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Heavy chain and light chain interactions.**

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For Sean and I

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ABBREVIATIONS

α -MEM	α -modified Eagle's minimal essential media
aa	amino acid
BCA	bicinchoninic acid
BSA	bovine serum albumin
CCD	charged cooled device
cDNA	complementary deoxyribonucleic acid
CMV	chicken mosaic virus
cs	coverslip
CY2	carboxymethylindocarbocyanine
CY3	indocarbocyanine
ddH ₂ O	double distilled, deionized water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC	embryonal carcinoma
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid disodium salt
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol-bis (β -aminoethyl ether) tetraacetic acid
FCS	fetal calf serum
GTP	guanosine triphosphate
HMW	high molecular weight

HRP	horseradish peroxidase
kb	kilobase
kDa	kilodalton
LB	Luria Bertani
LC	light chain
LMW	low molecular weight
mAB	monoclonal antibody
MAB	microtubule assembly buffer
MAP	microtubule-associated proteins
MES	N-morpholinoethanesulfonic acid
mRNA	messenger ribonucleic acid
Mt	microtubule
MTOC	microtubule organizing center
MW	molecular weight
NC	nitrocellulose
ON	overnight
PBS	phosphate buffered saline
PC	phosphocellulose
PEFA	p-aminoethylbenzenesulfonyl fluoride
PEM	PIPES/EGTA/MgCl ₂ buffer
PGK	phosphoglycerate kinase
PIPES	piperazine-N.N'-bis-2-ethanesulfonic acid

RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
TIFF	tagged image file format
UV	ultraviolet

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ABSTRACT

Microtubule (Mt)-associated proteins (MAPs) have been shown to play a role in Mt stability in axons and dendrites, in determining neuronal shape and in regulating the balance between rigidity and plasticity in neuronal processes.

MAP1A is the most abundant MAP in adult brains, and in neurons is localized in axons and dendrites. MAP1A is represented by a protein complex of one heavy chain (HC) and three light chains (LC1, LC2, LC3). Understanding MAP1A-Mt-LC interactions may give insight into the neuronal cytoskeleton, which is often involved in neuronal diseases.

To determine which area of the MAP1A HC is essential in Mt binding, parts of the MAP1A HC were expressed in HeLa cells. Through fluorescence microscopy, we determined that MAP1A colocalized with Mts, but did not alter the Mt network. Also, an *in vitro* binding assay, where MAP1A HC fragments were added to taxol stabilized Mts, determined that the N-terminus of MAP1A is involved in Mt binding. To determine the effect of the LCs on Mts, they were also expressed in HeLa cells. By the same *in vivo* and *in vitro* experiments, all three LCs were shown to bind Mts. LC1 and LC2 also conferred to Mts a wavy distribution, especially in the perinuclear region and increased Mt stability as shown by increased resistance to nocodazole. However, Mts in cells transfected with LC3 were not altered and showed no increased Mt stability. Whenever both MAP1A HC and LC2 were expressed in HeLa cells, the altering effect of LC2 was reduced, suggesting that the MAP1A HC might regulate LC2 activities.

RESUME

Les protéines associées aux microtubules (Mts) (MAPs) jouent un rôle à stabiliser les Mts dans les axones et les dendrites, à déterminer la morphologie neuronale et à régulariser la différence entre la rigidité et la plasticité dans les processus neuronaux. MAP1A est la MAP la plus abondante dans les cerveaux adultes et est localisée dans les axones et dendrites. MAP1A fait partie d'un complexe protéique composé de une chaîne lourde (HC) et trois chaînes légères (LC1, LC2, LC3). L'interaction MAP1A HC-Mts-LCs peut nous informer sur le cytosquelette neuronale qui est souvent impliqué avec les maladies neuronales.

Pour déterminer quel section de MAP1A HC est important dans l'interaction MAP1A HC-Mts, des sections de MAP1A HC ont été exprimées par transfection transiente dans des cellules HeLa. La microscopie fluorescente a démontrée que MAP1A HC se lie aux Mts mais ne modifie pas leurs distribution. Ainsi, des expériences *in vitro* ont démontrées que c'est la région du terminus N de MAP1A HC qui est impliquée dans la liaison aux Mts. Pour déterminer les effets des LCs sur les Mts, ils ont aussi été exprimées dans les cellules HeLa. Les mêmes techniques *in vivo* et *in vitro* ont démontrées que les trois LCs se lient aux Mts. LC1 et LC2 ont produit une distribution ondulées des Mts causant des Mts stables, résistantes à la drogue nocodazole. Cependant, les Mts dans les cellules exprimant LC3 n'ont pas été modifiées. Lorsque MAP1A HC et LC2 sont exprimées au même moment dans les cellules, l'effet de LC2 sur les Mts est minimisé, ce qui suggère que MAP1A HC régularise les activités de LC2.

INTRODUCTION

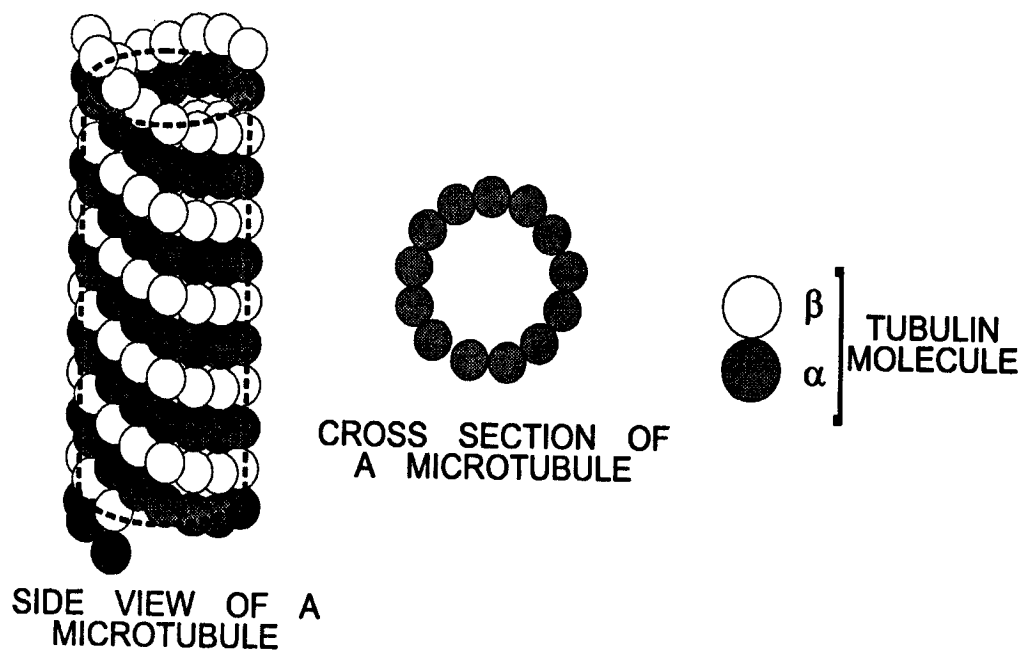
Microtubules

The cytoskeleton is a three-dimensional network formed inside the cell by microfilaments, intermediate filaments and microtubules (Mts). Mts play an important role in maintaining and generating cell shape, organelle organization, and chromosome segregation during mitosis and meiosis. Mts are also essential components of extremely stable structures such as ciliary and flagellar axonemes as well as axons and dendrites in neurons (reviewed by Chapin and Bulinski, 1992, Laferriere et al., 1997, Wade and Hyman, 1997 and Downing and Nogales, 1998).

Mts are composed of 50 kDa α and β -tubulin subunits. $\alpha\beta$ tubulin heterodimers (100 kDa) associate in a head to tail fashion to form protofilaments. When thirteen protofilaments align together laterally, they form the 24 nm diameter Mt as shown in **Figure 1**. Mts are nucleated within the Mt-organizing centre (MTOC) located near the nucleus. The Mt minus ends, or slow growing ends, are proximal and usually attached to the MTOC, while the Mt plus ends, or fast growing ends, are distal to the MTOC and grow into the cytoplasm. This Mt polarity ensures that the fast plus ends emanate out from the centre of the cell towards its periphery (reviewed by Oakley, 1994, Wade and Hyman, 1997, Laferriere et al., 1997, Downing and Nogales, 1998 and Joshi, 1998).

A particular characteristic of Mts is their ability to have different stability states. These different stability states are defined by the dynamic instability

Figure 1: Diagram showing the tubulin heterodimer and its assembly into Mts.
(Reproduced from Vaillant, 1997).



model proposed by Mitchison and Kirschner (1984). This model proposed that Mts are labile polymers existing in either an elongation phase by the addition of tubulin molecules or in a shortening phase by the loss of tubulin molecules from the Mt ends. The transition from the elongation phase to a shortening phase (catastrophe) and the transition from a shortening phase to an elongation phase (rescue) allows the Mts to participate in their many cellular processes (reviewed by Margolis and Job, 1994).

Catastrophe and rescue transitions are modulated by a variety of factors. Expression of multiple α and β tubulin isotypes, encoded by distinct genes, and post-translational modifications of these tubulin subunits can influence Mt dynamics. Post-translational modifications of tubulin include phosphorylation, acetylation, tyrosination/detyrosination, polyglutamylation and polyglycylation. Mt-associated proteins (MAPs) also influence Mt dynamics. Phosphorylation of these MAPs is another factor which can affect Mt dynamics. MAPs are now considered to be the main modulator of Mt dynamics (reviewed by Chapin and Bulinski, 1992, Hirokawa, 1994 and Laferriere et al., 1997).

Microtubule-associated proteins

Tubulin purified from brain through repeated cycles of temperature-dependant Mt assembly and disassembly always co-purifies with MAPs (reviewed by Ludueña et al., 1992). There are two groups of MAPs: structural MAPs and motor MAPs. These two groups are separated based on whether they

are nucleotide sensitive, the motor MAPs, or nucleotide insensitive, the structural MAPs (reviewed by Chapin and Bulinski, 1992).

Motor MAPs associate with Mts, but do not recycle with them during Mt assembly and disassembly cycles *in vitro*. Motor MAPs generate movement along the Mts, allowing for intracellular traffic, by using the chemical energy of nucleotide hydrolysis. Motor MAPs include the dynein and kinesin families (reviewed by Ludueña et al., 1992, Maccioni and Cambiazo, 1995 and Mandelkow and Mandelkow, 1995).

Structural MAPs

Structural MAPs are fibrous molecules ranging from 50 nm to 185 nm in length that project from the Mt surface. These MAPs are particularly prominent in the nervous system and have a particular developmental and expression pattern. These MAPs have two functional regions: an acidic projection domain and a basic Mt binding domain as depicted in **Figure 2**. MAPs are thought to bind Mts on the acidic carboxy terminus of both α and β tubulin through ionic interaction (Vallee, 1982). The projection domain is involved in cross-linking Mts to each other, to other cytoskeletal elements or to organelles (reviewed by Chapin and Bulinski, 1992 and Hirokawa, 1994).

Most structural MAPs are able to stimulate assembly of Mts and stabilize Mts *in vitro*. MAPs are thought to stimulate Mt assembly by interacting with the carboxy terminus region of tubulin. The carboxy terminus of tubulin is an endogenous inhibitor of Mt assembly. Therefore, MAP binding to this region

promotes Mt assembly. It is thought that this MAP/ Mt interaction allows Mts to determine neuronal cell shape (reviewed by Matus, 1988, Chapin and Bulinski, 1992, Hirokawa, 1994 and Laferriere et al., 1997). Since the structural MAPs have been cloned and sequenced, they have been classified into two groups based on their sequence homology and function: the tau-MAP2-MAP4 family and the MAP1 family (reviewed by Chapin and Bulinski, 1992 and Ludueña et al., 1992). Comparisons between the two families are detailed in **Table 1**.

The tau-MAP2-MAP4 family

Tau, MAP2 and MAP4 proteins have been grouped into a family based on several similarities. First, they all have their Mt binding domain located at their carboxy terminus while their projection domain is located near their amino terminus. Also, their Mt binding domains consist of three similar repeats of 18 amino acids and the regions flanking these repeats. These proteins also have an unusual resistance to elevated temperatures. Finally, all three proteins show heterogeneity at the protein level that is subject to developmental regulation. Their different isoforms (**Table 2**) arise from alternative splicing at the mRNA level (reviewed by Vallee, 1990, Chapin and Bulinski, 1992, Matus, 1994 and Mandelkow and Mandelkow, 1995).

MAP2, except for MAP2d (Doll et al., 1993), and tau are almost exclusively expressed in neurons. MAP2, except for MAP2c, which has been found in axons, is located primarily in dendrites and somata; while tau is found

Figure 2: Diagram showing the association of structural MAPs with its two binding domains to a Mt. (Reproduced from Vaillant, 1997).

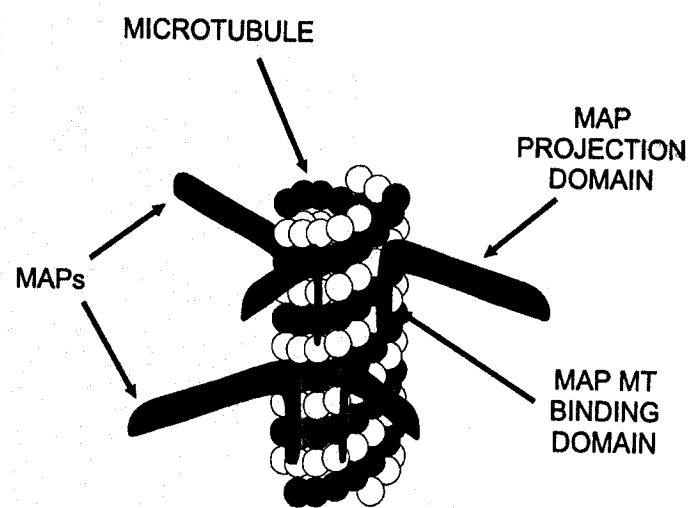


Table 1: The two principal structural MAP families.

Family	MT -Binding Domain	Reference
Tau-MAP2-MAP4	- located near C-terminus of each molecule; - consists of two regions: 1) three to four 18-amino acid repeats; 2) the regions flanking these repeats	Gustake et al., 1994; Goedert et al., 1996.
MAP1	- located near N-terminus of each molecule; - consists of two regions: 1) eleven /twenty-one repeats of (K/R) (K/R) (E/D) motif ; 2) the regions flanking these repeats	Noble et al. ,1989; Vaillant et al.,1998.

Table 2: MAP isoforms of the nervous system: localization, and expression (adapted from Riederer, 1990, Doll et al., 1993, and Schoenfeld and Obar, 1994)

Species	Isoforms*	MW (kDa)	Nervous System Localization	Expression During Neuronal Differentiation
MAP1A		350	dendrites, soma & axons	late
MAP1B		330	dendrites, soma & axons	early
MAP2	MAP2a	280	dendrites & soma	late
	MAP2b	260	dendrites & soma	early & late
	MAP2c	70	dendrites, soma & axons	early
	MAP2d	74	glial cells	late
tau	multiple (6)	45-65	preferentially in axons	early (juvenile forms) late (adult forms)

*They are generated by alternative mRNA splicing

mainly in axons (Berhardt et al., 1985 and reviewed by Lee, 1990, Vallee, 1990, Matus, 1994 and Goedert et al., 1994).

The different isoforms of both MAP2 and tau are developmentally controlled. MAP2a and most tau isoforms are found only in mature neurons, while MAP2c and juvenile tau are found only in developing neurons. MAP2b is expressed continuously throughout life (Riederer and Matus, 1985 and reviewed by Matus, 1994, Goedert et al., 1994). MAP2a and tau have been suggested to have a role in Mt stabilization which is necessary for the formation of cytoplasmic processes. In MAP2 and tau transfected cells, Mts become bundled into extensive parallel arrays as seen during neuronal differentiation (reviewed by Vallee, 1990). Also, the fact that MAP2c and juvenile tau are continually expressed in the adult mammalian olfactory system suggests that they play a role in the growth of neuronal processes by promoting Mt assembly (reviewed by Lee, 1990, Matus, 1994 and Goedert et al., 1994).

MAP4 consists of four different isoforms (190-255 kDa) and was first purified from HeLa cells. It is present in almost all non-neuronal cells. It is also present in developing neurons, but is found only in small amounts in adult neurons. Therefore, it is suggested that MAP4 has an important role in mitosis, cell motility, organelle distribution/movement and cellular morphogenesis by controlling Mt dynamics in dividing cells (reviewed by Chapin and Bulinski, 1992 and Bulinski, 1994).

The MAP1 family

The MAP1 family has two members: MAP1A (350 kDa) and MAP1B (320 kDa) (**Table 2**). MAP1A and MAP1B are both thermolabile fibrous molecules that can promote Mt assembly *in vitro* (reviewed by Müller et al., 1994 and Hammarback, 1997). Electron microscopic analysis of the neuronal cytoskeleton reveals that the MAP1 proteins form cross-bridges between Mts and between Mts and other cytoskeletal elements (Shiomura et al., 1987 and Sato-Yoshitake et al., 1989). These structural elements are thought to organize and strengthen the axonal cytoskeleton by multiple Mt and actin binding sites.

The MAP1 proteins have been found in a variety of cell and tissue types, but are predominant in neuronal cells. In neurons, MAP1 proteins have been found in both axons and dendrites as well as in somata. In prenatal brain, MAP1A cannot be detected while in postnatal brain, MAP1A abundance accumulates steadily as neuronal processes are growing and reaches a plateau when axon and dendrite formation are complete (reviewed by Matus, 1988, Chapin and Bulinski, 1992, Müller et al., 1994 and Hammarback, 1997). This pattern of expression is complementary to the expression of MAP1B which appears during the initial phase of neuronal differentiation and decreases steadily as the neuron matures (Langkopf et al., 1992). Due to this developmental regulation of these proteins, it was suggested that MAP1B plays a role in regulating the dynamic assembly of the Mts during neurite outgrowth, whereas

MAP1A plays a morphological role in stabilizing the Mts in mature axons and dendrites (reviewed by Müller et al., 1994).

Several studies have examined the role of MAP1B in neuritogenesis. Studies on cultured PC12 cells and cultured neurons in which MAP1B expression was inhibited showed that MAP1B plays a crucial role in axon formation (Brugg et al., 1993 and Gonzalez-Billault et al., 2001). Also, transgenic MAP1B mutant mice have been studied and despite some differences in the severity of the effects, such studies demonstrated that MAP1B deficient mice have impairment of brain development (Edelman et al., 1996, Takei et al., 1997 and Gonzalez-Billault et al., 2000). However, similar studies have not been performed with MAP1A.

The two MAP1 members are encoded by separate genes and are protein complexes composed of one high molecular weight polypeptide (heavy chain, HC) and up to three low molecular weight subunits (light chains, LC): LC1 (34 kDa), LC2 (30 kDa) and LC3 (18 kDa). The presence of the LCs was first discovered when immunoprecipitation of the HCs coprecipitated the low molecular weight subunits (Schoenfeld et al., 1989). Further investigations showed that all three LCs were present in the MAP1A immunoprecipitates and that only LC1 and LC3 were present in the MAP1B immunoprecipitates (Schoenfeld et al., 1989, Kuznetsov et al., 1986 and reviewed by Müller et al., 1994 and Hammarback, 1997).

Subunit stoichiometry of the LCs to the MAP1 HCs have been studied by two groups (Schoenfeld et al., 1989 and Pedrotti et al., 1994 and Pedrotti and Islam, 1995). Due to differences in the procedures employed by the two groups, the exact stoichiometry is unknown. However, it was suggested by both groups that the MAP1 HCs could bind more than one copy of each LC (reviewed by Hammarback, 1997).

Comparison of the cDNA sequence encoding the conserved carboxy terminus region of MAP1A and MAP1B with the amino acid sequence of the MAP1 LCs revealed that LC1 is encoded in the 3' end of the open reading frame of MAP1B (Hammarback et al., 1991); whereas, LC2 is encoded in the 3' end of the open reading frame of MAP1A (Langkopf et al., 1992). It is hypothesized that the single open reading frame of MAP1A or MAP1B mRNA is translated into a corresponding single polypeptide which is cleaved during or after translation to yield one HC and LC2 or LC1, respectively (**Figure 3**) (reviewed by Hirokawa, 1994, Müller et al., 1994 and Hammarback, 1997).

Mann and Hammarback (1994, 1996) found that the LC3 cDNA is not found in the MAP1A/LC2 or MAP1B/LC1 polypeptide cDNA but is expressed by a separate gene. However, LC3 is always co-expressed with either MAP1A or MAP1B during brain development.

Through cDNA and amino acid sequence comparison, MAP1A and MAP1B are found to be structurally related with high sequence homology (Langkopf et al., 1992 and Fink et al., 1996). It was established through

transfection experiments using truncated forms of both MAP1A and MAP1B and through *in vitro* Mt binding assays that for MAP1 family members, the Mt binding domain is located at the amino terminus of the HC, while the projection domain is located near the carboxy terminus.

Their Mt binding domain consists of two regions: a (K/R) (K/R) (E/D) repeat region and the regions flanking these repeats. This basic motif is repeated 11 times in MAP1A and 21 times in MAP1B (**Figure 4**). Noble et al. (1989) showed that binding of MAP1B occurred even in the absence of the repeat domain as long as the flanking regions were present. Similar results were obtained by Vaillant et al. (1998) who showed that MAP1A bound Mts *in vitro* and *in vivo* even in the absence of the basic repeat regions. Biochemical studies performed by Vaillant et al. (1998) also suggested that the Mt binding regions that flank the basic repeats in MAP1A showed higher affinity for the Mts in the absence of the basic repeats. This suggests that the affinity of MAP1A and MAP1B to Mts may be modulated by the basic repeats as seen with the Mt binding region of tau (Gustke et al., 1994).

It is known that the MAP1 HCs can bind to Mts, but there are two hypotheses regarding LC binding to Mts. The first hypothesis suggests that LCs are not able to bind independently to Mts, but interact with the MAP1 HCs, which in turn bind Mts. This hypothesis was suggested by preliminary studies of Vaillant (1997) which showed that the LCs do not colocalize with Mts after transfection into cultured cells. The second hypothesis suggests that LCs are

Figure 3: Diagram showing the proteolytic processing of MAP1A/LC2 and MAP1B/LC1 to give a heavy chain and a light chain (Adapted from Muller et al., 1994).

MAP1 - LIGHT CHAIN PROCESSING

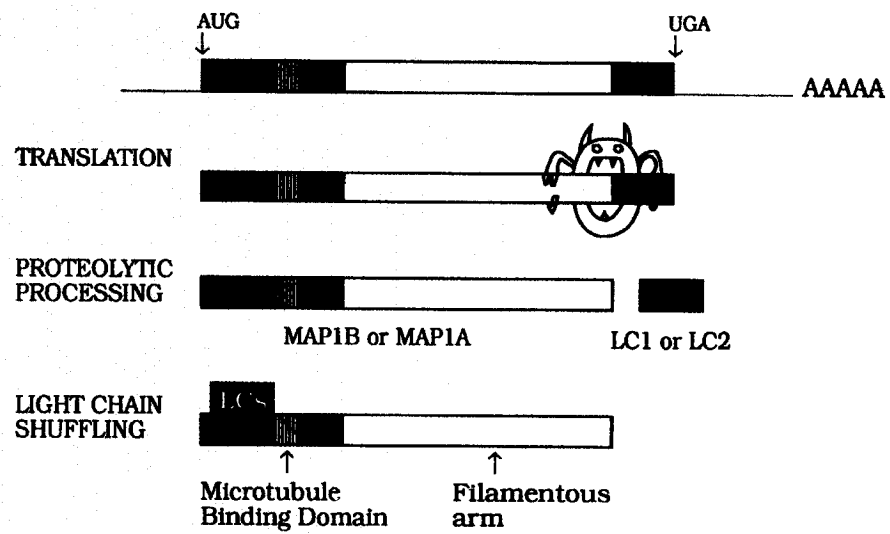


Figure 4: Schematic representation of the MAP1 family members: MAP1A/LC2, MAP1B/LC1 and LC3. (Reproduced from Vaillant, 1997).

MAP1a



LC2

MAP1b



LC1

□ = basic repeat

■ = flanking domains

▨ = acidic repeat

⋈ = putative MT binding domain (Cravchik *et al.*, 1994)

1 142
LC3

capable of binding Mts independently of the MAP1 HCs. This hypothesis is supported by Zauner et al. (1992) who showed that bacterial synthesized LC1 bound to Mts *in vitro* and by Tögel et al. (1998) who showed that LC1 transfected into cells colocalized with Mts even in the absence of MAP1B HC.

The MAP1 protein complexes, including the HCs and the LCs, have previously been shown to have microfilament binding activities (Fujii et al., 1993 and Pedrotti et al., 1994 and Pedrotti and Islam, 1995 and 1996). More recently, LC1 (Tögel et al., 1998) and LC2 (Noiges et al., 2002) have been shown to have microfilament binding activities and it is now possible to hypothesize that binding of the MAP1 HC/LC complex to microfilaments is due to the LCs. Whether LC3 associates with actin filaments remains undetermined.

There is no evidence to show that the MAP1 HCs associate with intermediate filaments (Bloom and Vallee, 1983), but Hosszu (2001) showed that LC2 might colocalize with vimentin filaments. These various LC activities suggest that LC content could regulate MAP1A and MAP1B activities.

Rationale for experiments

MAPs have been shown to play a role in Mt stability in axons and dendrites, in determining neuronal shape and in regulating the balance between rigidity and plasticity in neuronal processes (reviewed by Matus, 1988). MAP1A is the most abundant MAP in adult rat (Reiderer and Matus, 1985 and Garner et al., 1990), mouse (Matus, 1988) and human brains (Fink et al., 1996). Since it is

found in axons, dendrites and somata, changes in its expression would have global effects within the neuron.

As an increase in MAP1A expression parallels an increase in Mts in mature neurons, it has been suggested that MAP1A, through Mts, plays a role in maintaining neuronal morphology (reviewed by Müller et al., 1994). MAP1A expression reaches a maximum during the growth phase of brain neurons (Matus, 1988) and cultured neurons (Vaillant and Brown, 1995 and Vaillant, 1997).

Little is known of how the MAP1 LCs, especially LC2 and LC3, interact with the HCs during this growth phase. This present study tries to answer the following questions:

- 1) Are the MAP1 LCs capable of binding Mts in the absence of the MAP1 HCs?
- 2) Do the LCs affect Mt morphology/ distribution in the absence of the MAP1 HCs?
- 3) Does MAP1A have a regulatory role on LC2?
- 4) Do the MAP1 LCs interact with actin filaments?
- 5) What interactions do the MAP1 LCs have amongst themselves?

MATERIALS AND METHODS

EXPRESSION CONSTRUCTS

The PGK-6myc MAP1A, PGK-6myc MAP1A HC fragments and PGK-6myc LC expression vectors were previously constructed by A. Vaillant using three overlapping cDNAs spanning the entire mRNA for MAP1A (Langkopf et al., 1992) and cDNA for light chains 1, 2 and 3 (Dr. J. Hammerback, Bowman Gray School of Medicine, NC) (see **Figure 5** and detailed description in Vaillant, 1997 and Vaillant et al., 1998). The coding sequences for the full length MAP1A, the MAP1A HC fragments and the LCs were cloned into the pKJ1 Δ F-6myc vector (a gift from Dr. M. McBurney, University of Ottawa), which is a pUC19-based vector, containing the constitutively active mouse phosphoglycerate kinase (PGK) promoter driving the expression of 6 repeats of a 9 amino acid epitope from the human c-MYC protein.

The pMAP2c-EGFP expression vector (**Figure 6**) was previously constructed by K. Currie in our laboratory using a 1.4 kb Bgl II-Msc I fragment (containing the MT binding domain of MAP2c) from the pPGK-MAP2c plasmid, obtained by C. Addison (1997) also in our laboratory. This vector was constructed by ligating a 1.7 kb fragment containing the MAP2c cDNA (Kindler et al., 1990) (a gift from Dr. C. Garner, University of Alabama, Birmingham). The 1.4 kb fragment from it was ligated into a Bgl II-Sma I cut pEGFP-N1 commercially available vector (Clontech laboratories), which contains the CMV promoter driving the expression of the EGFP tag.

The pLC2-EGFP expression vector was previously constructed by N. Hosszu Ungureanu (see **Figure 7** and detailed description in Hosszu (2001)) using a 666 bp EcoRI – Bam HI fragment from the pPGK-6myc LC2 plasmid. This vector was constructed by ligating the 666 bp into an EcoRI – BamHI cut pEGFP-C1 commercially available vector (Clontech laboratories), which contains the CMV promoter.

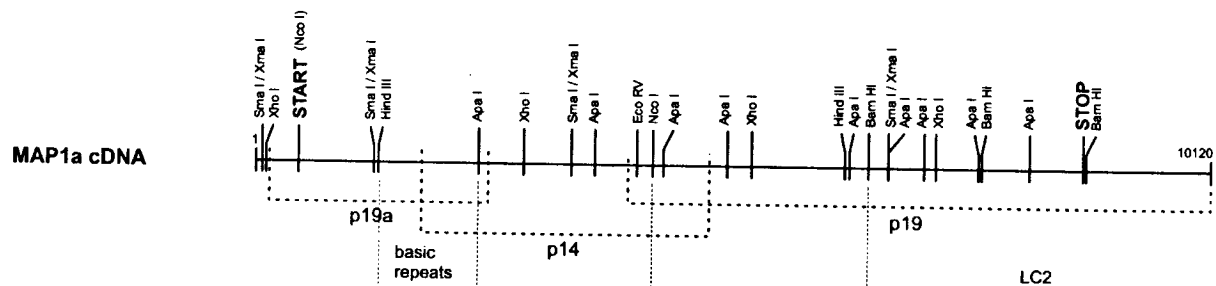
LARGE SCALE PRODUCTION OF PLASMID DNA FOR TRANSFECTION

Glycerol stocks of plasmid DNA were used to inoculate 5 ml of LB cultures with either 50 µg/ml of kanamycin (for the pEGFP based vectors) or 100 µg/ml of ampicillin (for the PGK-6myc based vectors) and grown ON at 37°C with vigorous shaking ($\cong$ 300 rpm). The following day, the cultures were diluted 1:500 in 500 ml LB broth with kanamycin or ampicillin (as above) and grown to saturation for 16 hours as above. Plasmid DNA was harvested from large cultures by centrifugation and isolated by alkaline-lysis using Qiagen Plasmid Mega Kit (25) (Qiagen). The plasmid DNA was then resuspended in nuclease free ddH₂O and stored at -20°C. DNA concentration and purity were determined by UV absorbance at 260 and 280 nm using a Genequant® spectrophotometer (Pharmacia).

TISSUE CULTURE

HeLa CCL-2 cells (ATCC) were kept semiconfluent in α -modified Eagles minimal essential medium (α -MEM) (Gibco BRL) supplemented with 10% fetal

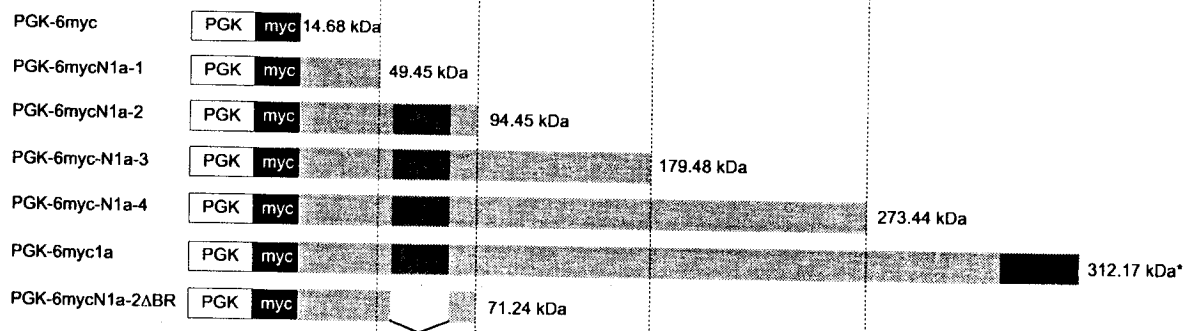
Figure 5: Diagram showing the PGK-6myc full length MAP1A, the PGK-6myc MAP1A heavy chain fragments and the PGK-6myc LC expression constructs obtained by A.Vaillant (1997). (Reproduced from Vaillant, 1997).



MAP1a PROTEIN



**MAP1a
EXPRESSION
CONSTRUCTS**



**LIGHT CHAIN
EXPRESSION
CONSTRUCTS**

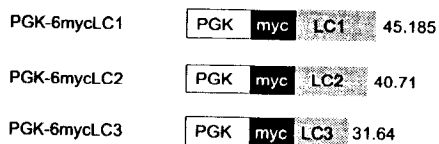


Figure 6: Diagram showing the MAP2C-EGFP expression construct obtained from K. Currie.

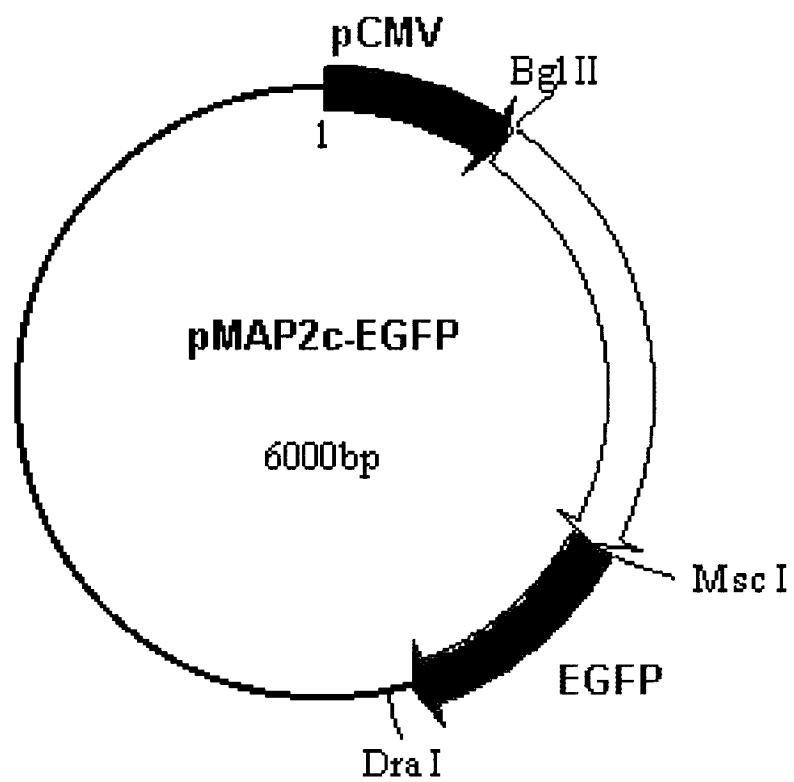
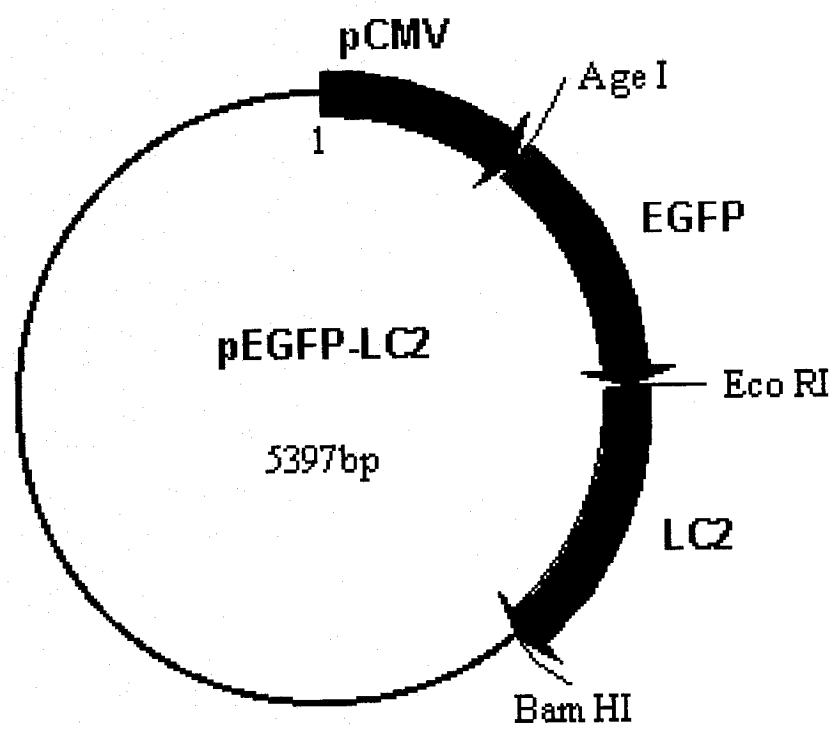


Figure 7: Diagram showing the EGFP-LC2 expression construct obtained from N.Hosszu (2001).



calf serum (Gibco BRL) and antibiotics (Gibco BRL). Cells were grown in a humidified incubator at 37°C and 5% CO₂ and passaged every two days.

TRANSFECTION OF HELA CELLS

Expression vectors were introduced into the cells using the multi-component lipid-based FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals). For protein extractions, cells were plated onto 60 mm dishes (Corning) at 1×10^5 cells/dish in 4 ml antibiotic-free media. For microscopy, cells were plated onto 24 mm² coverslips at 5×10^4 cells/ coverslip in 2 ml of antibiotic-free media. Cells were allowed to settle for 24 hours. The transfection and co-transfection mixtures were prepared, according to the manufacturer, with a FuGENE 6 reagent (μl): DNA (μg) ratio of 6:1 in 100 μl of serum and antibiotic free medium. The mixture was gently mixed and incubated for 20 minutes at RT. Drop wise, 100 μl of transfection mixture was added to each 2 ml of culture media. Cells were incubated at 37°C and 5% CO₂. The culture media was replaced with fresh culture media 24 hours after transfection. Cells were processed 48 hours after transfection.

DRUG TREATMENTS

Taxol (Sigma) was kept as a 0.01 M stock in DMSO at - 20°C. This stock was then diluted to 5 μM in α-MEM containing 10% FCS and 1% antibiotics to treat cell cultures for 7 hours. Nocodazole (Sigma) was kept as a 5 mg/ml stock in DMSO at -20°C and diluted to 8 μg/ml to treat the cells for 1 hour.

CELL FIXATION

Cells plated on glass coverslips were briefly rinsed in PBS and fixed at room temperature by three different protocols:

PRECIPITATION

(Stefanini et al., 1967) Coverslips were incubated for 1 h in Zamboni's fixative (14% picric acid (Fisher), 3.7% paraformaldehyde (JB EM Services Inc.) in 0.5 M Na_2HPO_4 , 0.5 M NaH_2PO_4 , pH 7.1) followed by a 3X5 min PBS (130 mM NaCl, 5mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4) wash, 5 min extraction with 0.5% TritonX-100 in PBS and a final 3X5 min PBS wash.

EXTRACTION / FIXATION

(Falconer et al., 1992) Coverslips were incubated 2 minutes in a pre-extraction solution of 0.2% Triton X-100 in PEM (PIPES, EGTA, MgCl_2 , followed by a 10 min fixation in 4% formaldehyde (JB EM Services Inc.), 0.25% glutaraldehyde (JB EM Services Inc.) and 0.5% Triton X-100 in PEM followed by a 3X5 min PBS wash.

METHANOL

Coverslips were quickly immersed in -20°C MeOH for 5 min. Coverslips were then re-equilibrated with 3X20 min PBS washes. This protocol was used for the anti-detyrosinated α -tubulin rabbit polyclonal antibody.

PROTEIN EXTRACTION

WHOLE CELL EXTRACTION (Figure 8)

This method was used to obtain extracts for the *in vitro* binding assay. Cells were rinsed briefly in cold MAB2 (0.1 M MES pH 6.4, 2.5 mM EGTA, 0.1 mM EDTA, 5 mM MgCl₂, Pedrotti et al., 1993) and then 250 µl of extraction buffer (MAB2 + 4 mM PEFA, 1mM 1,10-phenanthroline and 40 µg/µl each of aprotin, pepstatin A and leupeptin) was added. Cells were immediately scraped into an eppendorf and sonicated 15 seconds at 94 W, then immediately spun for 10 minutes at 10 000 rpm and 4°C. The supernatant was removed and stored at -84°C.

SDS WHOLE CELL EXTRACTION (Figure 9)

This method was used to obtain extracts for SDS-PAGE. Cells were rinsed briefly in PBS and then 250 µl of extraction buffer (25 mM Na₂HPO₄, pH 7.2, 400 mM NaCl, 0.5% SDS (w/v), 40 µM benzamidine HCl (Sigma), 4 mM PEFA (Centrichem), 1mM 1,10 phenanthroline (Sigma) and 40 µl of each aprotinin, pepstatin A and leupeptin (all from Sigma)) was added. The viscous lysate was immediately scraped into an eppendorf tube and placed in a boiling water bath for 10 min and then spun for 10 min at 10 000 rpm and 4°C. The supernatant was then removed and stored at -84°C.

CYCLING OF TUBULIN AND MAPS FROM BOVINE BRAIN (Figure 10)

This procedure is derived from Weingarten et al. (1975) and Collins and Vallee (1987). Three fresh adult bovine brains were obtained from Savage et Fils

Figure 8: Protocol for the whole cell extraction (Reproduced from Vaillant, 1997).

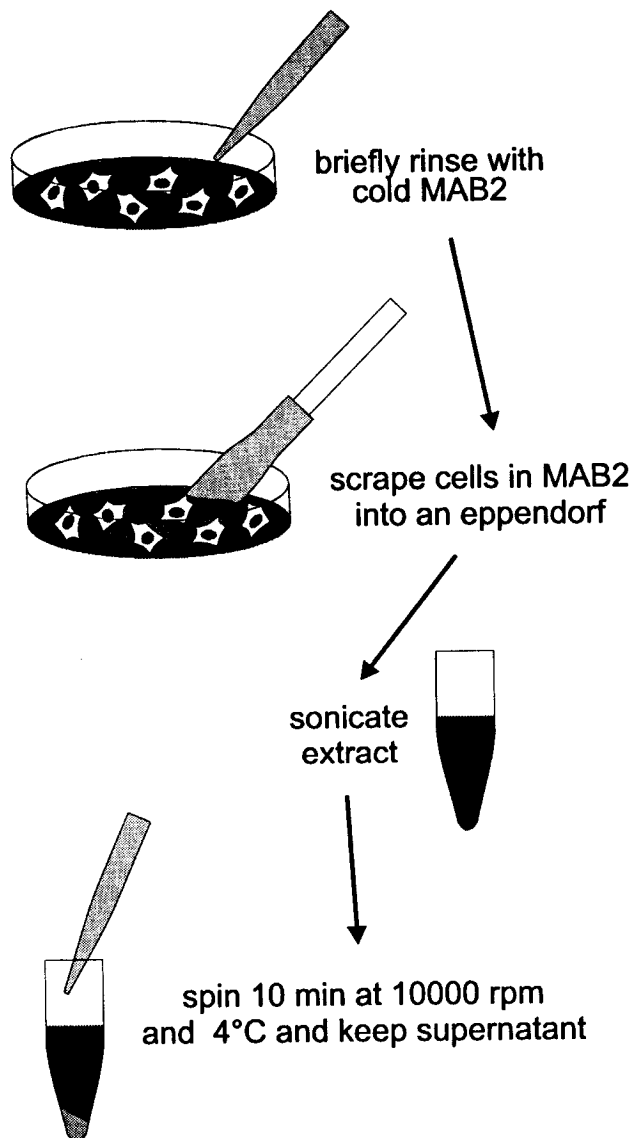
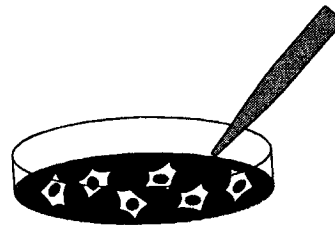
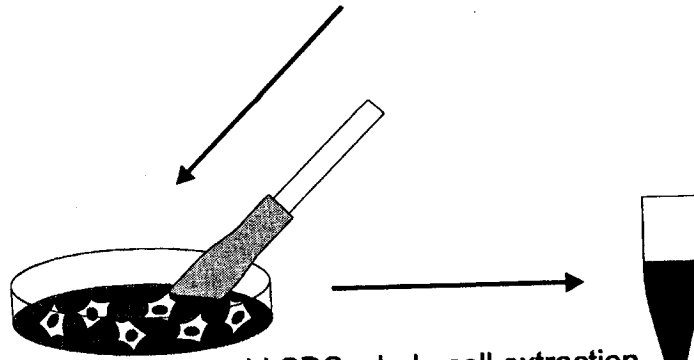


Figure 9: Protocol for SDS-whole cell extraction (Reproduced from Vaillant, 1997).



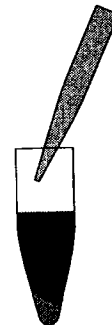
briefly rinse cells in
rinse buffer



add SDS-whole cell extraction
buffer and immediately scrape
into eppendorf

place eppendorf in boiling water
bath for 10 min

spin 10 min at 10000 rpm
and 4°C and keep
supernatant



slaughter house (St-Albert, Ontario) and kept submerged in ice cold MAB (0.1 M PIPES, pH 6.4, 1 mM EGTA, 1 mM MgCl_2 , 4 M glycerol and 0.1 mM GTP) while the connective tissue and meninges were removed. The brains were then homogenized in a Waring blender for 45 seconds in 70 % (v/w) chilled MAB and transferred to pre-chilled centrifuge bottles. The homogenate was then spun 10 minutes at 18 000 g and 4°C (10 000 rpm using a Sorval GSA rotor). The supernatant was transferred to new pre-chilled centrifuge tubes and spun 30 minutes at 36 000 g and 4°C (18 000 rpm using a Sorval SS-34 rotor). To this supernatant (designated S1), GTP was added to a final concentration of 1.8 mM and then incubated at 30 minutes and 37°C. The now viscous S1 extract was spun 30 minutes at 36 000 g and 37°C. The pellet was resuspended in chilled MAB (15 % of the S1 volume) and homogenized in a Dounce homogenizer on ice for 30-40 minutes. This homogenate was then spun 30 minutes at 36 000 g and 4°C. The supernatant (designated S2) was again brought to 1.8 mM GTP and incubated 30 minutes at 37°C. After spinning this supernatant as for S1, the pellet was resuspended in MAB (15% of S2 volume) and homogenized as for S1. The homogenate was again spun for 30 minutes at 36 000 g and 4°C. The supernatant was loaded onto a phosphocellulose column. After all polymerization steps, a small aliquot was taken and prepared for electron microscopy to verify the presence of Mts.

PHOSPHOCELLULOSE PURIFICATION OF TUBULIN (Figure 10)

The supernatant S3 was loaded onto a phosphocellulose column (P-11, Whatman) preequilibrated with 5 column volumes of MAB (- glycerol) and eluted with MAB (- glycerol). The presence of tubulin in collected fractions was determined by spectrophotometer readings. Tubulin containing fractions were pooled and concentrated using a 30 KDa cutoff ultrafree microconcentrator (Millipore). Protein concentrations were determined using the BCA assay and aliquots were stored at - 84°C.

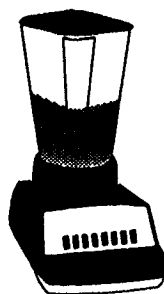
IN VITRO BINDING ASSAY (Figure 11)

Parts of this procedure were derived from the Mt-binding assay performed by Butner and Kirschner (1991). Taxol Mts were prepared from purified brain tubulin by sequentially adding 0.15, 1.0, 5.0, 20 and 40 μ M taxol (Sigma) at 5 min intervals to a tubulin solution of 5 mg/ml incubating at 37°C in MAB supplemented with 1 mM GTP. 20 μ l of protein extract was added to 200 μ g of taxol stabilized tubulin and incubated 20 min at 37°C. 80 μ l of the mixture was added to 160 μ l of 20% sucrose cushion made in MAB2 supplemented with 1 mM GTP and 10 μ M taxol. The sample was then ultracentrifuged at 60 000 rpm in a TL 100.1 rotor (Beckmann instruments) at RT. Supernatant was resuspended in an equal amount of 2X sample buffer (Laemmli, 1970). The pellet was resuspended in the same volume as the supernatant with 1X sample buffer. Aliquots of Mts before and after centrifugation were taken and prepared

Figure 10: Protocol for tubulin purification from bovine brain (Modified from Vaillant, 1997).

remove meninges and
connective tissue from
three cow brains

homogenize 45 sec in
chilled MAB



centrifuge 10 min at
18000g and 4°C



centrifuge supernatant
30 min at 36000g and
4°C;
keep supernatant (S1)



assemble tubulin in S1
supernatant 30 min at
37°C



centrifuge 30 min at
36000g and 37°C;
remove supernatant



homogenize pellet in
chilled MAB



centrifuge 30 min at
36000g and 4°C; keep
supernatant (S2)



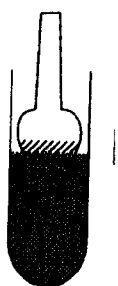
assemble tubulin in S2
supernatant 30 min at
37°C



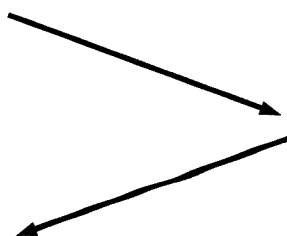
centrifuge 30 min at
36000g and 37°C;
remove supernatant



continued on page 41

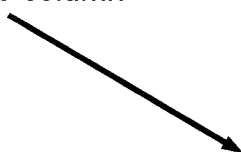
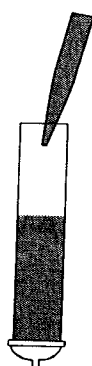


homogenize pellet in
MAB



centrifuge 30 min at
36000g and 4°C; keep
supernatant (S3)

load supernatant (S3) on
pre-equilibrated phos-
phocellulose column

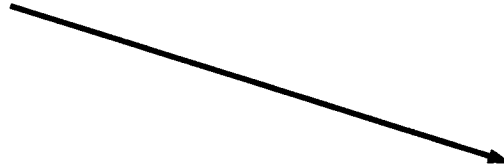


collect eluate and pool
tubulin containing
fractions

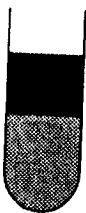
Figure 11: Protocol for the *in vitro* binding assay (Modified from Vaillant, 1997).



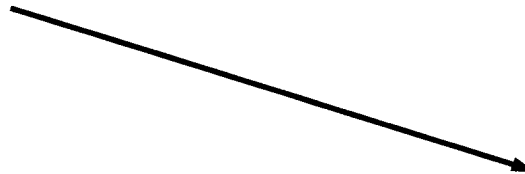
assemble phosphocellulose tubulin at
37 °C in increasing concentrations of
taxol



add transfected cell extract and incubate
for 20 min at 37 °C



add microtubule mixture to a sucrose
cushion and centrifuge for 15 min at
60 000 rpm



resuspend supernatant and microtubules
in sample buffer and analyze by Western
blot



for fluorescence microscopy to verify the presence of Mts. Samples were examined by Western Blot.

SDS-PAGE AND WESTERN BLOTTING

Protein samples diluted 1:1 in 2X sample buffer (Laemmli, 1970), biotinylated SDS-PAGE broad range standards (BioRad) diluted 1:10 in 2X sample buffer and prestained broad range standard (BioRad) undiluted were placed in a boiling water bath for 5 minutes. Samples were then loaded onto 7.5 or 12% polyacrylamide gels and separated using the BioRad minigel appartatus II. Proteins were electrotransferred onto nitrocellulose (Scleicher and Scheull) according to Towbin et al. (1979) and rinsed in PBS. Immunodetection of western blots was performed as follow: block 1 hour in 5% skim milk (Carnation) in PBS containing 0.05% Tween-20 and 4°C, 1 h incubation in primary antibody in 2% milk in PBS-Tween, 45 min incubation in biotinylated secondary antibody in 2% skim milk in PBS-Tween and 30 minutes incubation in biotinylated streptavidin-HRP (Amersham) diluted 1:4000 in PBS. A 3 X 5 min PBS wash was done between all antibody incubations. Antibody binding was detected by enhanced chemiluminescence (ECL) (Amersham) using Hyperfilm-ECL (Amersham). Except where noted all steps were performed at room temperature.

FLUORESCENCE MICROSCOPY

Following fixation, samples were quenched 3X4 min in 1 mg/ml NaBH₄ (Sigma) in PBS followed by 3X5 min PBS wash. All antibody incubations were for 45 min, except for the anti-detyrosinated α -tubulin antibody which had an

incubation time of 20 min, in PBS at room temperature with 3X5 PBS washes after each incubation. For all labelling, sequential primary and secondary incubations were used. Actin was stained with rhodamine phalloidin (Molecular Probes) diluted 1:30. Nuclei were visualized with the DNA stain Hoescht 33258 (Calbiochem®) diluted 1:3000. Cells were mounted in 50% glycerol-PBS containing 1% (w/v) paraphenylene-diamine (Sigma). Stained cells were visualized using a Zeiss Universal epifluorescence microscope equipped with a 50 W Hg burner. Images were digitally recorded with a Hamamatsu integrating CCD camera using Metamorph v4.01 (Universal Imaging). TIFF images were processed and assembled for publication using Adobe Photoshop v4.0 and Microsoft Publisher.

ANTIBODIES

Anti-tubulin

YL 1/2, a rat monoclonal IgG which recognizes α -tubulin [clone YL 1/2 (SeraLab)] was used at 1:300 for double immunolabelling.

DM1A, a mouse monoclonal Ig which recognizes α -tubulin [clone DM1A (Sigma)] was used 1:400 for immunolabelling.

Anti-MAP1A

HM-1, a mouse monoclonal IgG which recognizes MAP1A [clone HM-1 (Sigma)] was used 1:1000 for immunoblotting.

Anti-6myc

9E10, a mouse monoclonal IgG [clone 9E10, Evan et al., 1985] provided by Dr. C. Garner (University of Alabama, AL) and used undiluted for immunofluorescence microscopy and diluted 1:1 for immunoblotting.

Anti-GFP

JL-8, a mouse monoclonal IgG which recognizes GFP (clone JL-8) (Clontech) used diluted 1:1000 for immunoblotting.

Anti-MAP1B

AA6, a mouse monoclonal IgG which recognizes MAP1B (clone AA6) (Sigma) used diluted 1:1000 for immunoblotting.

Anti-LC3

LC-3, a rabbit polyclonal Ig (LC-3, Mann and Hammarback, 1994) provided by Dr. J. Hammarback and used 1:1000 for immunoblotting.

Anti-detyrosinated α -tubulin

Anti-E, a rabbit polyclonal Ig (anti-E, Xiang and MacRae, 1995) provided by Dr. T. MacRae (Dalhousie University, NS) and used 1:250 for immunofluorescence microscopy.

Anti-mouse

Donkey polyclonal IgG (H +L) conjugated to indocarbocyanine (CY3) (Jackson), crossabsorbed to rat and rabbit used at 1:400 for immunofluorescence microscopy.

Horse polyclonal IgG, biotinylated (Vector) and used at 1:1000 for immunoblotting.

Goat monoclonal IgG (H+L) conjugated to Alexa Fluor® 488 (Molecular Probes), highly cross-adsorbed used 1:400 for immunofluorescence microscopy.

Anti-rat

Goat anti-rat IgG (H + L) monoclonal antibody conjugated to Alexa Fluor® 350 (Molecular Probes) used at 1:200 for immunofluorescence microscopy.

Donkey anti-rat IgG (H+L) monoclonal antibody conjugated to carboxymethyl indocarbocyanine (CY2) (Jackson) used at 1:100 for immunofluorescence microscopy.

Anti-Rabbit

Goat anti-rabbit IgG (H +L), biotinylated (Vector) and used 1:1000 for immunoblotting.

Donkey polyclonal IgG (H+L) conjugated to indocarbocyanine (CY3) (Jackson) used 1:400 for immunofluorescence microscopy.

RESULTS

MAP1A is not detected in HeLa cells.

The presence of endogenous expression of MAP1A in HeLa cells was investigated by western blotting and was compared with the expression of MAP1A in adult bovine brain, used as a positive control. MAP1A was not detected in untransfected HeLa cells; therefore, investigation of exogenous expression of both heavy and light chains is permitted without the interference of endogenous MAP1A. As expected, MAP1A (**Figure 12***) was detected in adult bovine brain (reviewed by Muller et al., 1994). All lower molecular weight bands represent non-specific staining due to secondary antibody used during detection.

MAP1A heavy chain fragments are expressed in transiently transfected HeLa cells.

To ensure that the MAP1A HC fragments were being expressed correctly in HeLa cells, their expression was analysed by western blotting using an antibody against 6myc. All fragments (**Figure 13***) were detected within 10 kDa of their predicted molecular weight, except for 6myc1a (full length MAP1A). Even though MAP1A has a predicted molecular weight of 299 kDa, 6myc1a has a mobility of ~350 kDa due to anomalous mobilities seen during SDS-PAGE of filamentous proteins (Goedert et al., 1991). The amounts of individual fragments decreased as the size of the fragments increased; this corresponds to a decrease in transfection efficiency. The antibody also detected endogenous

human c-myc (**Figure 13**). All other bands represent non-specific binding of secondary antibody as seen in primary omission control in **Figure 13**.

MAP1A heavy chain fragments colocalize to Mts *in vivo*.

To confirm which MAP1A HC fragments colocalized with Mts, transiently transfected HeLa cells were observed by immunofluorescence microscopy (**Figure 14**). Cells were fixed by extraction fixation to observe which fragments bound to Mts. Vaillant et al. (1998) found that the MAP1A HC fragments bound to Mts *in vivo* if the basic repeat flanking region was present. The present study confirms these results since 6myc (**Figure 14a-a'''**) and 6mycN1a-1 (**Figure 14b-b'''**) do not colocalize with Mts after extraction fixation; however, all the other fragments do colocalize with Mts. The Mt distribution in the transfected cells was not altered, as assessed by comparison with untransfected HeLa cells in the same cell population.

To verify that in fact 6myc and 6mycN1a-1 were being expressed in the cell, HeLa cells transiently transfected with these fragments were fixed by precipitation. As expected, both these fragments were found in the cytoplasm (**Figure 15**). The secondary antibodies used for all fluorescence microscopy experiments did not show any unspecific binding as verified by primary omission experiments (data not shown).

MAP1A heavy chain fragments bind to Mts *in vitro*.

To confirm the previous microscopic analysis, an assay was devised to test the binding of MAP1A HC fragments to assembled Mts *in vitro*. (**Figure 16**).

Figure 12: Detection of MAP1A in S1, bovine brain extract, and in untransfected HeLa cell extract. MAP1A is not detected in HeLa cells, but is present in S1. 10 μ g of whole cell extract and S1 were separated by SDS-PAGE on a 7.5% gel. MAP1A was immunodetected on western blots using a mouse monoclonal antibody (clone HM-1) specific for MAP1A. Molecular weight markers are in kDa.

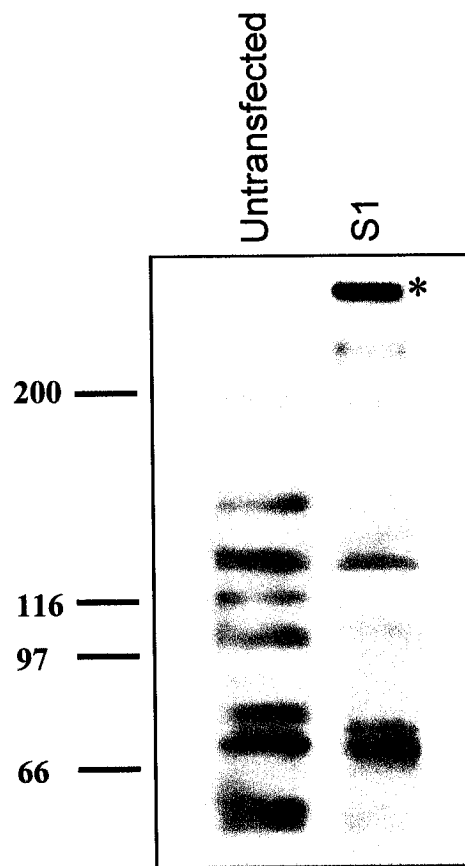


Figure 13: Detection of MAP1A HC fragments expressed in HeLa cells. 10 µg of untransfected whole cell extract and whole cell extracts of HeLa cells transfected with MAP1A HC fragments were separated by SDS-PAGE on a 12% gel. All fragments were immunodetected on western blots using a mouse monoclonal antibody (clone 9E10) against 6myc. Untransfected whole cell extract was blotted with the biotinylated mouse monoclonal antibody to show unspecific binding. Molecular weight markers are in kDa.

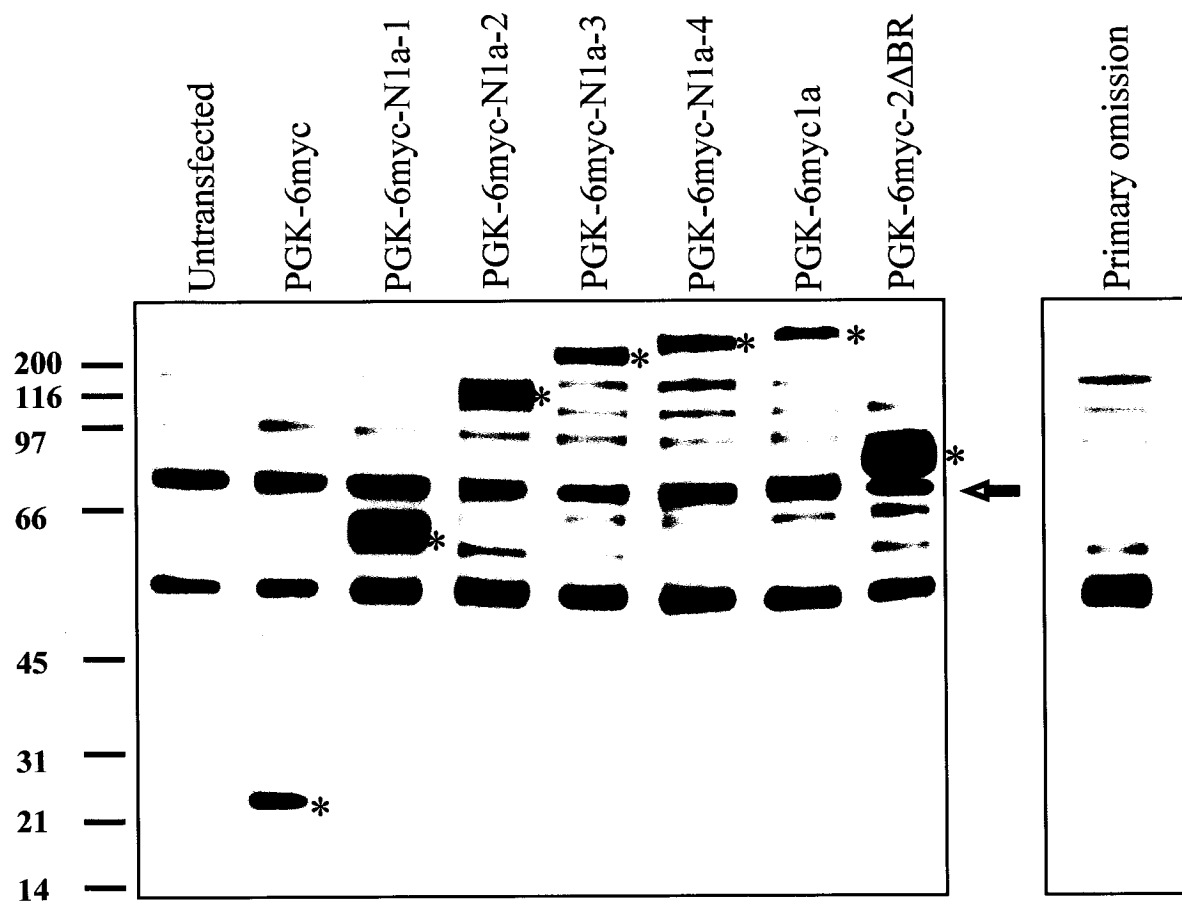


Figure 14: Hoescht staining of DNA (a-g) and double immunofluorescence labeling in HeLa cells transfected with 6myc (a-a'''), 6myc-N1a-1 (b-b'''), 6myc-N1a-2 (c-c'''), 6myc-N1a-3 (d-d'''), 6myc-N1a-4 (e-e'''), 6myc1a (f-f''') and 6myc-2 Δ BR (g-g'''). Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific against α -tubulin (a'-g') whereas the MAP1A HC fragments were visualized using a mouse monoclonal antibody (clone 9E10) specific for 6myc (a''-g''). The 6myc tag and 6myc-N1a-1 were removed from the cell during extraction fixation, whereas the remaining MAP1A HC fragments colocalized with the Mts and did not affect normal Mt distribution (yellow in merged images) (a'''-g'''). Bar = 10 μ M.

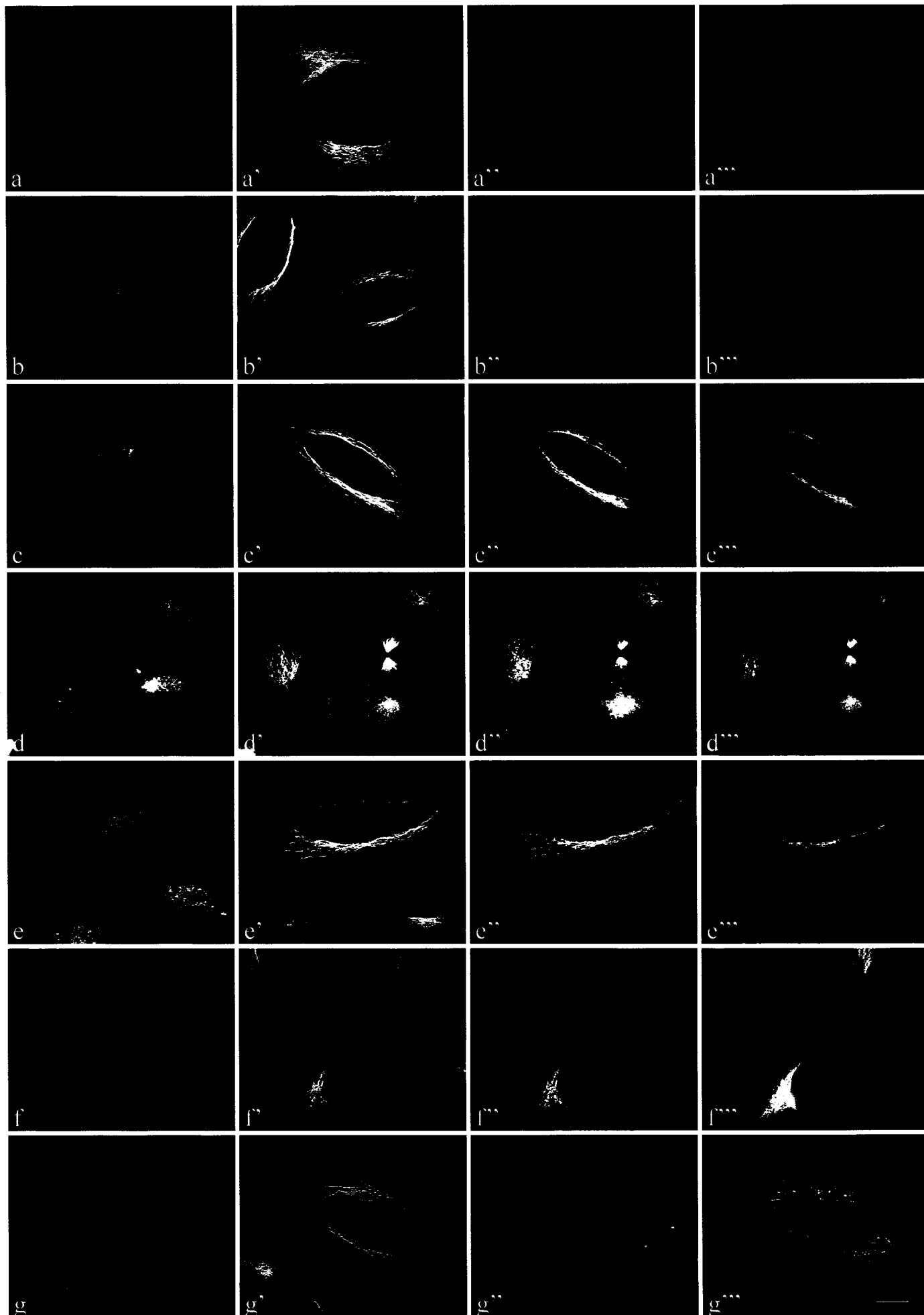


Figure 15: Hoescht staining of DNA (a-b) and double immunofluorescence labeling in HeLa cells transfected with 6myc (a-a'') and 6myc-N1a-1 (b-b''). Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific for α -tubulin (a'-b') while the 6myc tag and 6myc-N1a-1 were visualized using a mouse monoclonal antibody (clone 9E10) against 6myc (a''-b''). The 6myc tag and 6myc-N1a-1 were found in the cytoplasm of the cell when fixed by precipitation. Bar = 10 μ M.

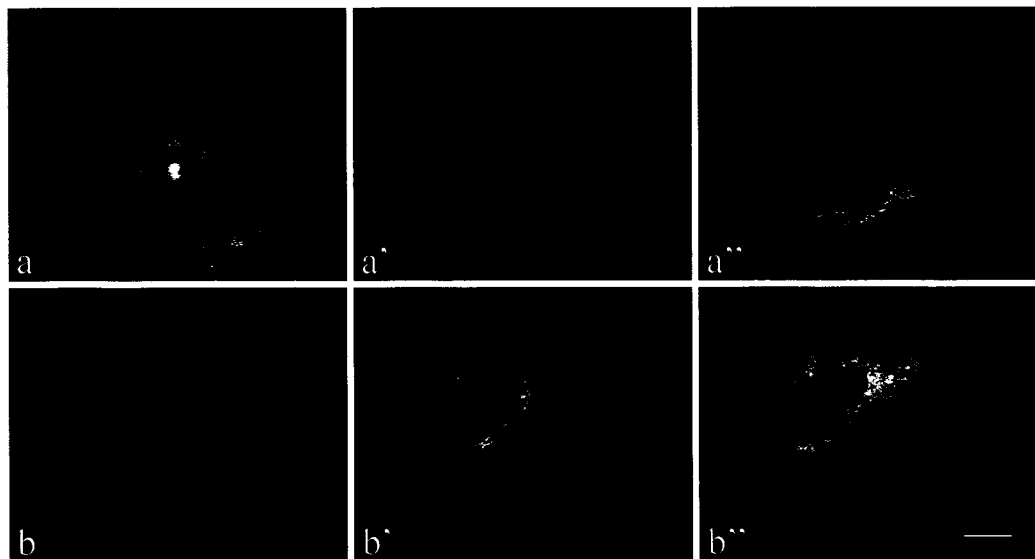
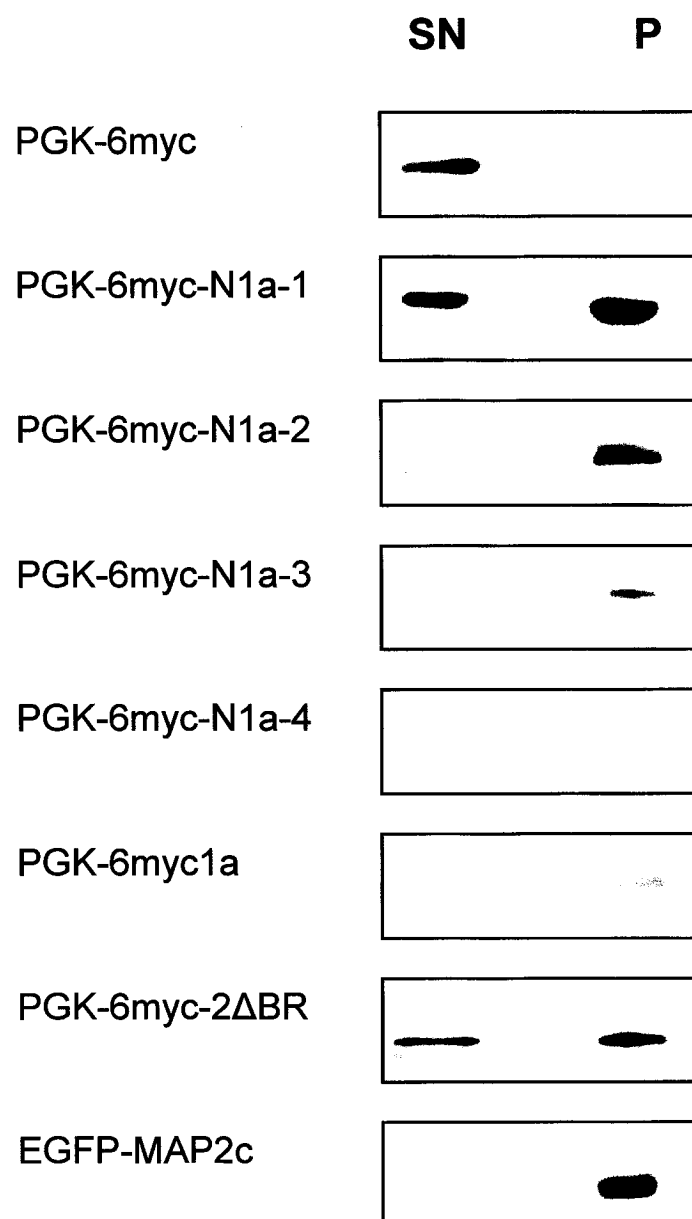


Figure 16: In vitro MAP binding assay. Equal volumes of sample were taken from the supernatant (SN) and the pellet (P) and separated by SDS-PAGE on 12% or 7.5% gels. MAP1A HC fragment samples were immunodetected on western blots using a mouse monoclonal antibody (clone 9E10) specific for 6myc. EGFP-MAP2c was immunodetected on a western blot using a mouse monoclonal antibody (clone JL-8) specific for GFP.



Proteins of interest were added to taxol stabilized Mts and centrifuged through a sucrose cushion; proteins that associate with Mts are found in the pellet. The validity of this assay was verified with the positive binding of EGFP-MAP2C. 6myc, a negative control, did not bind to the Mts. 6mycN1A-1 was found in both the pellet and the supernatant. This was unexpected since microscopic analysis showed that 6mycN1a-1 did not colocalize with Mts after extraction fixation. 6mycN1a-2, 6mycN1a-3, 6mycN1a-4 and 6myc1a were found in the pellet. 6mycN1a-2 Δ BR was found mostly in the pellet, but a significant amount was also found in the supernatant.

To verify that Mts were present before and after centrifugation, Mts were fixed, added to coverslips and immunolabeled with an anti α -tubulin antibody. Mts were found to be present throughout the procedure (**Figure 17**).

6mycN1a-1 binds to taxol stabilized Mts.

To determine whether the lack of detection of 6myc and 6mycN1a-1 after extraction fixation was due to low levels of fragments, cells were treated with taxol to induce Mt bundles. This has been shown to enhance detection of low levels of MAP1A in cells by concentrating the Mt-bound protein (Vaillant, 1998). Mts in the untransfected (**Figure 18a-a'''**) and transfected (**Figure 18b-e'''**) taxol treated cells were thick bundles and were found throughout the cytoplasm. 6myc (**Figure 18b-b'''**) was not found colocalized with the taxol stabilized Mts. 6mycN1a-1 (**Figure 18c-c'''**) was found colocalized with the Mts; however, it had a spotty distribution within the cell which is consistent with low expressing

proteins. This was compared to the colocalization of 6myc1a (**Figure 18d-d'''**) and 6mycN1a-2ΔBR (**Figure 18e-e'''**) with the taxol stabilized Mts.

EGFP-LC2 is expressed in transiently transfected HeLa cells.

To ensure that EGFP-LC2, constructed by Hosszu (2001), was being expressed correctly, its expression was analysed by western blotting using an antibody against GFP and was compared to the expression of the EGFP tag alone, used as a positive control. Both EGFP-LC2 and the EGFP tag were detected as single bands that correspond to their predicted molecular weights of 55 kDa and 27 kDa, respectively (**Figure 19***). The amount of EGFP present was higher than the amount of EGFP-LC2; this corresponds to higher transfection efficiency. All other bands represent non-specific binding due to the secondary antibody used during detection except for larger band below EGFP, which represents degradation products of EGFP.

EGFP-LC2 colocalizes to Mts *in vivo*.

To confirm that EGFP-LC2 colocalized with Mts *in vivo*, transiently transfected HeLa cells were observed by fluorescence microscopy (**Figure 20**). Cells were fixed by extraction fixation to observe the colocalization of EGFP-LC2 with Mts. Hosszu (2001) found that in HeLa cells transiently transfected with EGFP-LC2, 40% of transfected cells had the protein in the nucleus, 25% had the protein dotted along the Mts with bundling of Mts around the nucleus and 35 % had the protein located along the Mts, conferring to the Mts a wavy distribution as seen in **Figure 20b-b'''**. This was also found in the present study. Only

Figure 17: In vitro MAP binding assay. Samples of Mts before centrifugation (a) and from the pellet (b) were immunolabeled for tubulin using a mouse monoclonal antibody (clone DM1A) specific for α -tubulin to show the presence of Mts throughout the procedure. Bar = 5 μ M.

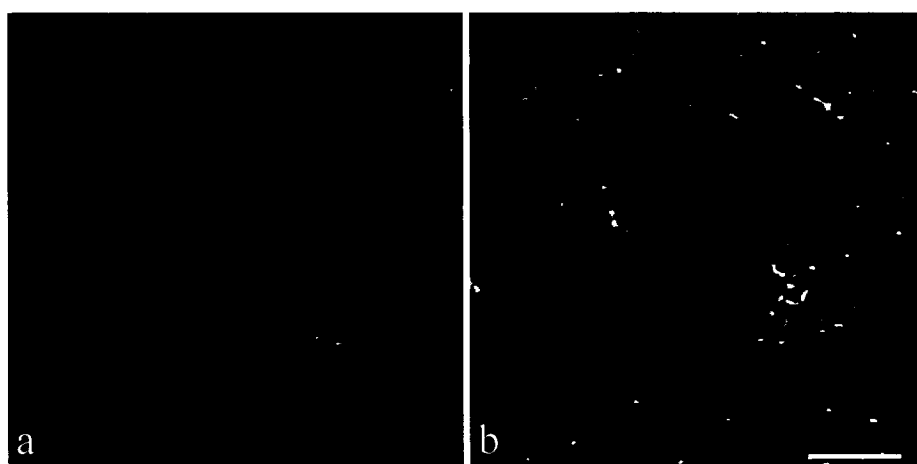


Figure 18: Hoescht staining of DNA (a-e) and double immunofluorescence labeling in taxol treated HeLa cells. Mts in untransfected cells (a-a''') and cells transfected with 6myc (b-b'''), 6myc-N1a-1 (c-c'''), 6myc1a (d-d''') and 6myc1a-2ΔBR (e-e''') were visualized using a rat monoclonal antibody (clone YL1/2) specific for α -tubulin (a'-e') while the 6myc tagged fragments were visualized using a mouse monoclonal antibody (clone 9E10) against 6myc (a''-e''). 6myc-N1a-1 was found to colocalize with Mts bundled by taxol (yellow in merged images) (a'''-e'''). Bar = 10 μ M.

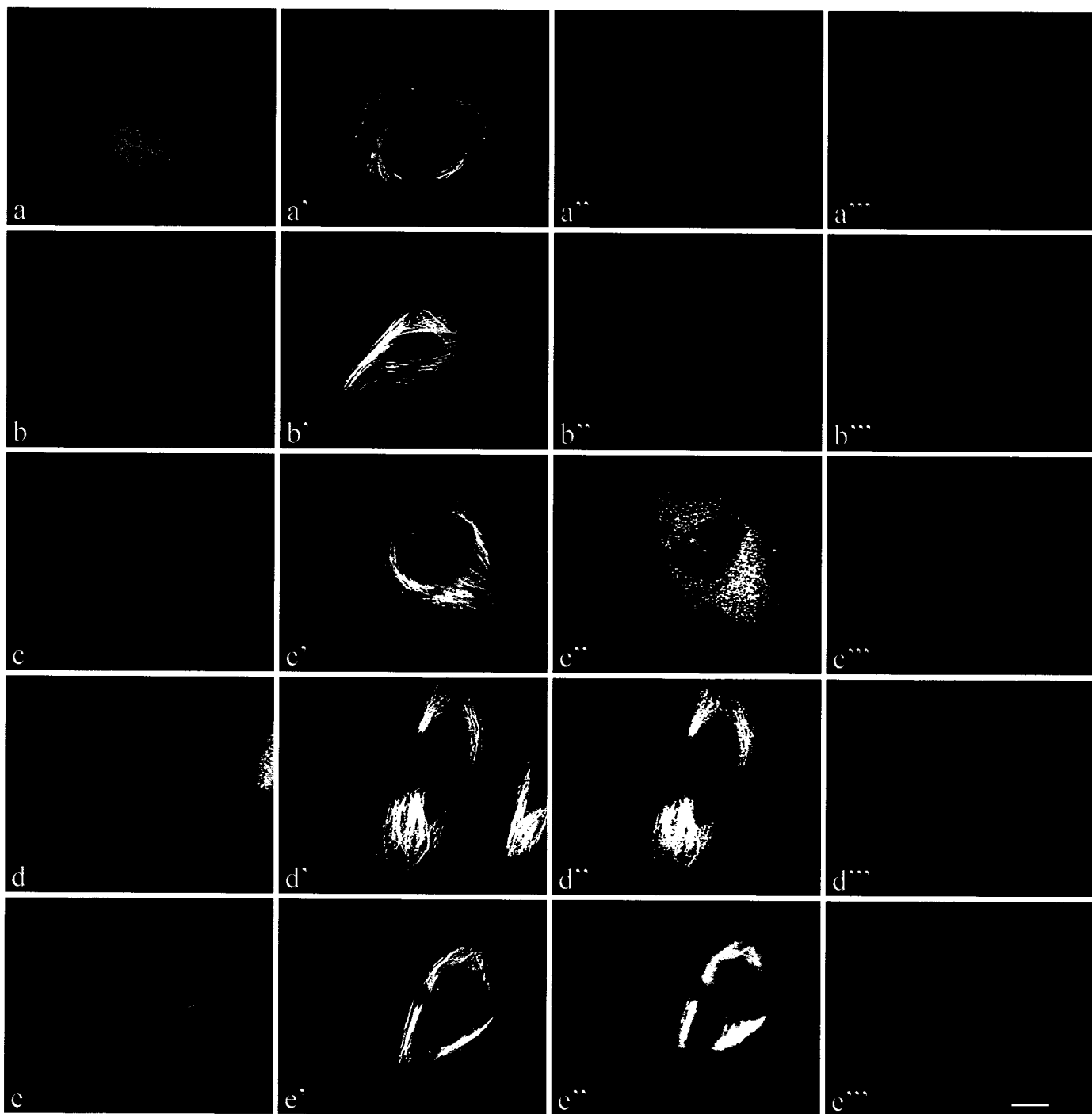


Figure 19: Detection of EGFP-LC2 and EGFP expressed in HeLa cells. 10 µg of untransfected whole cell extract and whole cell extracts of cells transfected with EGFP and with EGFP-LC2 separated by SDS-PAGE on a 12% gel. EGFP-LC2 and EGFP were immunodetected on western blots using a mouse monoclonal antibody (clone JL-8) specific for GFP. Molecular weight markers are in kDa.

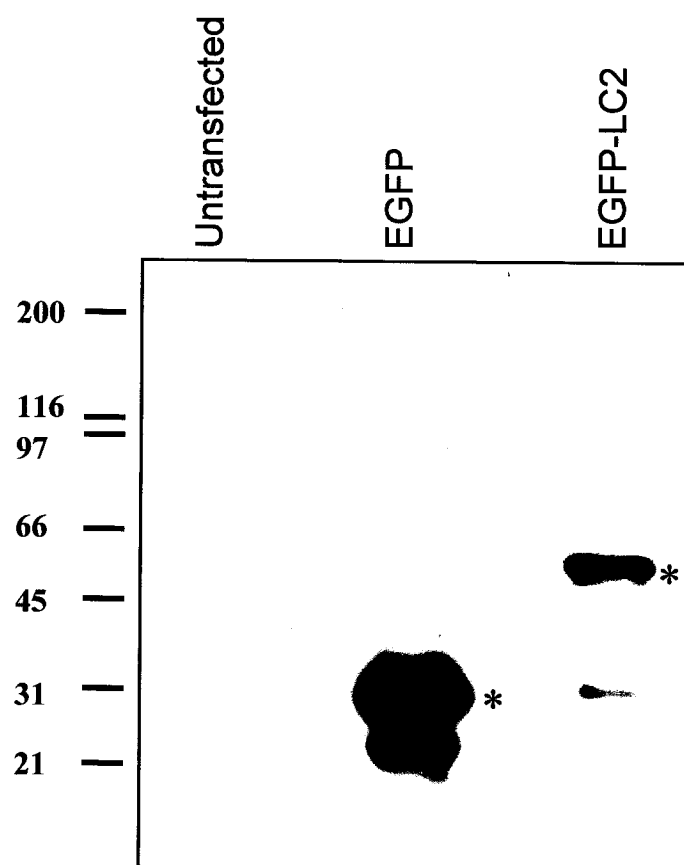
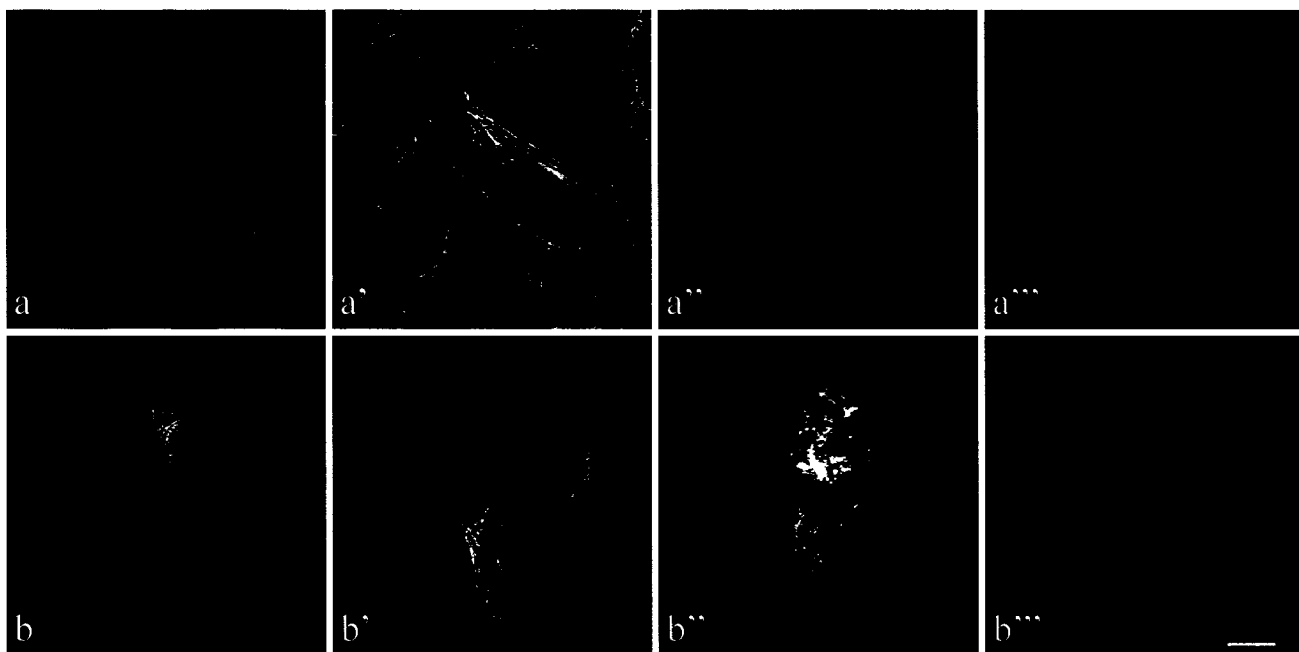


Figure 20: Hoescht staining of DNA (a-b) and immunofluorescence labeling of Mts in HeLa cells transfected with the EGFP vector alone (a-a'') and with EGFP-LC2 (b-b''). Mts were visualized using a mouse monoclonal antibody (clone DM1A) specific against α -tubulin (a'-b') while EGFP and EGFP-LC2 were visualized by fluorescence microscopy (a''-b''). The EGFP protein alone was found in the cell nucleus but did not affect normal Mt distribution whereas, EGFP-LC2 was found colocalized with Mts (yellow in merged images (a'''-b''')) and conferred to the MTs a wavy pattern. Bar = 10 μ M.



transiently transfected HeLa cells with EGFP-LC2 colocalized with Mts were observed in this study. The EGFP tag alone showed a faint nuclear staining (**Figure 20a''**) but did not alter the Mt network (**Figure 20a''**).

EGFP-LC2 binds Mts *in vitro*.

To confirm the microscopic analysis of EGFP-LC2 associating with Mts, the *in vitro* binding assay was also used to analyse EGFP-LC2 (**Figure 21**). The EGFP tag, used as a negative control, was not found to associate with Mts. EGFP-LC2 was found to associate with Mts independently of the MAP1A HC. The presence of EGFP-LC2 was found mostly in the pellet; however, a faint band was also detected in the supernatant.

MAP1A heavy chain fragments colocalize with EGFP-LC2.

Although it is known that MAP1A HC fragments can associate with Mts independently of EGFP-LC2 and vice versa, normally in brain Mt protein preparations, the HCs are always associated with the LCs. Therefore, experiments were carried out in which HeLa cells were co-transfected with both the MAP1A HC fragments and EGFP-LC2. 6myc (**Figure 22a-a'''**) and 6mycN1a-1 (**Figure 22b-b'''**) were not found to associate with Mts after extraction fixation even in the presence of EGFP-LC2 and Mts still had a wavy appearance. When all the other MAP1A HC fragments (**Figure 22c-g'''**) were present with EGFP-LC2, they were found to colocalize, and the wavy Mt distribution was not present; however, some bundling did occur around the nucleus. Bundling around the nucleus was especially found with the smaller

MAP1A HC fragments such as 6mycN1a-2 (**Figure 22c-c'''**), 6mycN1a-3 (**Figure 22d-d'''**) and 6mycN1a-2 Δ BR (**Figure 22g-g'''**). No disturbance in the Mt network is seen in cells containing both EGFP-LC2 and 6mycN1a-4 (**Figure 22e-e'''**) or 6myc1a (**Figure 22f-f'''**).

To verify that in fact the 6myc tag and 6mycN1a-1 were being expressed in the cell, HeLa cells transiently co-transfected with these fragments and EGFP-LC2 were fixed by precipitation. As expected, both these fragments were found in the cytoplasm (**Figure 23**).

MAP1B is not detected in HeLa cells.

The presence of endogenous expression of MAP1B in HeLa cells was investigated by western blotting and was compared with the expression of MAP1B in adult bovine brain, used as a positive control. MAP1B was not detected in untransfected HeLa cells; therefore, investigation of exogenous expression of its light chain, LC1, is permitted without the interference of endogenous MAP1B heavy and light chains. As expected, MAP1B (**Figure 24***) was detected in adult bovine brain (reviewed by Muller et al., 1994). All lower molecular weight bands represent non-specific staining due to secondary antibody used during detection except for larger band under MAP1B, which represent degradation products.

LC3 is not detected in HeLa cells.

The presence of endogenous expression of LC3 in HeLa cells was investigated by western blotting and was compared with the expression of LC3 in

Figure 21: : In vitro MAP binding assay. Equal volumes of sample were taken from the supernatant (SN) and the pellet (P) and separated by SDS-PAGE on 12% gels. EGFP-LC2 and EGFP samples were immunodetected on western blots using a mouse monoclonal antibody (clone JL-8) specific for GFP.

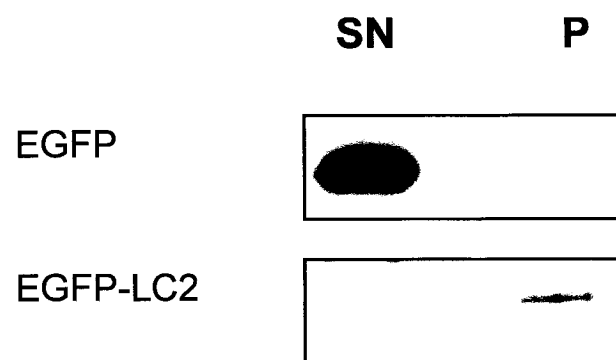


Figure 22: Double immunofluorescence labeling in HeLa cells co-transfected with EGFP-LC2 and 6myc (a-a'''), 6myc-N1a-1 (b-b'''), 6myc-N1a-2 (c-c'''), 6myc-N1a-3 (d-d'''), 6myc-N1a-4 (e-e'''), 6myc1a (f-f''') or 6myc-2 Δ BR (g-g'''). Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific against α -tubulin (a-g) whereas the MAP1A HC fragments were visualized using a mouse monoclonal antibody (clone 9E10) specific for 6myc (a''-g''). EGFP-LC2 was visualized by fluorescence microscopy (a'-g'). The 6myc tag and 6myc-N1a-1 were removed from the cell during extraction fixation, whereas the remaining MAP1A HC fragments colocalized with EGFP-LC2 (yellow in merged images) (a'''-g''') and returned to the Mts a normal distribution (a'''-g'''). Bar = 10 μ M.

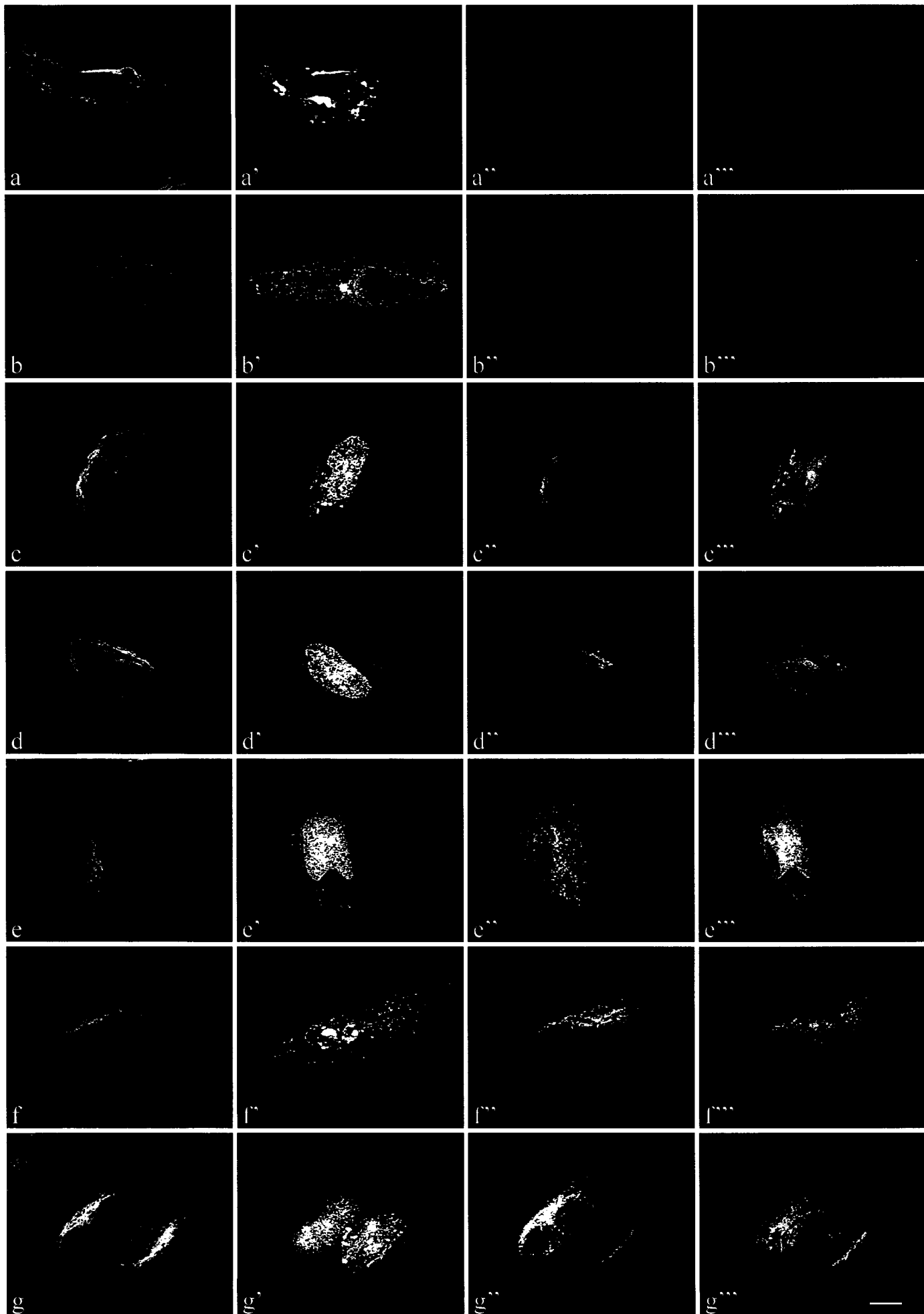


Figure 23: Double immunofluorescence labeling in HeLa cells co-transfected with EGFP-LC2 and 6myc (a-a'') or 6myc-N1a-1 (b-b''). Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific for α -tubulin (a-b) while the 6myc tag and 6myc-N1a-1 were visualized using a mouse monoclonal antibody (clone 9E10) against 6myc (a''-b''). EGFP-LC2 was visualized by fluorescence microscopy (a'-b'). The 6myc tag 6myc-N1a-1 were found in the cytoplasm of the cell when fixed by precipitation. Bar = 10 μ M.

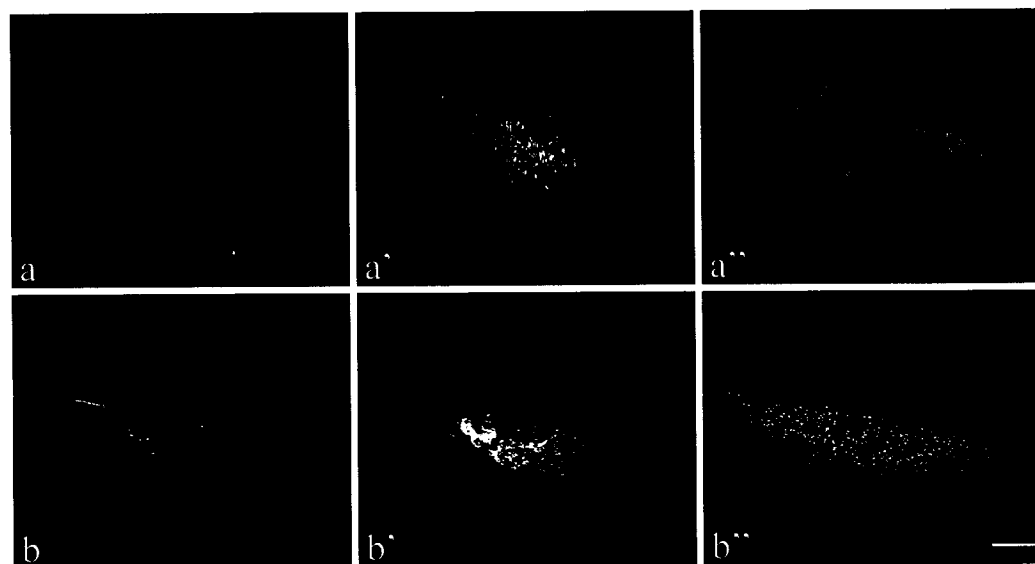


Figure 24: Detection of MAP1B in S1, bovine brain extract, and in untransfected HeLa cell extract. MAP1B is not detected in HeLa cells, but is present in S1. 10 µg of whole cell extract and S1 were separated by SDS-PAGE on a 7.5% gel. MAP1B was immunodetected on western blots using a mouse monoclonal antibody (clone AA6) specific for MAP1B. Molecular weight markers are in kDa.

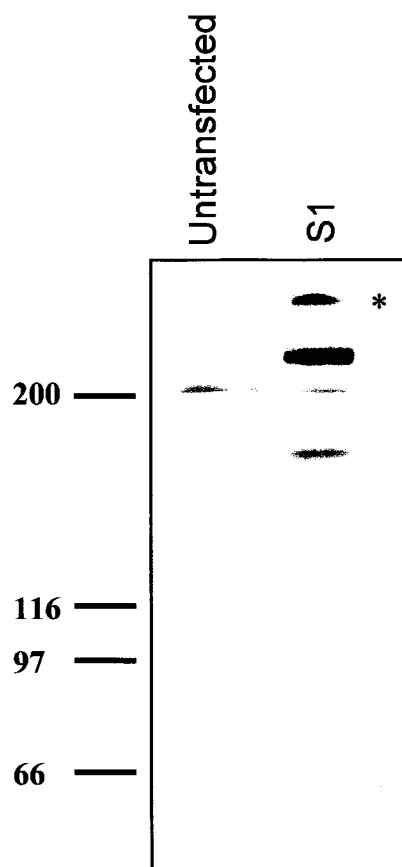
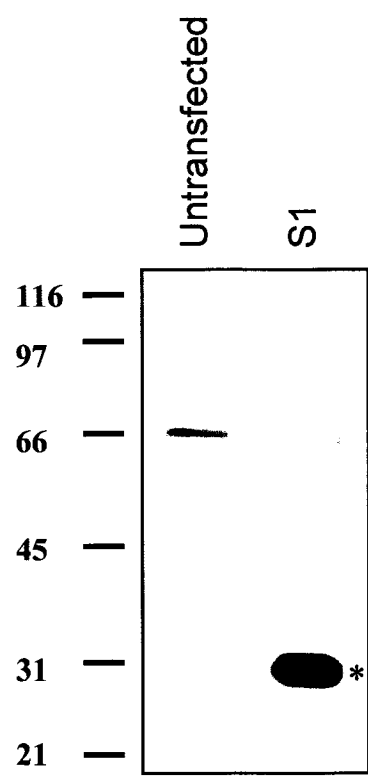


Figure 25: Detection of LC3 in S1, bovine brain extract, and in untransfected HeLa cell extract. LC3 is not detected in HeLa cells, but is present in S1. 10 µg of whole cell extract and S1 were separated by SDS-PAGE on a 7.5% gel. LC3 was immunodetected on western blots using a rabbit polyclonal antibody (clone LC-3) specific for LC3. Molecular weight markers are in kDa.



adult bovine brain, used as a positive control. LC3 was not detected in untransfected HeLa cells; therefore, investigation of exogenous expression of this light chain is permitted without the interference of endogenous LC3. As expected, LC3 (**Figure 25***) was detected in adult bovine brain (Kuznetsov and Gelfand, 1987). All other bands represent non-specific staining due to the secondary antibody used during detection.

MAP1A LCs are expressed in transiently transfected HeLa cells.

To ensure that the MAP1A LCs were being expressed correctly in HeLa cells, their expression was analysed by western blotting using an antibody against 6myc. All LCs (**Figure 26***) were detected within 10 kDa of their predicted molecular weight. All other bands represent non-specific binding due to the secondary antibody used during detection.

MAP1A LCs colocalize with Mts *in vivo*.

To verify which MAP1A LCs colocalized with Mts, transiently transfected HeLa cells were observed by immunofluorescence microscopy (**Figure 27**). Cells were fixed by extraction fixation to observe which LCs bound to Mts. All three LCs were found colocalized with Mts. Expression of all three LCs was low since they all have a spotty distribution within the cells as opposed to a continual distribution along the Mts. 6mycLC1 (**Figure 27a-a'''**) and 6mycLC2 (**Figure 27b-b'''**) conferred to the Mts a wavy pattern as seen with EGFP-LC2; however, 6mycLC3 did not appear to alter the Mt network.

Figure 26: Detection of 6myc tagged LCs expressed in HeLa cells. 10 µg of whole cell extracts of cells transfected with 6mycLC1, 6mycLC2 and 6mycLC3 were separated by SDS-PAGE on a 12% gel. The 6myc tagged LCs were immunodetected on western blots using a mouse monoclonal antibody (clone 9E10) specific for 6myc. Molecular weight markers are in kDa.

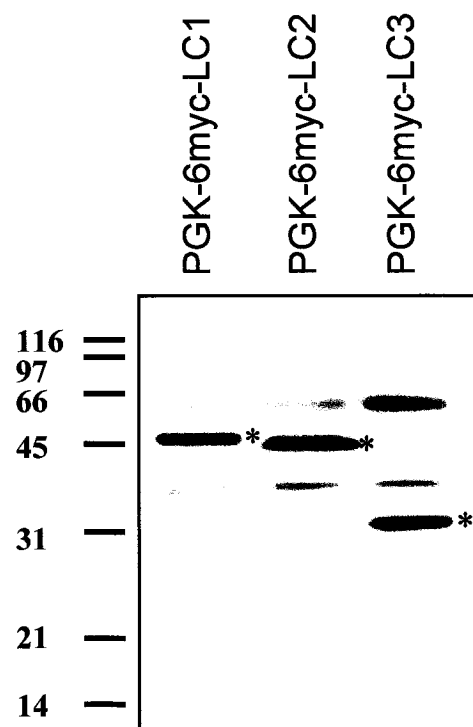
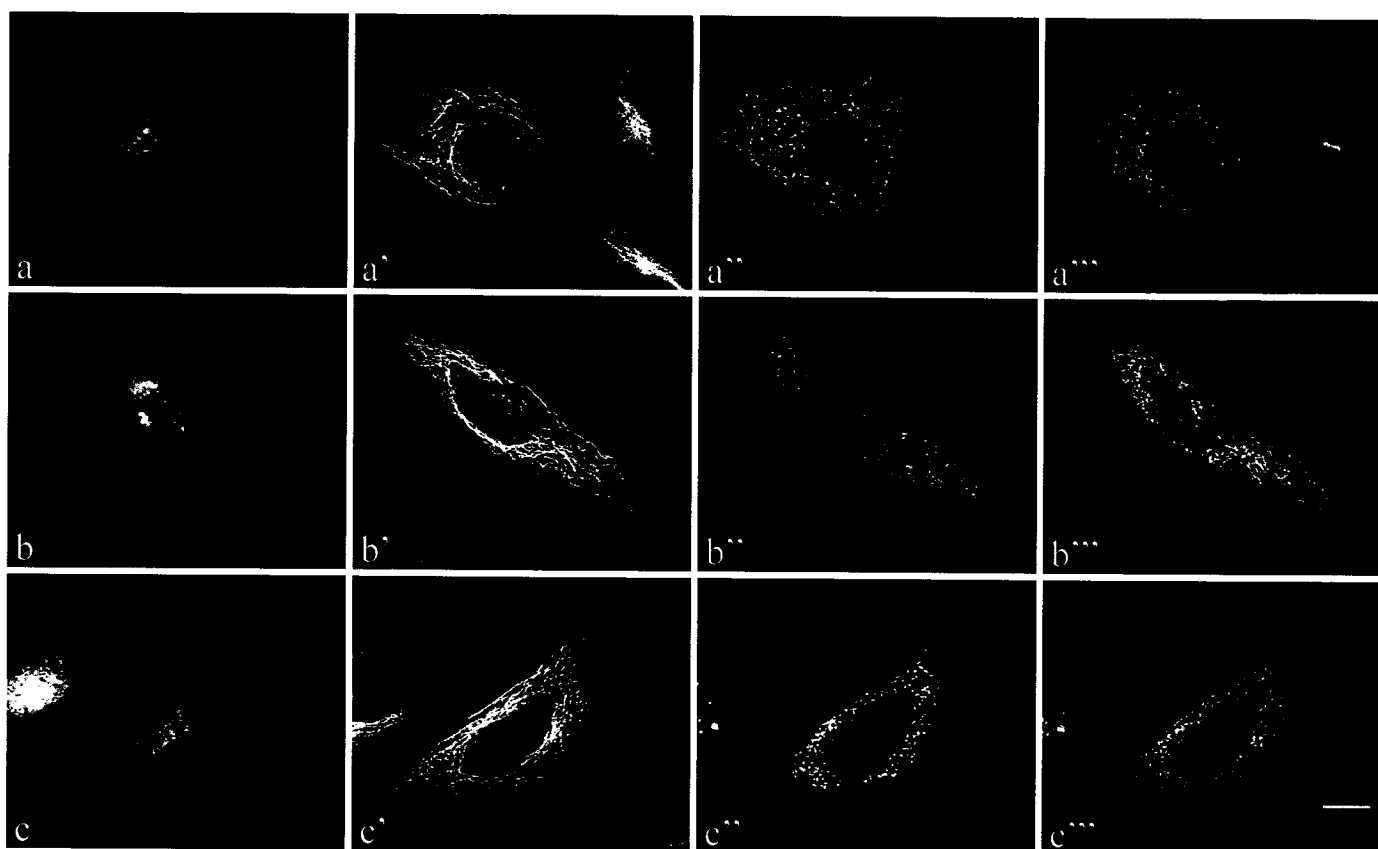


Figure 27: Hoescht staining of DNA (a-c) and double immunofluorescence labeling in HeLa cells transfected with 6mycLC1 (a-a'''), 6mycLC2 (b-b''') and 6mycLC3(c-c'''). Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific against α -tubulin (a'-c') whereas 6myc tagged LCs were visualized using a mouse monoclonal antibody (clone 9E10) specific for 6myc (a''-c''). All three LCs were found colocalized with Mts (yellow in merged images) (a'''-c'''). 6mycLC1 and 6mycLC2 conferred to the MTs a wavy pattern. Bar = 10 μ M.



MAP1A LCs bind to Mts *in vitro*.

To confirm the microscopic analysis of the 6myc tagged MAP1A LCs associating with Mts, the *in vitro* binding assay was used. All three MAP1A LCs were found to bind to Mts (**Figure 28**). 6mycLC2 was found in both the supernatant and the pellet as seen with EGFP-LC2.

6mycLC1 and 6mycLC2 stabilize Mts against the effects of nocodazole.

To determine if the binding of the MAP1A LCs had any stabilizing effects, MAP1A LC transfected cells were treated with the Mt depolymerising drug, nocodazole (**Figure 29**). In untransfected cells (**Figure 29a-a'''**), nocodazole caused the depolymerization of most Mts (**Figure 29a'**). By comparison, in cells expressing 6mycLC1 (**Figure 29b-b''**) and 6mycLC2 (**Figure 29c-c''**), Mts were largely resistant to nocodazole. No resistance to nocodazole was seen in cells expressing 6mycLC3 (**Figure 29d-d''**).

MAP1A LCs colocalize with a subset of Mts that contain detyrosinated tubulin.

To further investigate the stabilizing effects of 6mycLC1 and 6mycLC2, the presence of detyrosinated Mts was detected by fluorescence microscopy (**Figure 30**). Detyrosination represents a post-translational modification of tubulin that has been shown to be a biochemical marker of stable Mts with decreased dynamics (reviewed by Laferriere et al., 1997). In untransfected cells (**Figure 30a-a'''**), the detyrosinated Mt population was very faint. In cells transfected with 6mycLC1 (**Figure 30b-b'''**) and 6mycLC2 (**Figure 30c-c'''**), the detyrosinated

Figure 28: : In vitro MAP binding assay. Equal volumes of sample were taken from the supernatant (SN) and the pellet (P) and separated by SDS-PAGE on 12% gels. 6myc tagged LCs were immunodetected on western blots using a mouse monoclonal antibody (clone 9E10) specific for 6myc.

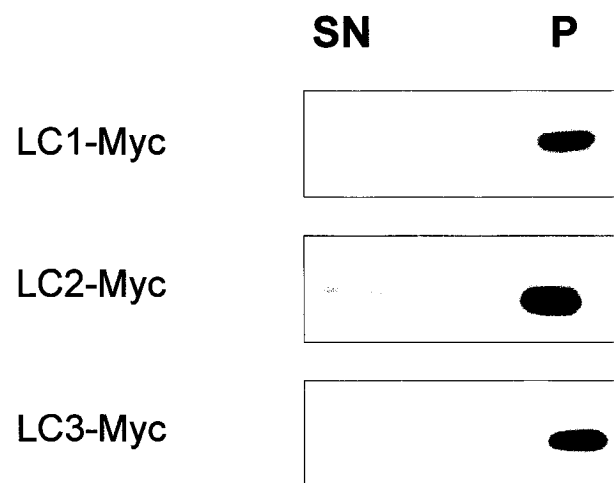


Figure 29: Hoescht staining of DNA (a-d) and double immunofluorescence labeling in untransfected (a-a'') HeLa cells and cells transfected with 6mycLC1 (b-b''), 6mycLC2 (c-c'') and 6mycLC3 (d-d'') and treated with nocodazole. Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific against α -tubulin (a'-d') whereas 6myc tagged LCs were visualized using a mouse monoclonal antibody (clone 9E10) specific for 6myc (a''-d''). Only 6mycLC1 and 6mycLC2 transfected cells showed resistance to the Mt depolymerizing drug. Bar = 10 μ M.

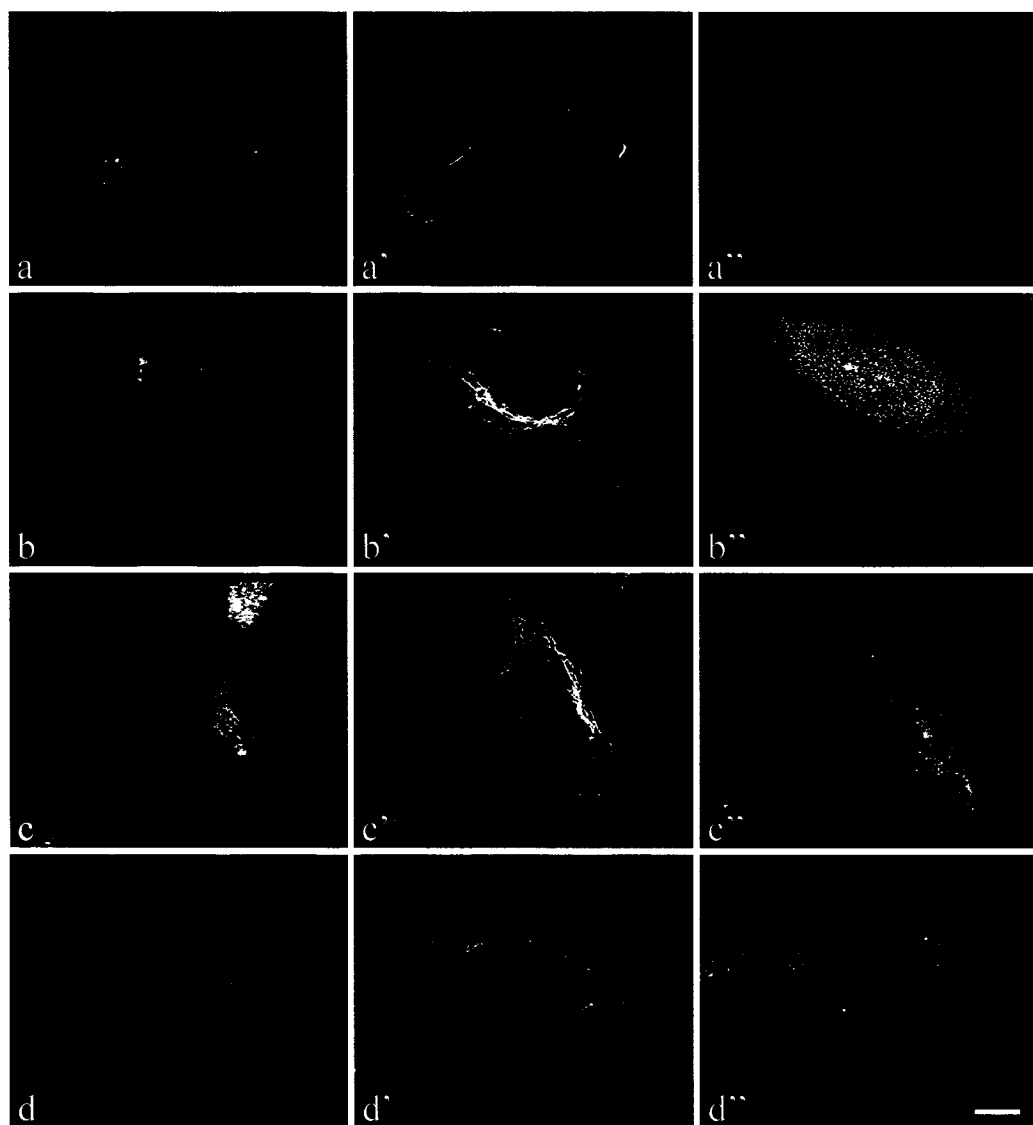
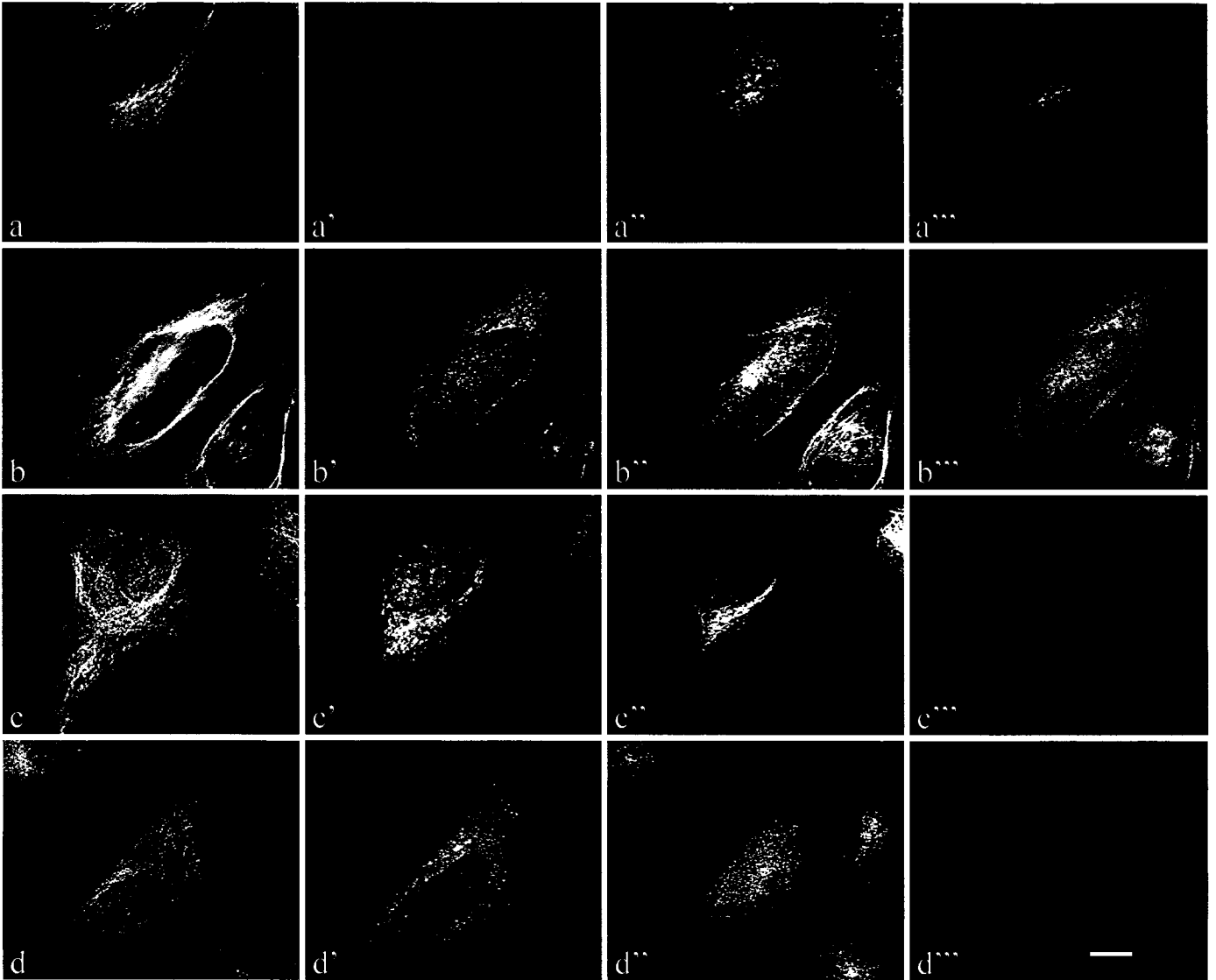


Figure 30: Triple immunofluorescence labeling in untransfected (a-a''') HeLa cells and cells transfected with 6mycLC1 (b-b'''), 6mycLC2 (c-c''') and 6mycLC3 (d-d'''). Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific against α -tubulin (a-d), 6myc tagged LCs were visualized using a mouse monoclonal antibody (clone 9E10) specific for 6myc (a'-d') whereas detyrosinated tubulin was visualized using a rabbit polyclonal antibody (clone anti-E) specific for detyrosinated α -tubulin (a''-d''). Detyrosinated α -tubulin colocalized with LCs (yellow in merged images) (a'''-d'''). Bar = 10 μ M.



Mts were much more abundant and colocalized with the wavy Mts. Surprisingly, there was also a large population of deetyrosinated Mts in 6mycLC3 (**Figure 30d-d'''**) transfected cells.

MAP1A LCs do not alter the distribution of actin.

Since it had been shown previously that LC1 was capable of interacting with actin filaments, actin distribution was observed by fluorescence microscopy in cells transfected with the MAP1A LCs. In untransfected cells (**Figure 31a-a''**), actin was found as stress fibers and networks (**Figure 31a''**). This actin distribution remained unchanged even in the presence of 6mycLC1 (**Figure 31b-b''**), 6mycLC2 (**Figure 31c-c''**) and 6mycLC3 (**Figure 31d-d''**) which did not even colocalize with the microfilaments.

EGFP-LC2 colocalizes with 6mycLC1 and 6mycLC2.

To determine if the different LCs colocalize, HeLa cells were co-transfected with EGFP-LC2 and one of the 6myc tagged LCs. Cells were fixed by extraction fixation. EGFP-LC2 colocalized with 6mycLC1 (**Figure 32a-a'''**) and 6mycLC2 (**Figure 32b-b'''**). When both LCs were expressed in the cell, the Mts had a definite wavy pattern. When EGFP-LC2 was present in the cells, 6mycLC3 (**Figure 32c-c'''**) did not remain bound to Mts, but was extracted from the cell.

Figure 31: Hoescht staining of DNA (a-d) and double fluorescence labeling in untransfected (a-a'') HeLa cells and cells transfected with 6mycLC1 (b-b''), 6mycLC2 (c-c'') and 6mycLC3 (d-d''). 6myc tagged LCs were visualized using a mouse monoclonal antibody (clone 9E10) specific for 6myc (a'-d') whereas actin was visualized with rhodamine phalloidin (a''-d''). 6myc tagged LCs did not alter microfilament distribution. Bar = 10 μ M.

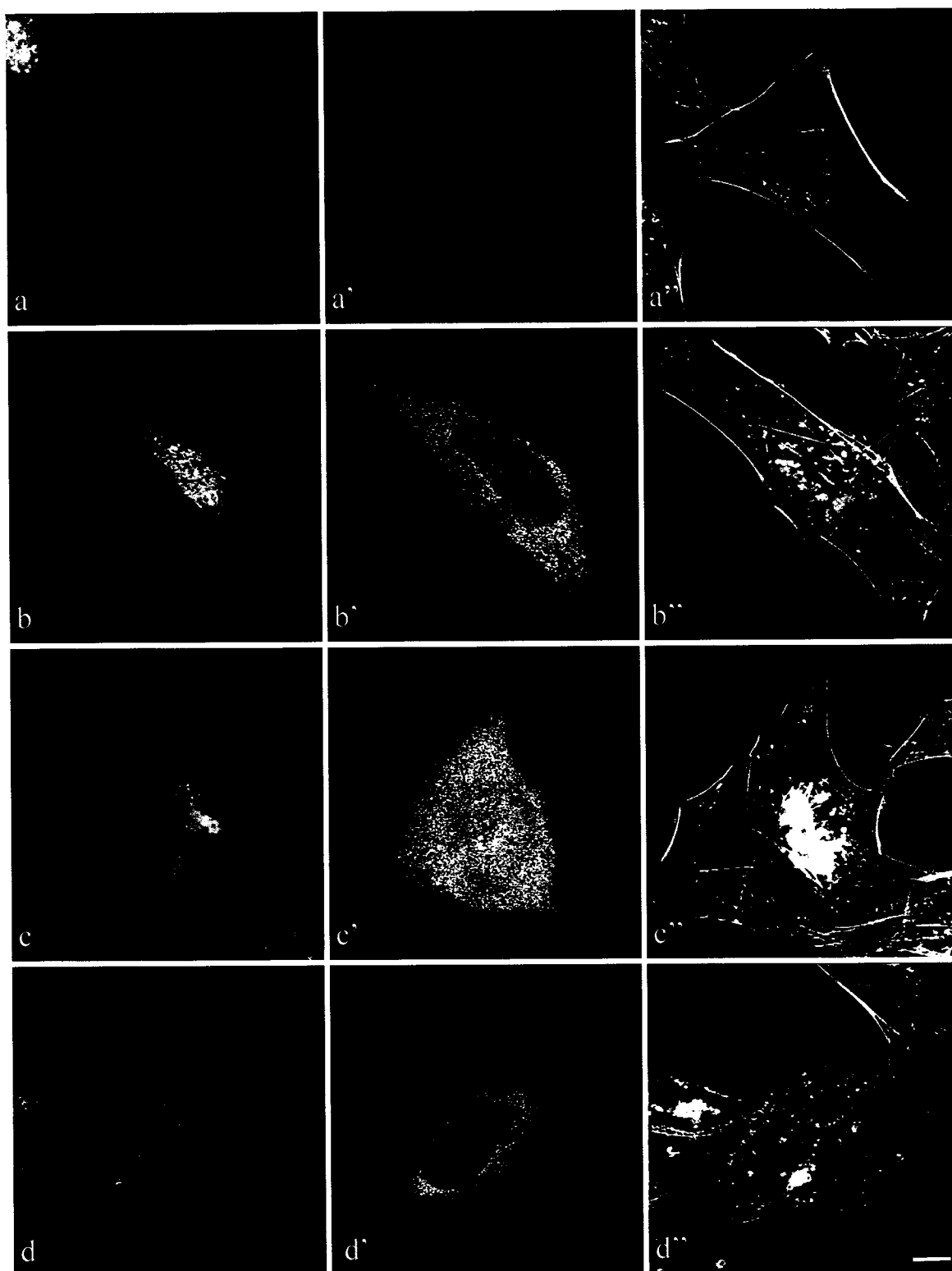


Figure 32: Double immunofluorescence labeling in HeLa cells co-transfected with EGFP-LC2 and 6mycLC1 (a-a'''), 6mycLC2 (b-b''') and 6mycLC3 (c-c'''). Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific against α -tubulin (a-c) whereas the LCs were visualized using a mouse monoclonal antibody (clone 9E10) specific for 6myc (a''-c''). EGFP-LC2 was visualized by fluorescence microscopy (a'-c'). 6mycLC3 was not found in the cell when EGFP-LC2 was present. 6mycLC1 and 6mycLC2 colocalized with EGFP-LC2 (yellow in merged images) (a'''-b''') and together they conferred to the Mts a wavy pattern. Bar = 10 μ M.

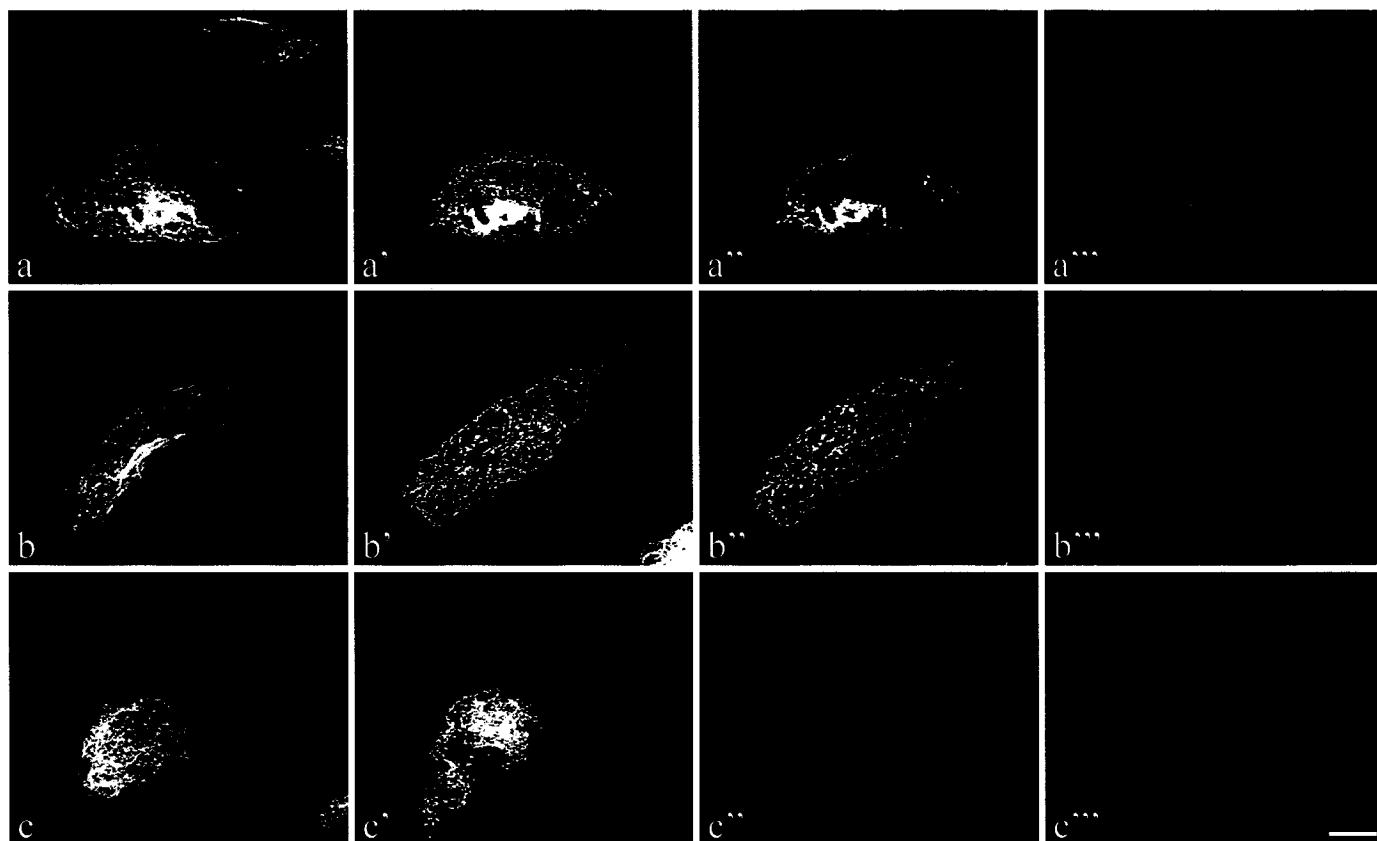


Figure 33: Double immunofluorescence labeling in HeLa cells co-transfected with EGFP-LC2 and 6mycLC3. Mts were visualized using a rat monoclonal antibody (clone YL1/2) specific against α -tubulin (a) whereas LC3 was visualized using a mouse monoclonal antibody (clone 9E10) specific for 6myc (a''). EGFP-LC2 was visualized by fluorescence microscopy (a'). 6mycLC3 was found in the cell simultaneously with EGFP-LC2 when cells were fixed by precipitation. Bar = 10 μ M.



To verify that 6mycLC3 was in fact expressed simultaneously with EGFP-LC2, cotransfected cells were fixed by precipitation (**Figure 33**). 6mycLC3 was then found in the cell cytoplasm (**Figure 33a''**) concurrently with EGFP-LC2 (**Figure 33a'**).

DISCUSSION

MAP1A HC binds to MTs *in vivo* and *in vitro* in the absence of the basic repeat region.

It was previously shown by Vaillant et al. (1998), that the MAP1A HC was capable of binding to Mts in the absence of LC2. It was further shown that fragments of the MAP1A HC which contained the basic repeats and the flanking regions (6mycN1a-2, 6mycN1a-3 and 6mycN1a-4) as well as fragments that contained the flanking regions but had the basic repeats deleted (6mycN1a-2 Δ BR) also bound to Mts. However, Vaillant et al. (1998) did not detect binding of the MAP1A fragment, 6mycN1a-1, (aa 1-281) either *in vivo* or *in vitro* even though this region contains part of the basic repeat flanking region.

In this present study, all fragments containing the basic repeat region and the flanking regions (6mycN1a-2, 6mycN1a-3, 6mycN1a-4 and 6myc1a), the basic repeat flanking regions (6myc-2 Δ BR) or part of the basic repeat flanking regions (6mycN1a-1) bound to Mts *in vivo* and *in vitro*. 6mycN1a-1 which showed binding to Mts *in vitro*, only showed *in vivo* binding to taxol stabilized Mts. 6mycN1a-1 was not detected along unbundled Mts because it is a low expressing protein which is characterized by its spotty distribution along the taxol stabilized Mts. Vaillant et al., (1998) probably did not detect binding of 6mycN1a-1 to Mts due to the low expression levels that occurred with their transient transfection procedure.

In vivo and *in vitro* binding of the MAP1A HC fragments to Mts is unlikely due to the 6myc tag since 6myc was never found colocalized along Mts *in vivo* and it was not detected to bind Mts *in vitro* either.

These results correlate with those found for MAP1B, which showed that the MAP1B binding domain consists of its basic repeat region or the basic repeat flanking regions (Noble et al., 1989). Only studies performed by Cravchik et al. (1994) contradicted our results where they found that the Mt binding domain for MAP1A was located in an acidic region near the middle of the protein (aas 1307-1606) (**Figure 4**). Also, Cravchik et al. (1994) demonstrated that their MAP1A Mt binding domain caused nocodazole resistant perinuclear Mts which was not observed by Vaillant et al. (1998) or in the present study. In fact the MAP1A HCs do not appear to alter the Mt network.

It has been suggested (Trinczek et al., 1995) that the basic repeat domains of tau and MAP2 also contribute to Mt binding albeit weakly (Ennulat et al., 1989 and Joly et al., 1989), but that strong binding requires the addition of the flanking regions (Kanai et al., 1992 and Lee and Rook, 1992). Different groups (Gustke et al., 1994 and Trinczek et al., 1995) have concluded that the flanking regions were important for placing tau on the Mt surface while the basic repeats acted as binding regions. This could be occurring in MAP1A and MAP1B since both their basic repeats and their flanking regions associate with Mts.

MAP1A LC2 binds to Mts *in vivo* and *in vitro* and alters the Mt distribution.

We know that the MAP1A HC can bind Mts both *in vivo* and *in vitro* and that this interaction does not require the presence of LC2. Hosszu (2001) constructed a LC2 plasmid tagged with EGFP. When this plasmid was transiently transfected into HeLa cells, it was found colocalized along Mts and it induced a bundled wavy pattern, especially in the perinuclear region, in a certain population of transfected cells. These results were confirmed by the present study. Also, *in vitro* binding of EGFP-LC2 was observed; therefore, LC2 has its own binding site along the Mts. *In vivo* and *in vitro* binding of LC2 to Mts is unlikely due to the EGFP tag since EGFP was never found colocalized along Mts *in vivo* and it was not detected to bind Mts *in vitro* either.

These results are similar to those previously obtained for LC1, which was also found to bind Mts both *in vivo* and *in vitro* (Zauner et al., 1992, Tögel et al., 1998 and Noiges et al., 2002). This is not surprising since LC1 and LC2 have 55% sequence homology (Langkopf et al., 1992 and Fink et al., 1996). The Mt wavy pattern seen induced by LC2 was also seen in cells expressing LC1 (Tögel et al., 1998). Since this wavy pattern is not seen in the presence of the MAP1A HC (present study, Vaillant et al., 1998) or the MAP1B HC (Tögel et al., 1998); therefore, it seems to be a characteristic of LC2 and LC1.

The Mt reorganization by LC2 might be because LC2 forms dimers. Tögel et al. (1998) showed by immunoprecipitation that LC1 molecules could dimerize.

These results are consistent with subunit stoichiometries demonstrating that the MAP1 HCs can bind more than one copy of each LC (Schoenfeld et al., 1989, Pedrotti and Islam, 1994 and Pedrotti et al., 1996). Since LC2 is structurally very similar to LC1, it is possible that LC2 also dimerizes. LC2 molecules bound to Mts could dimerize with LC2 molecules bound on neighbouring Mts. These Mts would then be brought closer together, generating the wavy bundled Mt appearance. Further studies are necessary to test whether LC2 is capable of dimerization.

Mt-MAP1A-LC2 interaction

It is shown in the present study that the MAP1A HC and LC2 can bind Mts independently, but in an adult brain they normally associate together. In the presence of the MAP1A HC fragments, LC2 does not appear to alter the Mt network, especially for the larger fragments (6mycN1a-4 and 6myc1a). However, some Mt bundling around the nucleus was seen with the smaller fragments (6mycN1a-2, 6mycN1a-3 and 6mycN1a-2 Δ BR). This suggests that the MAP1A projection domain has a regulatory effect on LC2. Perhaps the MAP1A projection domain interferes with LC2 dimerization between different Mts or along the same Mt. Similar results were obtained in cells co-expressing LC1 and full length MAP1B HC in which neither LC1 nor the Mts presented the wavy pattern (Tögel et al., 1998).

Both *in vivo* and *in vitro* studies have demonstrated that the LC1 binding domain of MAP1B is located in the first 508 aas of the amino terminus of MAP1B

HC (Tögel et al., 1998). MAP1A and MAP1B exhibit 63% similarity at their amino terminus (Langkopf et al., 1992), suggesting that the LC2 binding domain of MAP1A is located in this area. Since in this study, 6mycN1a-1 did not associate with Mts through LC2, the LC2 binding region to the MAP1A HC is not located in the first 281 aas.

MAP1A LCs bind to Mts *in vivo* and *in vitro*.

To compare with previous results found with LC1 and LC2 and to examine the binding of LC3, experiments with 6myc tagged LCs were performed. All three LCs were found to bind both *in vivo* and *in vitro* with Mts. It is thought that their expression was low since their distribution along the Mts was punctate. It has been suggested that this low expression is caused by the PGK promoter, which only drives low levels of LC expression (Vaillant, 1997 and Hosszu, 2001).

Binding of the 6myc tagged LCs was similar to that found for EGFP-LC2, which also bound to Mts both *in vivo* and *in vitro* (Hosszu, 2001 and the present study). These results also correlate with those obtained for LC1 which was found associated with Mts both *in vivo* and *in vitro* (Zauner et al., 1992, Tögel et al., 1998 and Noiges et al., 2002).

Even though the LCs were expressed at low levels, the Mt distribution was still altered in the presence of LC1 and LC2. The same wavy Mts were seen as with EGFP-LC2. Interestingly, no Mt disturbance appeared in the presence of LC3. If the MAP1A and MAP1B HCs have a regulatory role on LC2 and LC1,

respectively, then LC3, which has no corresponding HC probably has a different role in the neuronal cytoskeleton.

LC1 and LC2 have Mt stabilizing activity.

Previous studies have shown that MAP1A (Pedrotti et al., 1993 and Pedrotti and Islam, 1994) stabilizes Mts *in vitro*. Also, Bonnet et al. (2001) showed by an *in vitro* tubulin-binding assay that binding affinity of MAP1A to tubulin increases with an increase in polyglutamylation of tubulin, which occurs during neuronal maturation. This is consistent with the hypothesis that MAP1A, which is at its highest levels in mature brain, plays a role in stabilizing neuronal Mts (reviewed by Matus, 1988, Müller, 1994 and Hammarback, 1997). Since the whole MAP1A protein complex was used in these studies, it is unclear if the MAP1A HC or the LCs had stabilizing effects.

Vaillant et al. (1998) demonstrated that simultaneous expression of both the MAP1A HC and LC2 (6myc1a) did not alter the Mt network in transfected cells, nor did they protect against the effects of colchicine and taxol. The same results were obtained for the MAP1A HC fragments not expressing LC2 (6mycN1a-2, 6mycN1a-3, 6mycN1a-4 and 6mycN1a-2 Δ BR). However, in the same study, biochemical experiments on the effect of MAP1A/LC2 protein complex on α -tubulin modifications showed that this complex caused an increase in acetylation and detyrosination relative to control cells, in contrast to the MAP1A HC fragments which showed no increase in modified α -tubulin. This

suggests that LC2 is responsible for the increase in post-translational α -tubulin modifications, which are usually associated with stable Mts.

This theory is supported by results obtained by Faller (2003), which showed that Mts in the presence of almost full length MAP1A without LC2 (6myc-N1a-4) showed an increase in growth and Mts were more dynamic. On the other hand, Mts in the presence of the MAP1A/LC2 complex were much less dynamic. This suggests again that LC2 plays the stabilizing role in the neuronal cytoskeleton.

This is consistent with the results found in the present study where in 6mycLC1 and 6mycLC2 transfected cells the Mts were more resistant to nocodazole than in untransfected cells. Also, consistent with the Mt stabilizing action of LC1 and LC2, the amount of deetyrosinated α -tubulin in LC1 and LC2 transfected cells was increased. Similar results were obtained by Tögel et al. (1998), who showed that Mts in LC1 transfected cells were resistant to nocodazole and taxol treatments. Also, Hosszu (2001) showed that Mts in EGFP-LC2 transfected cells were resistant to nocodazole and colchicine and that these cells had an increase in both acetylated and deetyrosinated α -tubulin.

It is interesting that the Mts in 6mycLC3 transfected cells showed no increase in resistance to nocodazole and the Mts in the transfected cells were not bundled. However, there was still an increase in the deetyrosinated α -tubulin population. This data can be correlated with those obtained by Faller (2003) which showed by live cell observations that LC3 caused a decrease in the growth

rate as well as a decrease in shrinkage rates of Mts. This indicates that LC3 causes Mts to be less dynamic, but not necessarily resistant to Mt depolymerizing drugs.

MAP1 LCs do not alter actin filaments.

Previous *in vitro* studies have shown that MAP1A and MAP1B had actin-binding and cross-linking activities (Fujii et al., 1993 and Pedrotti et al., 1994). However, again it was unclear whether this MAP1 binding activity to actin was due to the MAP1 HCs or the LCs. LC1 has been shown to possess an actin-binding site at the carboxy terminus and a Mt binding site at the amino terminus (Tögel et al., 1998). A recent study (Noiges et al., 2002) has shown that full-length LC1 and LC2 show only *in vitro* binding to actin filaments, but that a truncated form of LC1 and LC2 containing only the carboxy terminus can bind actin stress-fibres both *in vivo* and *in vitro*. This data correlates with our results since microfilament distribution was not altered by the presence of full-length LC1, LC2 or LC3.

It has been suggested that the differences between the *in vivo* and *in vitro* results regarding LC1 and LC2 binding to microfilaments could be due to post-translational modifications. Post-translational modifications, such as phosphorylation, of the LCs could inhibit or regulate LC binding to actin. It has been shown that binding of the MAP1B/LC1 complex to actin requires previous treatment with alkaline phosphatase (Pedrotti and Islam, 1996). A similar mechanism might be in place for MAP1A and LC2.

No work has been done to determine if LC3 may have actin binding activity *in vitro*. However, since LC3 has no obvious sequence similarity to LC1 and LC2 (Mann and Hammarback, 1994), its actin-binding site will not necessarily be located in its carboxy terminus. The observations of LC3 distribution in transfected cells in the present study showed colocalization with Mts and no indication of binding of LC3 to actin stress fibers.

LC/LC interactions

It is known that the MAP1 HCs and the MAP1 LCs bind to Mts independently and that the LCs associate with the HCs. Cells transfected with both EGFP-LC2 and either 6mycLC1, 6mycLC2 or 6mycLC3 demonstrated that LC1 and LC2 colocalize together in a cell. This is not entirely surprising since it has been suggested previously that LC1 can dimerize (Tögel et al., 1998). Therefore, perhaps LC1 can dimerize with LC2 and vice versa, especially since they have high sequence homology (Langkopf et al., 1992 and Fink et al., 1996).

However, it is interesting that in the presence of EGFP-LC2, 6mycLC3 no longer colocalized with Mts. This may suggest that LC2 and LC3 compete for the same binding sites along the Mt and that LC2 has a much higher affinity towards Mts than LC3. MAP1B/LC1 protein levels are highest in embryonic brain and decline steadily in post-natal brain, while MAP1A/LC2 protein levels increase steadily in post-natal brain and reach a plateau in adult brain (reviewed by Müller et al., 1994). As discussed earlier, LC2 stabilizes Mts against depolymerizing drugs suggesting that these Mts are more stable. Meanwhile, LC3 protein levels

are higher in post-natal brain than in embryonic or adult brain (Mann and Hammarback, 1996). As seen earlier, LC3 tends to make Mts less dynamic, but still not completely resistant to Mt depolymerizing drugs. Therefore, perhaps LC3 plays a role in developing neuronal processes. When the circuitry of the mature brain is established, perhaps LC2 replaces LC3 along the neuronal cytoskeleton.

Concluding remarks

Vaillant et al. (1998) demonstrated that the MAP1A HC can bind to Mts independently of LC2 and that its basic repeat flanking regions were important for Mt binding. The present study confirmed these results through *in vivo* and *in vitro* experiments and further demonstrated that only part of the MAP1A HC basic repeat flanking regions is necessary for Mt binding.

The present study also confirmed, through *in vivo* and *in vitro* experiments, previous results (Hosszu, 2001 and Noiges et al., 2002) that LC2 could bind Mts independently of the MAP1A HC. These results are similar to those obtained for LC1 (Tögel et al., 1998).

However, in neurons, the MAP1A HC and its associated LCs (LC1, LC2 and LC3) are found together and it is their interaction with the cytoskeleton that is important. The present study offered the first information regarding MAP1A HC-LC2 interactions. It demonstrated that the MAP1A HC probably regulated LC2 activity. In the future, biochemical investigations into the MAP1A HC-LC2 interaction would provide us with the location of the LC2 binding domain of

MAP1A. However, from the current study, we do know that the LC2 binding domain of MAP1A is not located in the first 280 aas of its amino terminus.

The present study also demonstrated for the first time that LC3 could bind to Mts both *in vivo* and *in vitro*. A different gene encodes LC3, its expression increases steadily through neuronal differentiation, and it may have different functions than LC1 and LC2. In order to better understand how MAP1A functions in neurons, it is important to understand the roles of the LCs. Therefore, future work would determine whether LC2 and LC3 are able to dimerize. Also, biochemical investigations would be necessary to characterize the association between the LCs and what effects such associations might have on MAP1A binding and on Mt dynamics. Finally, it would be important to determine how the MAP1A complex interacts with other cytoskeletal proteins and what roles such interactions play in neuronal functions.

Increasing MAP1A/LC2 and LC3 expression in developing neurons parallels an increase in Mt stability, suggesting they play a role in maintaining neuronal morphology and are involved in the neuronal processes that require stable Mts (reviewed by Müller et al., 1994). For example, in neurons, stable Mts are implicated in vesicle transport, synaptic transmission (Wooten et al., 1975, Knowles et al., 1996 and Carson et al., 1997) and synaptic memory (Kohrmann et al., 1999). On the other hand, MAP1A is also expressed in regions of the brain where neuronal growth still persists such as the optic (McKerracher et al., 1989) and the olfactory nerves (Schoenfeld et al., 1989) suggesting that by

rendering Mts moderately dynamic, MAP1A is associated with neurite outgrowth and plasticity.

To conclude, the present study has provided the following answers to the questions previously posed:

- 1) MAP1A HC fragments can associate with Mts even in the absence of the basic repeats.
- 2) LC1, LC2 and LC3 can bind to Mts even in the absence of the MAP1 HCs.
- 3) LC1 and LC2 confer to Mts a wavy appearance, which is regulated by the presence of the MAP1A HC and have Mt stabilizing activities.
- 4) The LC2 binding domain of MAP1A is not located in the first 280 aas of its amino terminus.
- 5) The MAP1 LCs do not interact with actin filaments.
- 6) LC2 and LC3 appear to compete for the same binding site on Mts.

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