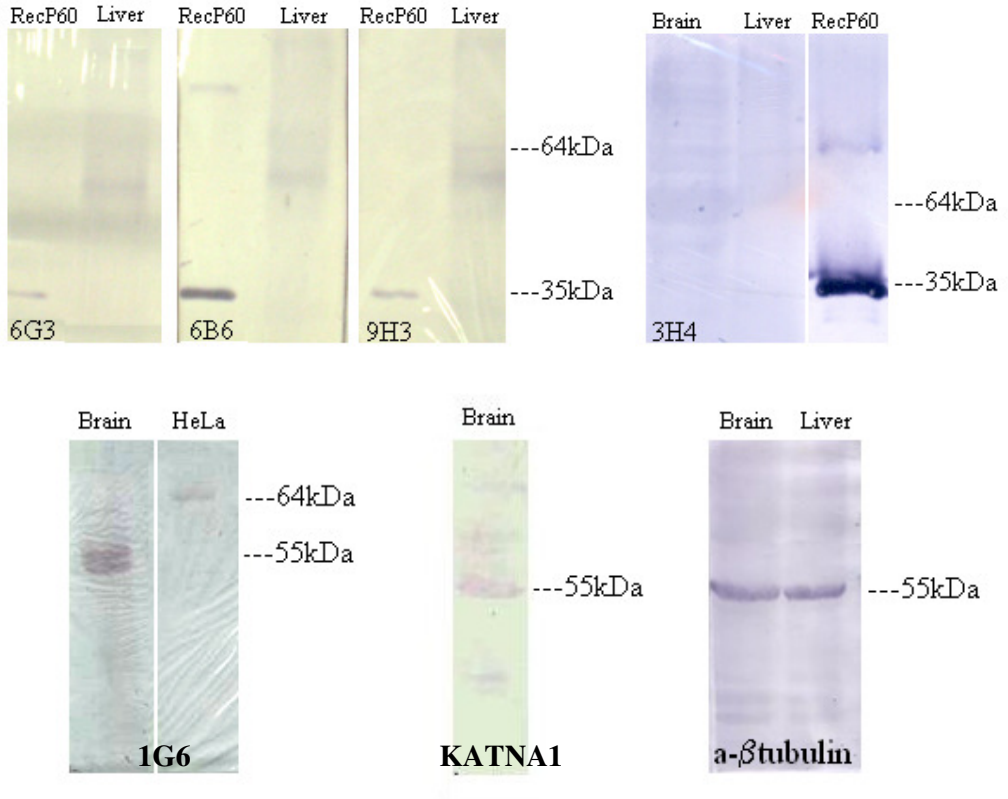

Figure 3.7 : Screening of monoclonal antibodies by Western-blotting.
They recognized no significant band on lysates of brain and liver tissues and HeLa cell line except 1G6. Anti-β tubulin an
used as a positive control for tissue lysates.

Because the method is more sensitive, chemiluminescant semi-dry Western-blotting was performed to examine 1G6 MAb recognition of P60-katanin in various tissues. In this method, 1G6 interaction with native P60-katanin in brain, brain stem, cerebellum, liver, lung, heart lysates of embryo and adult rat tissues were screened. As a result, 1G6 recognized a protein band at 55 kDa on brain, cerebellum and brain stem and 65 kDa on liver, lung, heart of adult and embryonic rats (Figure 3.8). Among the other MAbs, only 1G6 seemed to recognize P60 in all tissues. According to these results, supernatants of 1G6 were collected and 1G6 antibody was purified from the supernatant for further characterizations. After purification 1G6 was

examined again with Western-blotting, wide-cross-reactivity ELISA and immunocytochemistry. The same results were obtained with purified 1G6 that recognized P60-Katanin in tissues by Western-blotting. Wide-cross-reactivity-ELISA and immunocytochemistry results were told in the next sections 3.3.2 and 3.3.3.

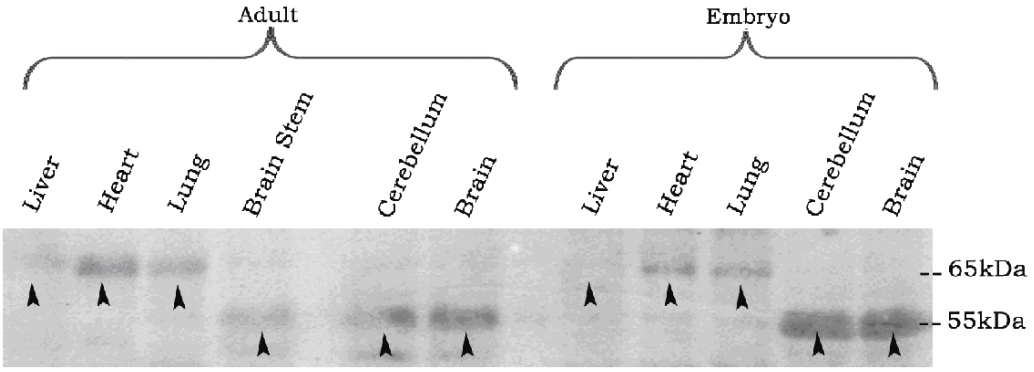

Figure 3.8 : 1G6 recognition of P60-katanin in various tissue lysates by chemiluminescant Western-blotting.
The protein bands at 55kDa on brain, cerebellum and brain stem and 65 kDa on liver, lung, heart of adult and embryo were shown by arrows.

3.3.2 Purification of 1G6 monoclonal antibody

Since 1G6 was found to recognize P60-katanin with Western-blotting, it was aimed to concentrate, store and purify 1G6 monoclonal antibody for further cross-reactivity ELISA tests and immunocytochemistry. For this purpose, 1G6 was cultued in large scale and its supernatnat was collected and protein precipitation by ammonium sulphate method was done. After dialysing the concentrated protein mixture of cell culture medium against PBS, 1G6 monoclonal antibody was purified by Protein-G Immunoaffinity Column Chromatography. 0,5 ml of fractions was collected after loading our sample into the column and absorbance was measured at 280 nm to follow the protein concentration changing during binding, washing and elution steps of purification. After purification, column fractions were also tested for ELISA against RecP60 to determine the tube number of the eluted antibody fractions and their antibody activity. 3H4 antibody was also purified with the same method as a control to compare their affinity alteration after purification.

At the end of the study, anti-RecP60 activity and protein concentration analysis showed us that 3H4 and 1G6 were purified succesfully. As seen on graphs, after elution buffer (EB) was loaded onto the column, 1G6 was obtained in three tubes,

15^{th}-18^{th} (0,5ml/tube) and 3H4 in 12^{th}-14^{th} tubes (1ml/tube) (Figure 3.9). The concentrations of purified antibodies were calculated by using absorbances of fractions at 280 nm and the equation of "1mg/ml solution of IgG antibody absorbances 1,43 at 280nm" in the formulas given below:

Protein absorbance / 1,43 (constant number) = Protein amount/ml **(3.1)**

Protein amount/ml x Volume of the fraction (ml) = Total Protein amount **(3.2)**

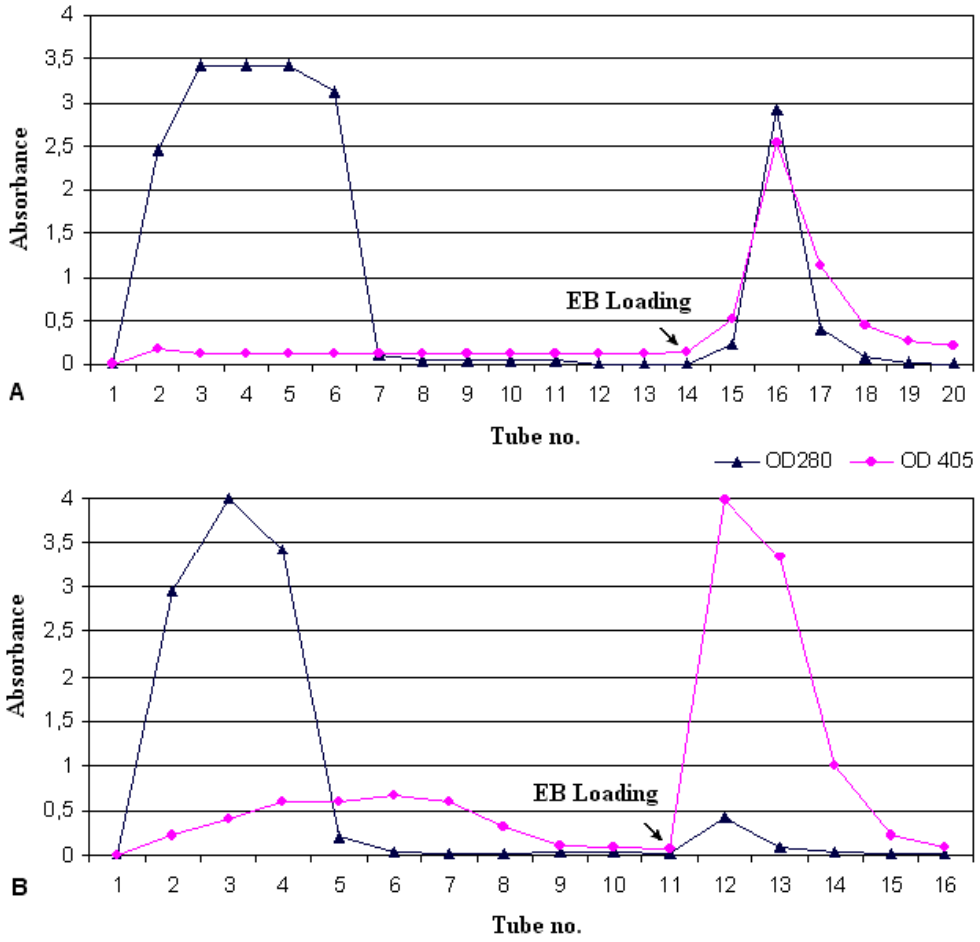

Figure 3.9 : 1G6 (A) and 3H4 (B) purification fractions by Protein-G Immuno-Affinity Column Chromatography.
OD 405 represents anti-RecP60 activity of column fractions assayed by ELISA and OD 280 represents protein absorbances. EB: Elution buffer.

The protein concentrations of purified 1G6 fractions were calculated as follows: 15th fraction, 0.08 mg; 16th fraction, 1.02 mg; 17th fraction, 0.14 mg; 18th fraction, 0.025 mg. The protein concentrations of purified 3H4 fractions was calculated as following: 12th fraction, 0.3 mg; 13th fraction, 0.06 mg; 14th fraction, 0.02 mg. According to calculations, pure 1G6 was obtained in 2 ml of total collected fractions at 0,63 mg/ml and 3H4 was obtained in 3 ml at 0,126 mg/ml.

As seen on the graph of 3H4 purification, there was some anti-RecP60 activity before EB loading fractions. This could become if the antibody was overloaded into the column. It was seemed that after elution, the activity and concentration of the 3H4 antibody increased, this shows us that elution was done on that step and 3H4 was obtained purely because in washing fractions before elution there were no trace amounts of proteins observed at 280nm.

For finding the optimum dilution of the newly purified antibody and incubation time of the substrate step of ELISA, it was necessary to examine different dilution ranges and substrate incubation times. To examine the activity of 1G6 and 3H4, cross-reactivity ELISA test was performed widely with proteins such as milk powder, tubulin, BSA and another recombinant peptide, nRecP60, designed from P60-Katanin. Tubulin was used to detect whether an activity of a monoclonal antibody was observed against this protein which P60-katanin interacts with. Since BSA and skim milk were used in ELISA assays as blocking agents, they were used in this assay to find out the better blocking agent for newly produced antibody. nRecP60, another antigen, was used to determine whether the antibody recognition part (epitop) of the antigen was common with its original antigen, RecP60. 1/100 and 1/30 dilution of purified 1G6 and 3H4 were also applied in the assay to analyse the affect of dilution. The absorbance of 405 nm was measured 30 minutes and 1 hour after substrate addition (Figure 3.10).

After obtaining the wide cross-reactivity test results firstly, 1G6 affinity to milk powder and RecP60 was controlled. After purification 1G6 activity to RecP60 was higher than the activity against skimmed milk powder (Figure 3.10 C) which was the same as the activity of unpurified 1G6 (Figure 3.6). No alteration of the recognition of RecP60 and skim milk was also observed for 3H4 after purification, which showed that the purification method was successful for 1G6 and 3H4.

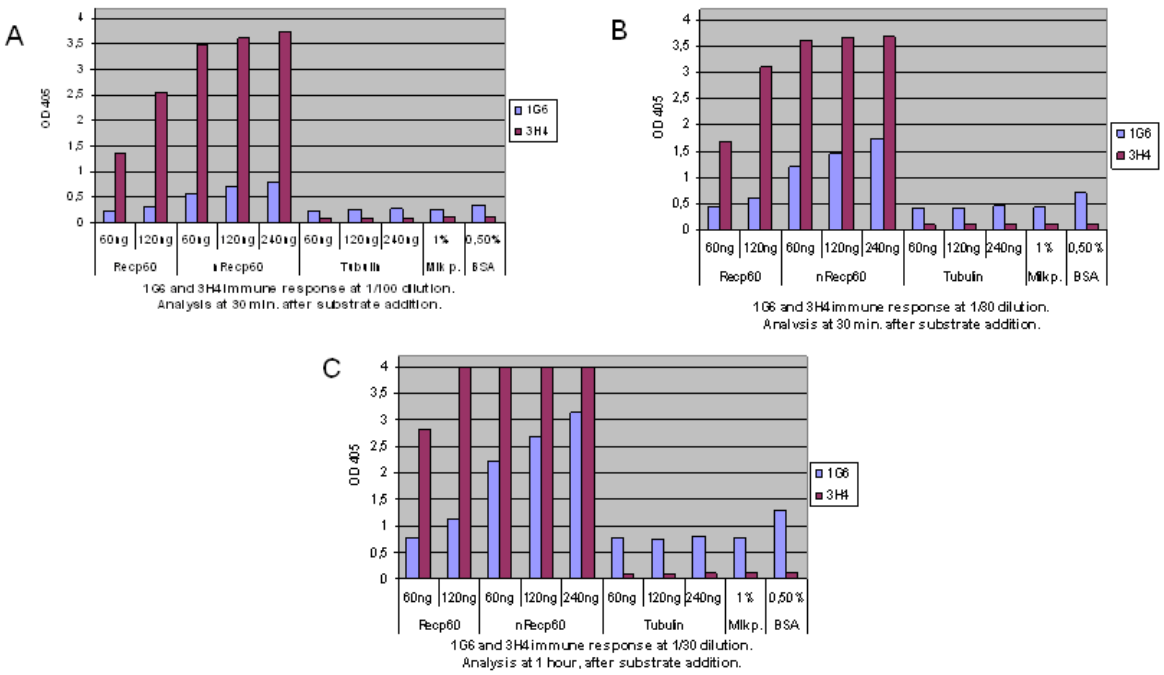

Figure 3.10 : Cross reactivitiy ELISA of 1G6 and 3H4 with several proteins.
Milk p.: skim milk powder

Then, the affinity of 1G6 to other proteins was controlled (Figure 3.10). Purified 1G6 reacted with RecP60 (60, 120ng) and nRecP60 (60,120, 240 ng) higher than skim milk and tubulin (60, 120, 240 ng). 1G6 activity against BSA was equal to 120 ng RecP60 activity, higher than 60 ng RecP60 activity, but lower than all concentrations of nRecP60 activity. When RecP60 and nRecP60 concentrations were increased (60, 120, 240), the activity of 1G6 also increased. When tubulin concentration was increased no activity of 1G6 increased. These results showed that 1G6 has high affinity to the two kinds of RecP60, designed from native P60-katanin and also interacts lower with skim milk and BSA but no significant activity was found with tubulin. According to these results it is suggested that 1G6 recognized the epitops of RecP60 and nRecP60 which were so similar to the specific epitop of native P60-katanin, the epitopic structure of BSA and skim milk was less similar to the epitop of P60 and there is no similarity with the epitop of tubulin. Purified 3H4 which was used as control only reacts with RecP60 and nRecP60 which showed us that 1G6 reacts with the epitops different from 3H4.

To analyse the affect of dilution factor to the antibodies affinity to different proteins, 1/100 and 1/30 dilution of purified 1G6 and 3H4 were applied in the assay. The absorbance of 405 nm was measured after 30 minutes and 1 hour after substrate

addition and compared to monitor the most active time of antibodies. To find the optimum dilution of the newly purified antibody and incubation time of the substrate step of ELISA for the diluted antibody, it was necessary to examine the combination of different dilution ranges and substrate incubation times. It was expected that when dilution of antibodies increased, the activity of antibodies would decrease. This affect was observed at 30^{th} minute of substrate addition and application of 1/100 and 1/30 dilution of 1G6 and 3H4 (Figure 3.10 A and B). In this examination, the increase of 1G6 activity is higher than 3H4 because the concentration affect to the activity was also important for the antigen recognition and it is changeable for different antibodies. For 1G6 1/30 dilution was optimum, for 3H4 1/100 dilution was optimum. In ELISA assays, the measurement for the binding activity of the antibodies increased when the incubation time after substrate addition increased. From our previous results, it was determined that 1 hour was optimum for our ELISA tests to catch the minimum activity. It was also observed in this examination that, at 1/30 dilution of each antibodies and on the 1^{st} hour of substrate addition their activities were higher then the 30^{th} min. This high measurement allowed us to compare the cognition patterns of different proteins with 1G6 because the difference between their measurements were more than the measurements at 30^{th} min. Therefore, 1 hour incubation time for substrate step and 1/30 dilution were suitable for purified 1G6 (Figure 10 C). The activity of 3H4 was not quantified when it reaches over 4 at this conditon (Figure 10 C) but its increasing activity was seen on increased dilution and dicreased substrate incubation conditions observed at Figure 10 A and B.

3.3.3 Immunoflourescence staining of P60-katanin in mitotic cells with 1G6

Obtained IgG type anti-RecP60 antibodies were examined whether they recognize P60-katanin in cells by immunocytochemistry. The method was performed on cell lines from different species. Human origin HeLa and SHSY-5Y neuroblastoma and mouse origin NIH/3T3 fibroblast cells were fixed with different types of fixation methods. Paraphormaldehyde, gluteraldehyde and methanol fixation methods were tried to find out the optimum method for our antibody stainings. MAbs were examined with their supernatant and also with their purified forms.

Flourescence labelled secondary antibodies, Alexa 488 anti-mouse IgG antibody; Alexa 546 anti-mouse IgG antibody; Cy5 anti-mouse IgG antibody, were used to

visualize our mouse originated monoclonal antibodies' affinity. In dividing cells, only one monoclonal antibody, 1G6, reacted with P60-katanin specifically. In Figure 3.11-13, 1G6 showed the expected P60 interaction with the microtubular structure, detected by previous studies (Karabay et. al., 2004; McNally and Thomas, 1998).

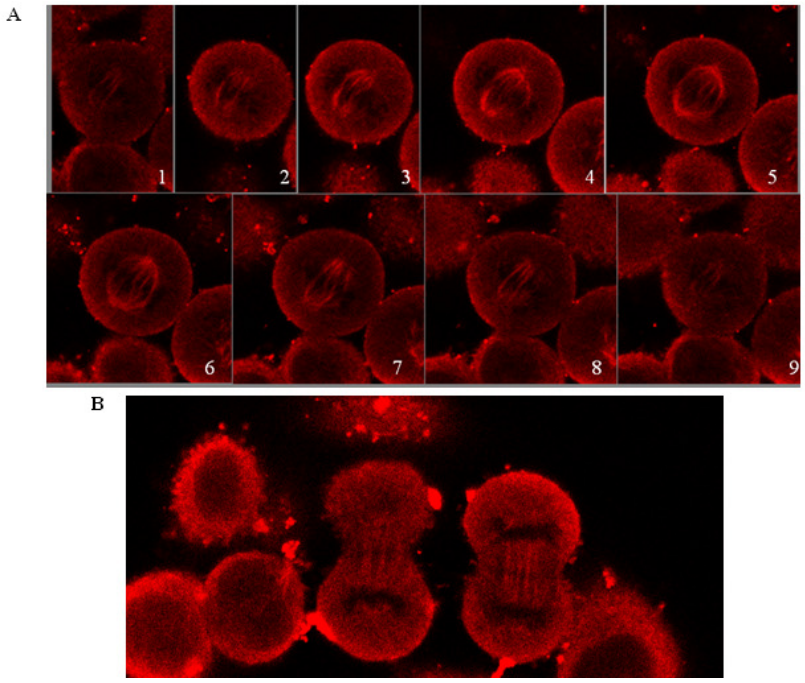

Figure 3.11 : Immunocytochemistry analysis of P60-katanin by 1G6.
Dividing HeLa cell was photographed at different z-positions and arranged as series (1-9). 1G6 staining was localized on the mitotic apparatus in every section (A). 1G6 stained the remains of spindle microtubules of HeLa cells at telophase (B). The fixation was done with paraphormaldehyde, supernatant of 1G6 and Alexa
sed as secondary antibody.

According to the results of single staining, 1G6 appeared to stain P60-katanin of HeLa, neuroblastoma and fibroblast cells showing the specific localization on the mitotic spindle, also on astral microtubules (Figure 3.11A), and on the remains of spindle microtubules at telophase (Figure 3.11B). In three kinds of fixation methods, there was no significant difference with 1G6 staining. Also three types of secondary antibody did not significantly change the visualization of 1G6 interaction (Figure 3.11, 3.12, 3.13).

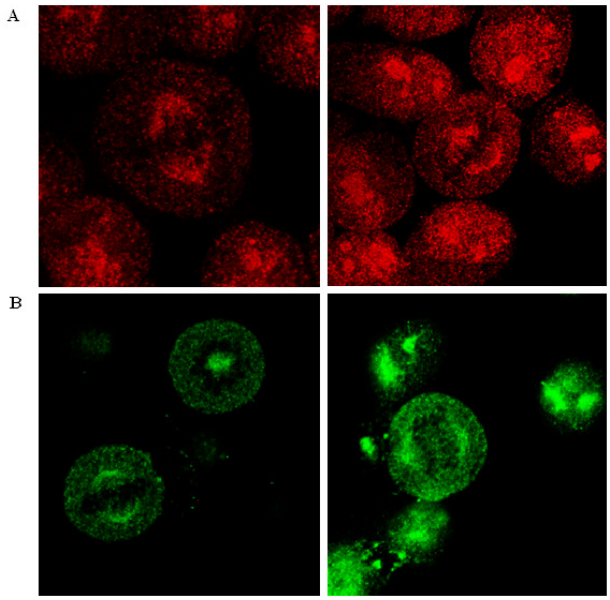

Figure 3.12 : 1G6 localization on dividing HeLa cells.
The fixation was done with glutaraldehyde and Alexa 546 anti-mouse IgG antibody (A) and Alexa 488 anti-mouse IgG antibody (B) were used as secondary antibodie

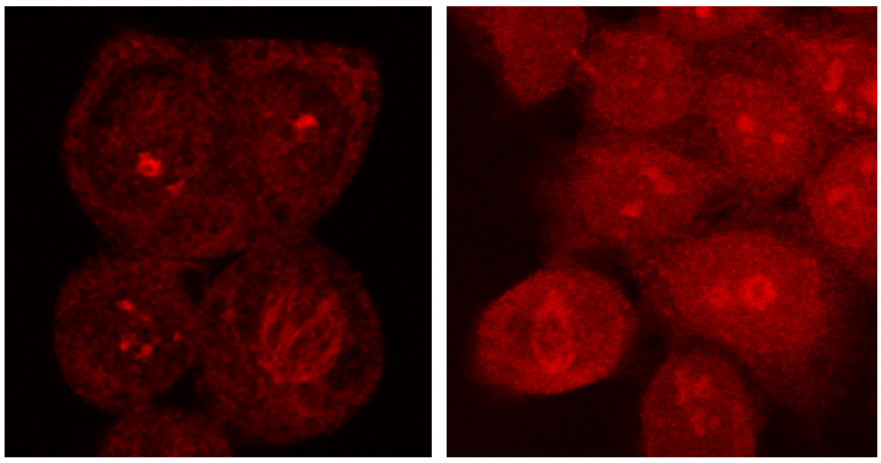

Figure 3.13 : Immunocytochemistry analysis of P60-katanin in different cell lines.
1G6 localized on the mitotic spindle of SHSY-5Y neuroblastoma (A) and NIH/3T3 fibroblast (B). In these studies, gluteraldehyde (A) and methanol fixation (B) and Cy5 anti-mouse IgG antibody was used.

Dual 1G6 and tubulin staining were also performed to show the known interactions of P60-katanin localization on microtubule. Methanol fixed HeLa cells were stained with 1G6 and rabbit anti-β tubulin antibodies. Alexa-488 anti-mouse IgG was used to

visualize 1G6 and Alexa-647 anti-rabbit IgG antibody was used to visualize β tubulin interaction.

As a result, 1G6 staining merged with β tubulin staining image was such that 1G6 was localized on the mitotic spindle and concentrated specifically on the microtubules towards the spindle poles; different from β tubulin staining which is distributed on the entire microtubules of mitotic spindle in dividing cells (Figure 3.14).

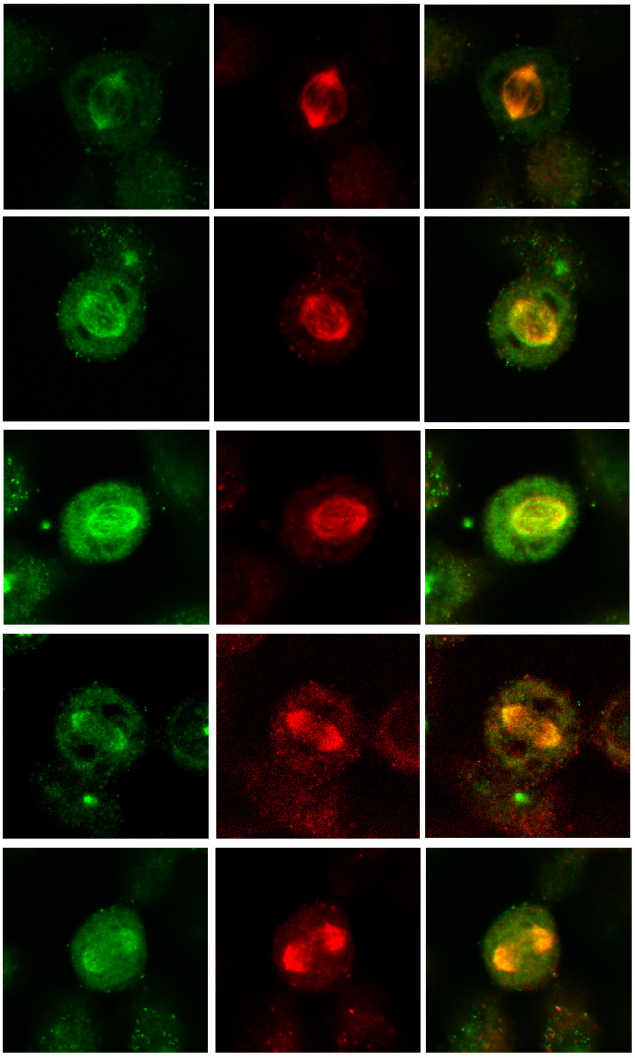

Figure 3.14 : Comparison of tubulin and P60-katanin localization on the mitotic spindle.

HeLa cells were stained with 1G6 (green, left panel) and anti-β tubulin antibodies (red, middle panel). The right panel shows the overlay of both signals.

According to the results, 1G6 showed the expected P60-katanin interaction with microtubules and recognized P60-katanin of two species, human and mouse. These results are also consistent with the information that P60-katanin was available on microtubules to control the length of microtubules which is necessary for the phases of cell division.

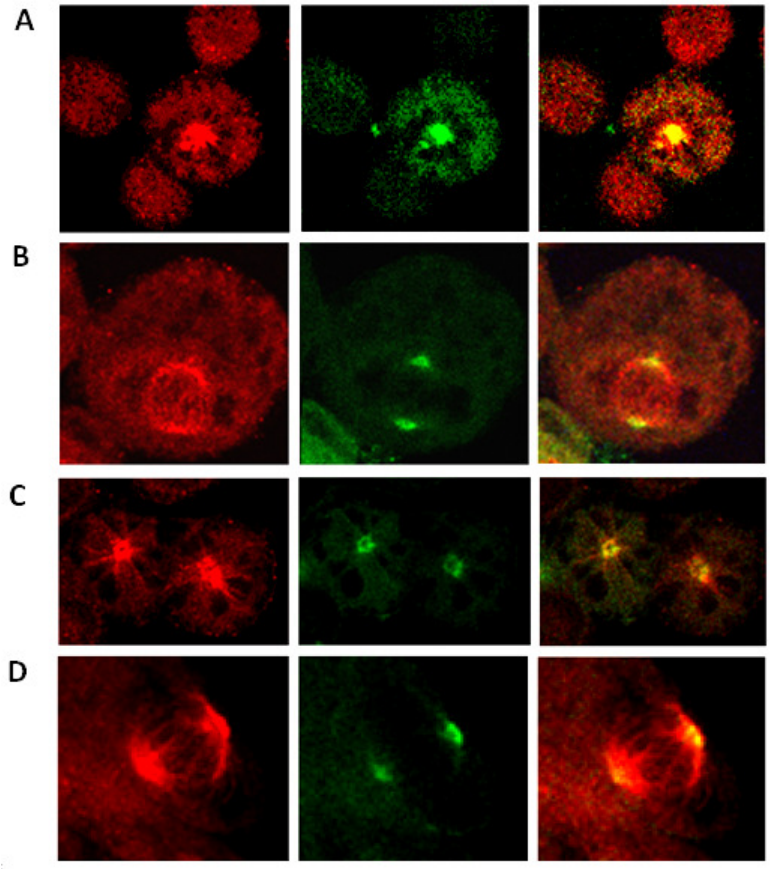

Figure 3.15 : Analysis of human P60-katanin expressed in dividing HeLa cells.
P60-GFP expression (green, middle panel) became dense at the poles of mitotic apparatus and merged with 1G6 staining (red, left panel). The right panel shows the overlay of both signals.

To observe the recognition pattern of overexpressed P60-katanin with 1G6 on extrinsic katanin expression, HeLa cells were transfected with human originated full P60-katanin which was previously cloned into pEGFP-C1 vector. After transfection, cells were plated and fixed-permeabilized with methanol at 4^{th}, 8^{th}, 12^{th}, 24^{th}, 48^{th} and 72^{th} hours of incubation to determine different expression levels of P60. After staining with 1G6 and chicken anti-GFP antibody, Cy5 conjugated anti mouse-

antibody and FITC conjugated anti-chicken IgY antibody was used for secondary antibody to visualize staining. The forced overexpressed P60-katanin seemed to specifically concentrate on the spindle poles (Figure 3.15) preferentially at first, which is expected with P60-katanin's known function.

4. DISCUSSION

Microtubule severing is an important mechanism for cellular activities. In mitosis and meiosis, microtubule severing on the spindles is thought to contribute to spindle reorganization (Ahmad and Baas, 1995). In neurons, all neuronal microtubules are suggested to be severed to relocate to populate in growing processes such as axons and dendrites (Baas et al., 2005). P60-katanin is the microtubule severing subunit of the heterodimeric katanin protein which has also P80 subunit that enhances the severing activity (Hartman et al., 1998). Katanin is concentrated at a microtubule-dependent structure at mitotic spindle poles, suggesting katanin may sever microtubules from their centrosomal attachments in non-neuronal cells (McNally and Thomas, 1998). In neurons, the level of P60-katanin was found to be very high in axons actively growing toward their targets, but then it decreased when the axon reached its target and stopped growing (Yu et al., 2005).

In this study, we produced the first monoclonal antibody against P60-katanin, using rat sequence based RecP60 (Recombinat P60-katanin) protein as antigen. Soluble RecP60 was prepared succesfully. Hybridoma technology was performed and 10 MAbs were produced. Cross-reactivity ELISA tests, Western-blotting and immunocytochemistry were performed for selection of the specific antibody. Only, 1G6 recognized native P60-katanin showing the specific cellular localization and protein migration.

4.1 Properties of Recombinant P60-Katanin Protein

In this study, our aim was firstly to overexpress P60-katanin for anti-P60-katanin monoclonal antibody production. In order to obtain accurate folded P60-Katanin protein, an amino acid sequence named as RecP60, was selected from full rat P60-katanin protein sequence regarding the inclusion of specific and soluble antigenic determinants. To achieve the specificity, the conserved C-terminal AAA domain was excluded from the full length of P60. To solve the solubility and folding problem,

hydrophobic regions and cystein residues were excluded. In other words, the amino acid sequence of N-terminus part (amino acids 1-194) of the full length rat P60-katanin was selected to make the soluble N-terminal His_6-tag recombinant P60 protein (Figure 4.1).

```
  1 MSLLMITENV KLAREYALLG NYDSAMVYYQ GVLDQINKYL YSVKDTHLHQ KWQQVWQEIN
 61 VEAKHVKEIM KTLESFKLDS TSLKAAQHEL PSSEGEVWSL PVPVERRPLP GPRKRQSTQH
121 SDPKPHSNRP GAVVRAHRPS AQSLHSDRGK AVRSREKKEQ SKGREEKNKL PAAVTEPEAN
181 KFDSTGYDKD LVEALERDII SQNPNVRWYD IADLVEAKKL LQEAVVLPMW MPEFFKGIRR
241 PWKGVLMVGP PGTGKTLLAK AVATECKTTF FNVSSSTLTS KYRGESEKLV RLLFEMARFY
301 SPATIFIDEI DSICSRRGTS EEHEASRRVK AELLVQMDGV GGASENDDPS KMVMVLAATN
361 FPWDIDEALR RRLEKRIYIP LPSAKGREEL LRISLRELEL ADDVNLASIA ENMEGYSGAD
421 ITNVCRDASL MAMRRRIEGL TPEEIRNLSR EEMHMPTTME DFEMALKKVS KSVSAADIER
481 YEKWIVEFGS C
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Figure 4.1: The amino acid sequence of full length rat P60-katanin (Gene Bank, the European Molecular Biology Laboratory, accession No. AY621629). Expressed RecP60 is selected from N-terminus part (underlined). Conserved C-terminal AAA domain (bold); Non-polar (Hydrophobic) aminoacids (blue). Extensive hydrophobic parts are marked in gray. Cystein residues are marked in yellow. Point mutation that changed glutamine (Q) to arginin (R) is marked in green.

According to SDS-PAGE analysis, overexpressed RecP60 was detected as 35 kDa. Molecular weight of RecP60 must have been about 22 kDa (22167.08 Da) which was calculated from its amino acid sequence by using Expasy PI tool (http://expasy.org/tools/pi_tool.html). When RecP60 was expressed in the pET30a construct His-tag, thrombin, S-tag, enterokinase and 5 proline residues and some additional amino acid sequences were fused hence its weight becomes 35 kDa.

Mutation resulting in amino acid change can affect activity or folding of the expressed protein. After cloning of selected P60-katanin cDNA sequence into pET-30a expression vector, no shift mutation was observed according to nucleotide sequencing results, but a point mutation (purine-purine) was found. The mutation resulted in CGA instead of CAA that changed the encoding for glutamine to arginin. Antigen fits to its antibody due to complementarity in shape over an area of contact by non-covalent bonding. Protein folding proceeds by moving the hydrophobic parts of the protein inwards, and the hydrophilic ones outwards. RecP60 structure should not be altered significantly by the mutation because glutamine and arginine are both hydrophilic amino acids, so the surface exposition of that amino acid would supposedly not change.

The best conditions for obtaining RecP60 were determined considering the factors that affect the protein solubility. The amino acid sequence of a recombinant protein determines the physical properties of the protein, including its stability, p*I*, hydrophobicity, and molecular weight, any or all of which might directly affect the expression and solubility levels and accuracy of the folding process (Tsunoda et al., 2005). Cystein residues that are responsible for disulfide bond formation in protein folding process were also excluded because any possibility for wrong disulfide bond formation might result in wrong folding (Baneyx, 1999). After SDS-PAGE of total cell protein analysis, it was observed that the overexpressed RecP60 was in soluble fraction.

Overexpressed soluble RecP60 was purified from the bacterial culture to further use in mice immunization. Natural or denaturating conditions is possible for protein purification by Ni-NTA resin. Since expressed RecP60 was obtained soluble, purification under natural conditions was choosen. Moreover, such purification condition does not distort protein conformation which is important for immunization efficiency.

4.2 Monoclonal Antibody Production

In this part RecP60 was used as antigen and produced MAbs against RecP60 by hybridoma technology. MAb 1G6 was the only antibody that recognized P60-katanin on mitotic apparatus in dividing cells. MAb 1G6 was found to be specific for P60-katanin and able to show the cellular P60-katanin and can be used to investigate its functions.

Our hybridoma technology study started by the generation of RecP60 specific B cells via immunization of female BALB/C mice. Immunized female mice generated strong IgG response after second immunization which means that immunization procedure was done successfully. At some methods, addition of carrier proteins or methods for conjugation of antigen was recommended for achieving immunization. 35 kDa of our antigen was enough when dissolved in PBS and mixed with Freund's adjuvant as an injection material for immunization.

In the next step, the fusion of immunized spleen B cells, which with myeloma cells, and selection of the specific hybridoma clone by ELISA were performed. After the

fusion step, increasing the number of colonies which were observed in fusion culture plates would be proportional to increase the chance for obtaining the specific antibodies. In other words, the obtained hibridoma cell number was important. The problems obtaining low hybridoma cell number was solved by changing several factors of the method. Since the cells were starting to die after sacrification of mice, the fusion step must be performed rapidly to maintain cell viability. The chemical fusion process would also make cells sensitive. Therefore, starting from the sacrification to fusion process, every step were performed rapidly and gently to maintain cell surviving by gaining experience. Cell culturing conditions were also changed. Culture media was prepared from powdered DMEM and CO_2 concentration of the incubator was calibrated to 5 %. After all optimization procedures, the yield from the fusions were increased and it reached to the top in the last fusion which resulted in qualified antibody 1G6.

Hybridoma cells appeared after 10 days from the fusion and their media was replaced with the fresh one in around every ten days. Each antibody activity of replaced media was tested by ELISA. Because the secreted antibody was in a low amount which were produced from little colony of cells at the beginning, ELISA tests were done for two times for each cell to catch antibody producing cells. In addition, to determine antibody producing cells in their early growing period, the most antigen coating amount which was 120 ng was used in these screening method and cross-reactivity ELISA tests. This technique acquires us most of the antibody producing cells correlated with the number of proliferated hibridomas. From the 4^{th} fusion only one, from the 7^{th} fusion 12 anti-RecP60 antibody producing hybridoma cells were obtained. Each antibody producing cells was screened by ELISA to test if their antibody producing property continued. 10 hybrid cells maintained their antibody producing activity and 3 of them lost their activity, the reason could be releated with the chromosome loss in the hybrid line.

Screening monoclonal antibodies only against RecP60 is not enough to decide if they are specific or not. In the immune response of the organism, all the antibodies secreted into serum of the RecP60 immunized mouse showed polyclonal anti-RecP60 activity. However, when their sources were isolated and cultured individually, they had different antigen affinity properties. Antibody-antigen affinity belongs to epitop interaction and there can be common epitops in different proteins.

To investigate a certain protein, its specific recognition by the antibody is needed which requires the recognition of its epitop, that is not present in other proteins, only belonging to the certain protein. Also in screening ELISA tests, milk powder was used as blocking agent. The antibody activity could be against milk powder rather than RecP60. Therefore, cross-reactivity ELISA tests were performed for understanding recognition properties of obtained antibodies. 3 of the antibodies highly cross reacted with RecP60, cellular lysate of BL21 and skim milk powder. 6 of the monoclonal antibodies including 1G6 reacted mostly with RecP60 than BL21 lysate and skim milk. 1 antibody differently recognized BL21 lysate higher than RecP60. The 6 antibodies reacted mostly with RecP60 were potential specific anti-RecP60 antibodies.

Since the aim was producing monoclonal antibody that recognize native P60-katanin to investigate its function in further methods, their recognition properties in Western-blotting and immunocytochemistry were also need to be determined because the epitop that was presented at the surface of the protein also may change due to the fact of conditions of the method the antibody would be tried. Therefore, all the 10 antibodies were tested for these methods because there can be similar epitops recognized on these three proteins exposed in the conditions of ELISA, but specific when exposed on the native P60-katanin in the conditions of immunocytochemistry and Western-blotting methods. These further characterization methods would be discussed in Section 4.3.

Resultant hybridomas must be single-cell cloned and then expanded as individual clones, which secrete only one antibody to be monoclonal antibodies. Therefore, cloning them by “limiting dilution” and the up-scaling of MAb production was done.

Isotypes of the obtained antibodies were also determined and 5 of them including 1G6 were IgG type. The majority of monoclonal primary antibodies for use in immunocytochemistry (ICC) and Western-blotting are of the IgG Isotype because recognition of the antigen by IgG monoclonal antibodies tend to be more specific than other types of antibodies. Therefore, obtaining monoclonal antibodies of IgG isotype is an advantage and this can be achieved by generating secondary immunization against the antigen.

4.3 Characterization of Anti-RecP60 Monoclonal Antibodies

In this part recognition properties of all 10 MAbs in Western-blotting and immunocytochemistry were determined, although there were only 6 potential antibodies including 1G6 that would recognize native P60 specifically according to cross-reactivity test. Due to the conditions of cross-reactivity ELISA, common epitops of the proteins could be recognized by one antibody but the same epitop could be specific when exposed on the native P60-katanin in the conditions of immunocytochemistry and Western-blotting methods. Therefore, to understand the monoclonal antibody specificity to P60, it is neeeded to be tried in Western-blotting and immunocytochemistry methods which P60-katanin staining and migration patterns were known.

Western-blotting studies were performed to test whether obtained MAbs would recognize 55-65 kDa native P60-katanin in tissues. Western-blotting of P60-katanin with 1G6 on cell lines and tissues yielded protein bands at 65kDa and 55kDa indicating that there may be alternative spliced forms of P60-katanin depending on tissue types. A 65 kDa band was obtained on liver, heart, lung of adult and embryonic rat tissues and HeLa, SHSY-5Y and NIH/3T3 cell lines; which were all mitotic. A 55kDa band was shown to be present in non-mitotic cerebellum and brain tissues of adult and embryonic rat tissues and also in the brain stem of adult rat tissue. From these results, it is possible to think that 1G6 MAb interacts with the common epitop of alternative spliced forms of P60-katanin of both tissues.

In the wide-cross-reactivity ELISA, purified 1G6 recognition was examined with proteins such as milk powder, tubulin, BSA and another recombinant P60 protein nRecP60 (Figure 4.2). 1G6 activity to RecP60 and nRecP60 was higher than skim milk and BSA, but no significant activity of 1G6 was found with tubulin. According to results, it was suggested that 1G6 reacts with the similar epitop of native P60-katanin and RecP60, nRecP60 and epitop structure of BSA and skim milk was less similar with P60-katanin in ELISA. Antibodies show activity by interacting with the epitope region of the antigen. In different methods, proteins may fold differently and may present different epitopic structures. The recognition of the antigen by antibody specifically depends on the presentation of the specific epitop of the antigen in the applied method, so each antibody may not be suitable for each method. In ELISA,

the monoclonal antibodies that show affinity to RecP60, may also show some activity against BSA and milk powder. In addition, the same antibodies may be specific only to P60-katanin in immunocytochemistry and Western-blotting methods. According to this information and the results of wide-cross-reactivity ELISA, it was suggested that presented epitops of RecP60 and nRecP60 in ELISA which were recognized by 1G6 were so similar to P60-katanin's specific epitope and 1G6 showed less activity to BSA and skim milk suggested that the epitopic structure of the latter proteins has a less similarity with the specific epitop of P60.

The wide-cross ELISA results were also consistent with the cross ELISA results obtained with supernatants of the antibodies. This showed that after purification there were no alteration about the antibodies' recognition.

```
  1 MSLLMITENV KLAREYALLG NYDSAMVYYQ GVLDQINKYL YSVKDTHLHQ KWQQVWQEIN
 61 VEAKHVKEIM KTLESFKLDS TSLKAAQHEL PSSEGEVWSL PVPVERRPLP GPRKRQSTQH
121 SDPKPHSNRP GAVVRAHRPS AQSLHSDRGK AVRSREKKEQ SKGREEKNKL PAAVTEPEAN
181 KFDSTGYDKD LVEALERDII SQNPNVRWYD IADLVEAKKL LQEAVVLPMW MPEFFKGIRR
241 PWKGVLMVGP PGTGKTLLAK AVATECKTTF FNVSSSTLTS KYRGESEKLV RLLFEMARFY
301 SPATIFIDEI DSICSRRGTS EEHEASRRVK AELLVQMDGV GGASENDDPS KMVMVLAATN
361 FPWDIDEALR RRLEKRIYIP LPSAKGREEL LRISLRELEL ADDVNLASIA ENMEGYSGAD
421 ITNVCRDASL MAMRRRIEGL TPEEIRNLSR EEMHMPTTME DFEMALKKVS KSVSAADIER
481 YEKWIVEFG
```

Figure 4.2: The aminoacid sequence of nRecP60.
The amino acid sequence of RecP60 (red and blue) and nR was marked in full sequence of rat P60-katanin.

Obtained IgG type anti-RecP60 antibodies were examined whether they would recognize P60-katanin in cell lines by immunocytochemistry. The results of immunocytochemistry studies with 1G6 also showed the evidence that P60-katanin was available on microtubules for its regulation to control the length of microtubules which is necessary for the phases of cell division (McNally K, 2006).

The immunocytochemistry studies also indicate that 1G6 MAb is specific to P60-katanin of two species, mouse and human, because it recognized native P60-katanin of human origin HeLa, SHSY-5Y Neuroblastoma cells and mouse origin NIH/3T3 Fibroblast cells. 1G6 was obtained by immunizing mouse with RecP60 which was expressed based on the sequence of rat P60-katanin. Aminoacid sequences of human P60-katanin and mouse P60-katanin were blasted with rat P60-katanin and gave a high homology of 93 % with human and 95 % with mouse. This indicated that it is

possible for 1G6 to recognize P60-katanin of three types of organisms, and this observation was confirmed with our immunocytochemistry and Western-blotting studies.

5. CONCLUSION

P60-katanin is a microtubule severing protein. Katanin promotes spindle-shortening and severing processes in mitotic and meiotic spindles. In neurons, the level of P60-katanin was found to be very high in actively growing axons and at the tips of growing neuronal processes and in dendritogenesis. Katanin is one of the key protein for organizing microtubular structure of a variety of cell types.

In this study, we produced 1G6, the first monoclonal antibody against P60-katanin. RecP60, which was produced based on a specific region of rat P60-katanin, was expressed in *Escherichia coli* and used as antigen. 10 MAbs were produced by hybridoma technology. Only, 1G6 recognized native P60-katanin in Western-blotting and immunocytochemistry in which monoclonal antibody would increase the range of applications. 1G6 is the first monoclonal antibody, showing the specific cellular localization of P60-katanin and can be used to investigate P60-katanin's functions. This antibody would also extend the range of applications, especially would enable multiple stainings. Since P60-katanin polyclonal antibodies have been used in studies until now, obtaining the hybridoma cell, the unlimited source of a monoclonal antibody against P60, results in a great advantage to investigate P60-katanin's functions.

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APPENDIX

APPENDIX A. Approval of Animal Care and Use Committee.

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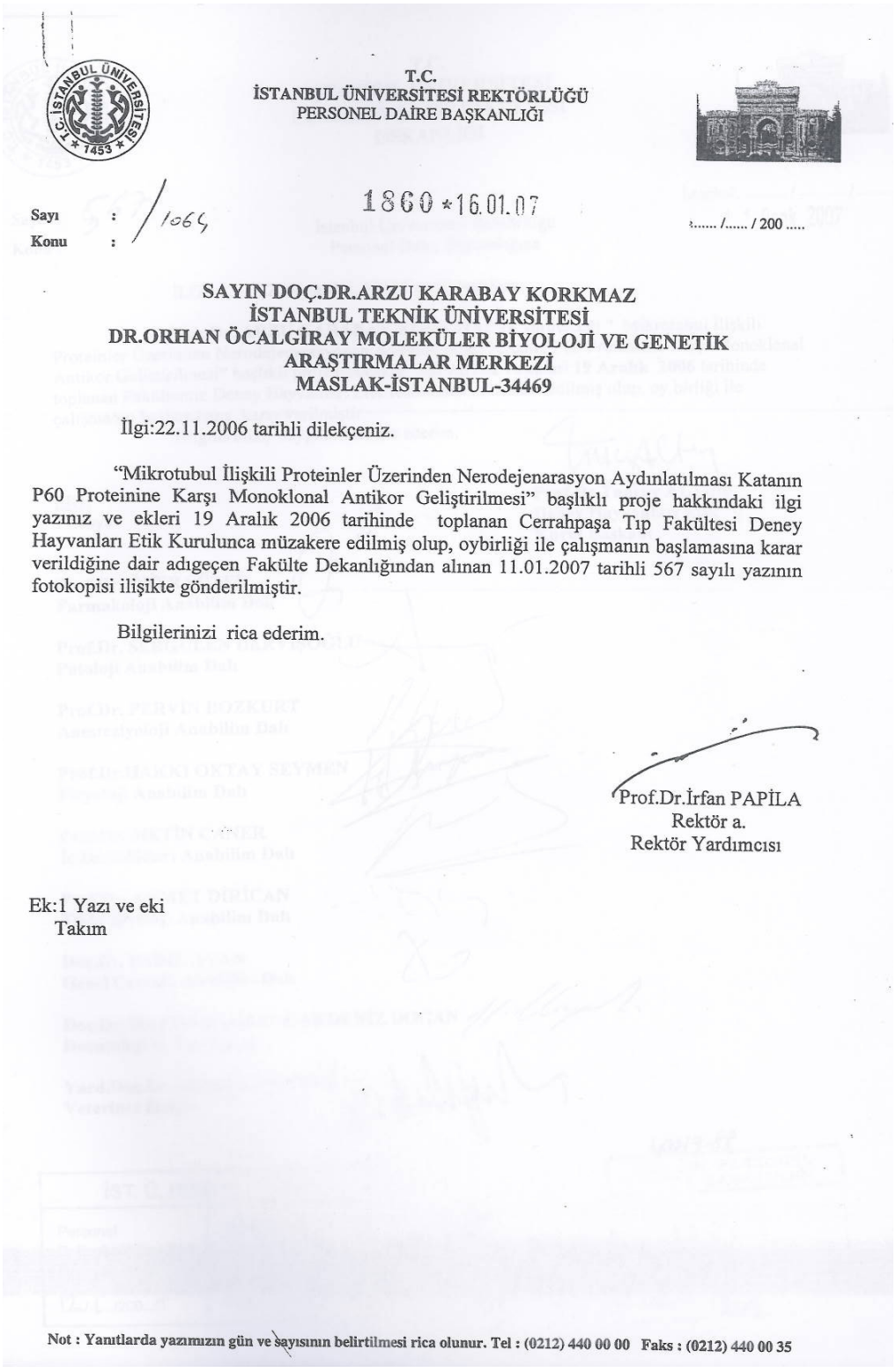

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İlgi:22.11.2006 tarihli dilekçeniz.

"Mikrotubul İlişkili Proteinler Üzerinden Nerodejenarasyon Aydınlatılması Katanın P60 Proteinine Karşı Monoklonal Antikor Geliştirilmesi" başlıklı proje hakkındaki ilgi yazınız ve ekleri 19 Aralık 2006 tarihinde toplanan Cerrahpaşa Tıp Fakültesi Deney Hayvanları Etik Kurulunca müzakere edilmiş olup, oybirliği ile çalışmanın başlamasına karar verildiğine dair adıgeçen Fakülte Dekanlığından alınan 11.01.2007 tarihli 567 sayılı yazının fotokopisi ilişikte gönderilmiştir.

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Doç.Dr. ARZU KARABAY KORKMAZ'ın yürüteceği " Mikrotubul İlişkili Proteinler Üzerinden Nerodejenarasyonun Aydınlatılması Katanın P60 Proteinine Karşı Monoklonal Antikor Geliştirilmesi" başlıklı proje hakkında ilgi yazınız ve ekleri **19 Aralık 2006** tarihinde toplanan Fakültemiz Deney Hayvanları Etik Kurulunca müzakere edilmiş olup, oy birliği ile çalışmanın başlamasına karar verilmiştir.

Bilgilerinizi, saygılarımla arz ederim.

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