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Heterotypic complex formation between subunits of microtubule-associated proteins 1A and 1B is due to interaction of conserved domains

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Abstract

The microtubule-associated proteins MAP1A and MAP1B are related but distinct multi-subunit protein complexes that consist of heavy and light chains. The predominant forms of these complexes are homotypic, i.e. they consist of a MAP1A heavy chain associated with MAP1A light chains or a MAP1B heavy chain associated with MAP1B light chains, respectively. In addition, MAP1A and MAP1B can exchange subunits and form heterotypic complexes consisting of a MAP1A heavy chain associated with MAP1B light chains which might play a role in a transition period of neuronal differentiation. Here we extend previous findings by confirming that heterotypic MAP1B heavy chain-MAP1A light chain complexes also exist in the developing murine brain. We show that these complexes form through interaction of homologous domains conserved in heavy and light chains of MAP1A and MAP1B. Likewise, conserved domains of the MAP1A and MAP1B light chains account for formation of light chain heterodimers. By yeast 2-hybrid analysis we located the light chain binding domain on the heavy chain to amino acids 211–508, thereby defining a new functional subdomain.
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Keywords: Microtubule-associated protein; Multimeric protein complex; Protein–protein interaction; Neuronal cytoskeleton; Brain development

1. Introduction

The high molecular mass microtubule-associated proteins 1A (MAP1A) and 1B (MAP1B) are expressed in mammalian brain in a reciprocal pattern, MAP1B being elevated during early stages of neuronal development in the brains of embryos and newborn mice and MAP1A reaching its peak in the adult brain, when neuronal differentiation is complete and MAP1B levels decrease [1,2]. MAP1B is essential for normal brain development [3–6] and plays a role in axon guidance and elongation [6–9]. Much less is known about the function of MAP1A which is thought to replace MAP1B in mature neurons.

MAP1A and MAP1B are multi-subunit protein complexes consisting of one heavy chain (HC) and at least one light chain (LC). In each case, heavy and light chains are generated by proteolytic cleavage of a polyprotein precursor [10,11], HC2 and LC2 from the MAP1A precursor and HC1 and LC1 from MAP1B. MAP1A and MAP1B share 3 domains of sequence homology [11] which we call MAP1 homology (MH) 1, 2, and 3 (Fig. 1A). These are located at the N- and C-termini of the heavy chains (MH1 and 2, respectively) and in the C-terminal half of the light chains (MH3). MH3 has 3 important functions: it can bind to F-actin [12,13] and might thus be responsible for the observed interaction of MAP1A and MAP1B with microfilaments [14–16], it mediates dimerisation of LC1 [12], and it contains the domain through which the light chain binds to the MH1 domain of the heavy chain [12,17]. The N-termini of LC1 and LC2 each contain a microtubule binding domain which is not homologous in sequence and has distinct effects on microtubule organization and stability [12,13]. These and other findings suggest that LC1 and LC2 are active subunits of the MAP1 complexes and determine the distinct functional properties of MAP1A and MAP1B [12,13,18]. The heavy chains might act as regulatory subunits [12].

The structural relationship of MAP1A and MAP1B in crucial functional domains, the similar organization of the multi-

Abbreviations: HRP, horse radish peroxidase; MAP, microtubule-associated protein; MH, MAP1 homology; HC, heavy chain; LC, light chain; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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subunit protein complexes, and the fact that during certain periods in brain development both proteins are expressed at equal levels [1,2] and even in the same cells [18] raise the possibility that not only homotypic complexes (HC1+LC1 or HC2+LC2), but also heterotypic complexes (HC1+LC2 or HC2+LC1) could be formed. Indeed, the existence of heterotypic complexes consisting of HC2 and LC1 has been reported [1,19]. Since LC1 and LC2 have distinct functional properties these heterotypic complexes might be of special importance during the transition from developing to mature neurons, but it is unclear how they are formed.

Here we report that the light chains of MAP1A and MAP1B, LC2 and LC1, can form heterodimers and that LC2, in addition to LC1, can interact directly with the heterologous heavy chain of MAP1B. We propose that each of these heterologous interactions can provide a mechanism for the formation of heterotypic MAP1 complexes. We also could narrow down the binding domain for LC1 and LC2 in the N-terminus of the heavy chain, thus defining a functional subdomain of MH1.

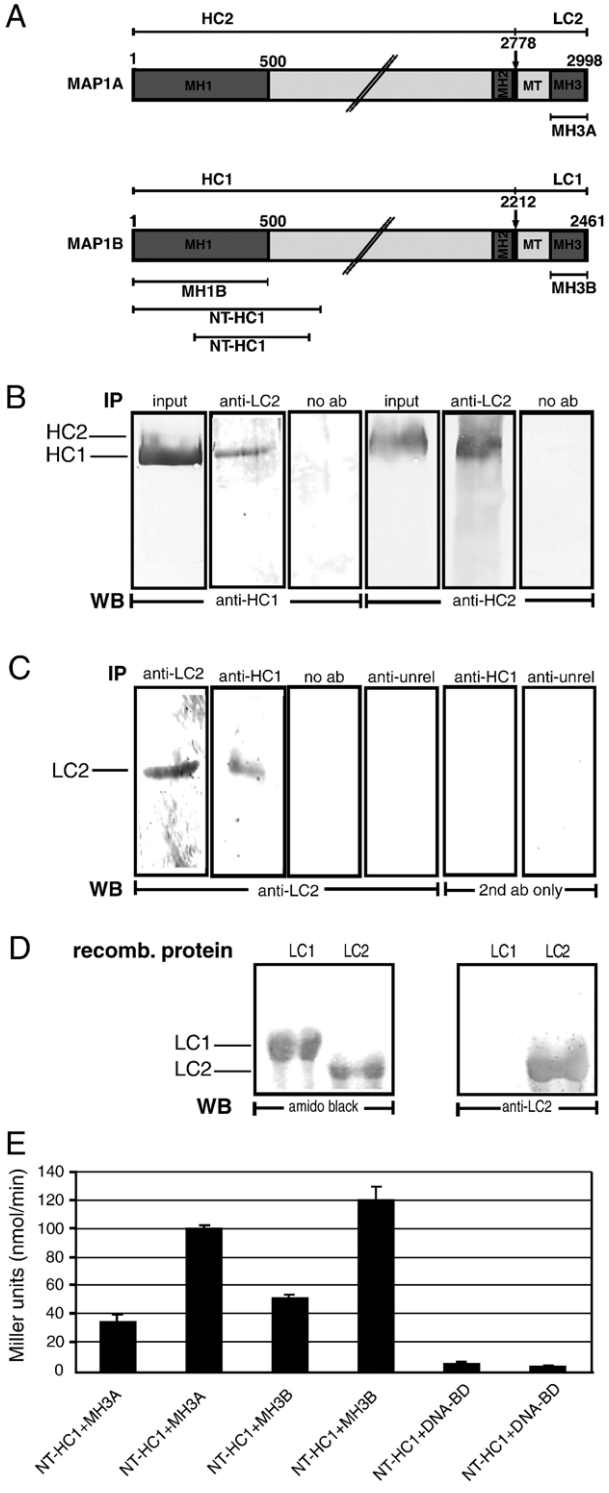

Fig. 1. The MH3 domain of LC2 can bind to the MH1 domain of MAP1B. (A) The MAP1A and MAP1B polyprotein precursors and the corresponding heavy and light chains (*HC2* and *LC2* for MAP1A; *HC1* and *LC1* for MAP1B) are indicated, as well as the sites of cleavage of the polyprotein precursors (arrows), the microtubule binding domains of the light chains (*MT*), and the domains of sequence homology (*MH1*, *MH2*, *MH3*, dark shading). Numbers indicate amino acid positions for rat MAP1A [11,24] and MAP1B (according to database entry XM_215469). Constructs (amino acids): *LC1* (2212–2461); *LC2* (2778–2998); *MH1B* (1–508); *NT-HC1* (1–731); Δ*NT-HC1* (211–692); *MH3B* (2337–2461); *MH3A* (2874–2998). (B) Whole brain protein lysates of 13-day-old mice (*input*) were immunoprecipitated (*IP*) with an antibody against the MAP1A light chain (*anti-LC2*) or without antibody (*no ab*) as negative control. Precipitates were fractionated on 4.5% gels, blotted and probed (*WB*) with antibodies against the MAP1B (*anti-HC1*) or MAP1A (*anti-HC2*) heavy chain. The position of the respective heavy chain on the blot is indicated. A fraction of MAP1B heavy chain present in the lysate can be coimmunoprecipitated with anti-LC2. (C) Whole brain protein lysates of 21-day-old mice were immunoprecipitated (*IP*), fractionated on 15% gels, and analysed by immunoblotting (*WB*) with the indicated antibodies. The position of LC2 on the blot is indicated. The MAP1A light chain was precipitated from brain lysates with anti-LC2. LC2 was also coimmunoprecipitated with anti-HC1. To rule out that the LC2 signal after precipitation with anti-HC1 was merely due to reaction of the secondary antibody with the light chain of the anti-HC1 antibody which is similar in size to LC2, controls with secondary antibody only (*2nd ab only*) were carried out. No signal was detected in these cases. The antibody against an unrelated protein (*anti-unrel*) is specific for the chicken FIP-2 protein [22]. (D) Approximately 1 μg of recombinant LC1 or LC2 were fractionated on a 15% gel, blotted and probed with amido black to reveal the presence of the 2 proteins on the blot or with anti-LC2 which exclusively reacted with LC2. (E) Interaction of MAP1A and MAP1B domains. Yeast cells expressing the indicated combinations of activation domain-fusion proteins of the MAP1B heavy chain (*NT-HC1* or Δ*NT-HC1*) and the MH3 domains of LC1 (*MH3B*) or LC2 (*MH3A*) fused to a DNA-binding domain were analyzed for β-galactosidase activity. Cells containing plasmids encoding the DNA-binding domain (*DNA-BD*) instead of the MH3/DNA-binding domain fusion proteins were used as negative controls. Bars represent the mean values of β-galactosidase activity determined in Miller units. Results were measured in triplicates in three independent experiments.

2. Materials and methods

2.1. Constructs

cDNA constructs for expression of the various MAP1A and MAP1B domains in PtK2 cells have been described previously [12,13]. For yeast 2-hybrid β-galactosidase assays based on the Matchmaker LexA system (Clontech) we inserted fragments containing MH3A and MH3B into the bait-fusion vector pLexA to yield constructs for the expression of the respective MH3 domain fused to the DNA binding domain of the 2-hybrid system. Briefly, the multiple cloning site of the bait-fusion vector pLexA (Clontech) was modified by inserting the double-stranded oligonucleotide 5′-AATTCCC-CATGGGGGCCC-3′ between the *Eco*R1 and *Xho*1 sites to yield pLexARN. This vector encodes the LexA DNA binding domain (DNA-BD) of the 2-hybrid system. A construct containing myc-tagged MH3B (Fig. 1A; [12]) was cut with *Hpa*1 and *Apa*1 to remove the C-terminal myc-tag and re-ligated using the

double-stranded oligonucleotide 5′-GTAGTCTAGAGGGCCA-3′. Subsequently, a fragment containing MH3B now flanked by *Nco*1 and *Apa*1 sites was excised and cloned into the *Nco*1 and *Apa*1 sites of pLexARN to yield a construct for the expression of the MH3B domain fused to the transcription factor DNA binding domain of the 2-hybrid system in yeast. The corresponding MH3A domain of MAP1A (Fig. 1A) was amplified by PCR using a construct containing LC2 [13] as template and primers 5′-GATCGAATTCCCCA-CAGCCTTGGGCTCCAAG-3′ and 5′-GATCCTCGAGCTAGAACT-CAATCTTGCAGG-3′, cut with *Eco*R1 and *Xho*1 and inserted into the *Eco*R1 and *Xho*1 sites of pLexARN to yield a construct for the expression of the MH3A domain fused to the transcription factor DNA binding domain of the 2-hybrid system in yeast. Constructs expressing NT-HC1 and ΔNT-HC1 fused to the activation domain of the transcription factor of the 2-hybrid system were obtained in a 2-hybrid screen performed using MH3B as bait and a cDNA library prepared from 19-day mouse embryos (Clontech) according to the manufacturer's recommendations. For expression of recombinant 6xHis-tagged proteins in *E. coli*, cDNA fragments were cloned into a derivative of pET15b (Novagen). LC2 was also cloned into pET32a (Novagen) to obtain the S-tagged variant. The correct sequence of all constructs was confirmed.

2.2. Immunofluorescence microscopy

Cell culture, DNA transfection and immunofluorescence microscopy of PtK2 cells were carried out as described [13]. Primary antibodies: affinity purified polyclonal rabbit anti-myc antibody [20] at a concentration of 1 μg/ml; monoclonal rat anti-HA antibody 3F10 (Boehringer Mannheim) at a dilution of 1:1000; monoclonal mouse anti-tubulin antibody B-5-1-2 (Sigma) at 1:500; a mixture of monoclonal mouse anti-β-actin antibodies AC-15 and AC-74 (Sigma) each at a dilution of 1:200. Secondary antibodies were Alexa Fluor 488 labeled anti-rabbit, Alexa Fluor 568 labeled anti-mouse and Alexa Fluor 350 labeled anti-rat secondary antibodies (Molecular Probes) used at dilutions of 1:500–1:1000.

2.3. β-galactosidase assay

β-galactosidase assays were based on the Matchmaker system (Clontech). Three colonies of each transformant were grown overnight at 30 °C in 2 ml dropout-induction medium (6.7 g/l yeast nitrogen base, 1× dropout supplements, 2% (w/v) galactose; 1% (w/v) raffinose, 26 mM Na_2HPO_4, 25 mM NaH_2PO_4). 4 ml of fresh induction medium were inoculated with 1 ml overnight culture, grown at 30 °C to an OD_{600} of 0.5 to 0.8. Exact OD_{600} was recorded, cells were collected by centrifugation, washed with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0), and resuspended in 100 μl of Z-buffer. 700 μl of Z-buffer with 0.27% (v/v) β-mercaptoethanol, one drop of 0.1% SDS and two drops of $CHCl_3$ were added, mixed thoroughly and equilibrated for 15 min at 30 °C. Enzyme reaction was started by addition of 160 μl freshly prepared ortho-nitrophenyl-β-D-galactopyranoside (4 mg/ml). Reactions were stopped by addition of 400 μl 1M $NaCO_3$ when samples turned yellow and time was recorded. Samples were centrifuged for 15 min at 14000 rpm and the OD_{420} was recorded. β-galactosidase activity was calculated in Miller units: $1U = 1000 \times OD_{420}/t \times V \times OD_{600}$).

2.4. Coimmunoprecipitation

Whole brains (500 mg wet weight) of C57BL/6 mice were homogenized in 1 ml of ice-cold TEN buffer (100 mM Tris/HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.1 mM DTT) containing a mixture of protease inhibitors (Roche) with a Potter–Elvehjem homogenizer with a motor driven Teflon pestle and incubated on ice for another 30 min. Immunoprecipitations were carried out on ice as described [12]. Finally, samples were eluted by boiling in 30 μl SDS sample buffer containing 5% 2-mercaptoethanol, fractionated on denaturing gels by SDS-PAGE, blotted, and analyzed as described [21]. Antibodies: polyclonal rabbit anti-LC2 serum [13] at a dilution of 1:100 in precipitation and 1:1000 in blot analysis; affinity purified polyclonal rabbit anti-HC1 antibody [6] at 1:25 in precipitation and 1:800 in blot analysis; monoclonal mouse anti-HC2 antibody HM-1 (Biogenesis) at 1:500; affinity purified polyclonal rabbit anti-FIP-2 antibody [22] at 1:25.

2.5. Blot overlay assay

For detection of protein interaction by blot overlay, recombinant proteins were expressed in *E. coli*, purified as described [13], fractionated by SDS-PAGE, and blotted onto nitrocellulose membranes (BA83, 0.2 μm pore size, Whatman, Schleicher and Schuell). Blots were stained with Ponceau Red to verify that equal amounts of recombinant proteins were loaded on the gel and present on the blot and subsequently cut into strips each containing only one protein. Strips were handled individually in the following procedures. Strips were blocked in buffer A (150 mM NaCl, 0.25% Tween 20 in PBS) containing 2% bovine serum albumin for 1 h, washed 3 times for 5 min in buffer A, incubated with or without 10 μg/ml LC2 with S-tag in buffer A containing 1% bovine serum albumin for 2 h, washed again, probed with INDIA HisProbe-HRP (Pierce; 1:7000) or horse radish peroxidase (HRP) conjugated S-protein (Novagen; 1:40000) for 1 h, and developed according to the manufacturer's recommendation.

3. Results

3.1. The light chain of MAP1A can bind to the heavy chain of MAP1B: heterologous interaction of conserved domains MH1 and MH3

Studies carried out on MAP1A and MAP1B revealed that the respective light chains can interact with N-terminal domains of the heavy chains [17,19]. We showed for MAP1B that the crucial domains for this interaction are MH1B and MH3B [12]. MH1 and MH3 are (by definition) conserved in MAP1A and heterologous complexes consisting of HC2 and LC1 have been detected in the brain [1,19]. Evidence for the existence of the complementary complex, HC1 + LC2 was limited due to the low level of expression of this complex [1]. To confirm that such complexes exist in the brain we performed coimmunoprecipitation assays using either anti-LC2 or anti-HC1 antibodies and brain protein lysates from 13- to 21-day-old mice. At this age MAP1B expression is still sufficiently high despite its decline from perinatal levels and MAP1A levels have already increased to about 50% of adult brain so that both proteins are expressed at easily detectable levels. At these stages we detected a small fraction of MAP1B heavy chain HC1 to be associated with LC2 (Fig. 1B and C), both when immunoprecipitations were carried out with anti-LC2 (Fig. 1B) or with anti-HC1 antibodies (Fig. 1C). A number of controls were performed to ensure specificity of these coimmunoprecipitations. Coimmunoprecipitating HC1 or LC2 were not detected in negative control precipitations carried out either without antibodies or with antibodies against an unrelated protein (Fig. 1B and C). To rule out that the 30-kDa LC2 signal in precipitates of anti-HC1 was merely due to reaction of the secondary antibody used in immunoblotting with the light chain of the anti-HC1 antibody used in the precipitation (which is similar in size to LC2), controls with secondary antibody only were carried out. No signal was detected in these cases (Fig. 1C). Moreover, the specificity of the anti-HC1 antibody was demonstrated by its lack of interaction with any proteins in extracts of MAP1B−/− mice [6] and the specificity of the anti-LC2 serum was shown by exclusive reaction with recombinant LC2 but not LC1 (Fig. 1D). These results confirm earlier indications [1] that heterotypic HC1 + LC2 complexes are expressed in the brain.

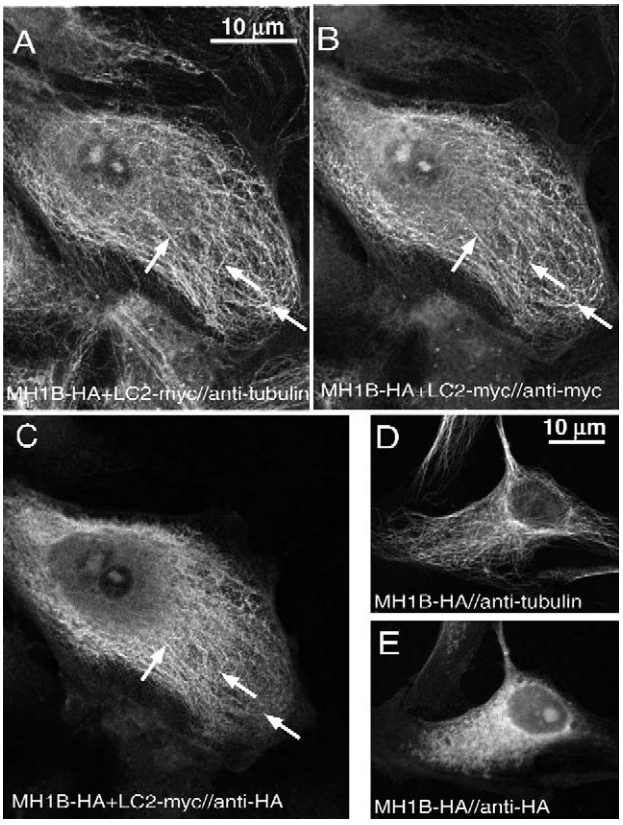

Fig. 2. LC2 can bind to the MH1 domain of MAP1B in vivo. (A–C), PtK2 cells co-expressing HA-tagged MH1B (*MH1B-HA*) and myc-tagged LC2 (*LC2-myc*) were analyzed by triple immunofluorescence microscopy using antibodies against tubulin (A, *anti-tubulin*) the myc-tag (B, *anti-myc*), and against the HA-tag (C, *anti-HA*). In the presence of LC2, MH1B was found to co-localize with LC2 on microtubules (arrows). (D and E), cells expressing MH1B only displayed a diffuse distribution of the protein throughout the cytoplasm (compare panels D and E).

We next tested the possibility that the formation of these heterologous complexes can be mediated by MH1B and MH3A using the yeast two-hybrid system. Plasmids encoding fusion proteins comprising MH1B fused to the activation domain of the transcription factor were co-transfected with constructs encoding the MH3 domains of LC1 or LC2, MH3B and MH3A, respectively, fused to the DNA-binding domain of the transcription factor into yeast cells harboring a *lacZ* reporter gene. Transformants were analyzed for reporter gene expression by measuring β-galactosidase activity. The MH3 domains of both LC1 and LC2 were efficient in driving reporter gene activity when expressed in the presence of a MAP1B heavy chain fragment (Fig. 1E). Our analysis further showed that amino acids 1–210 of the heavy chain are dispensable for homotypic and heterotypic HC/LC interaction, thus defining a functional subdomain of MH1. While we consistently observed higher reporter gene activity with ΔNT-HC lacking amino acids 1–210, indicating that this region could influence HC/LC interaction, we cannot rule out that increased reporter gene activity was due to slightly higher expression of the ΔNT-HC construct. The specificity of the assay was shown by analyzing transformants containing plasmids encoding the DNA-binding domain only (instead of the MH3/DNA-binding domain fusion proteins) which resulted in background levels of β-galactosidase activity. Auto-activation of MH1B and MH3 fragments was excluded (not shown).

To confirm binding of LC2 to the MH1 domain of MAP1B in vivo, we transiently expressed LC2 and MH1B in PtK2 cells and analyzed the intracellular localization of the two proteins. Non-neuronal PtK2 cells were chosen, because they contain only low levels of endogenous MAP1B protein [12], which cannot interfere with the proteins ectopically expressed. Myc-tagged LC2 and HA-tagged MH1B were found to co-localize with cellular microtubules (Fig. 2A–C). In contrast, cells expressing MH1B alone revealed a diffuse cytoplasmic distribution of the protein, demonstrating that this domain was not sufficient for microtubule binding (Fig. 2D and E). Thus, microtubule binding of MH1B in the presence of LC2 could be attributed exclusively to the microtubule binding activity of LC2 and demonstrated that LC2 can simultaneously bind to microtubules and MH1B in cultured cells. This is consistent with the fact that LC2 binds to microtubules with its N-terminal domain [13] and to MH1B with the C-terminal MH3A domain (Fig. 1E). Further confirmation of direct interaction of LC2 with MH1B was obtained in blot overlay assays using purified recombinant proteins (Fig. 3). A purified protein fragment comprising MH1B on the blot was specifically detected by the LC2 probe. Together, our results

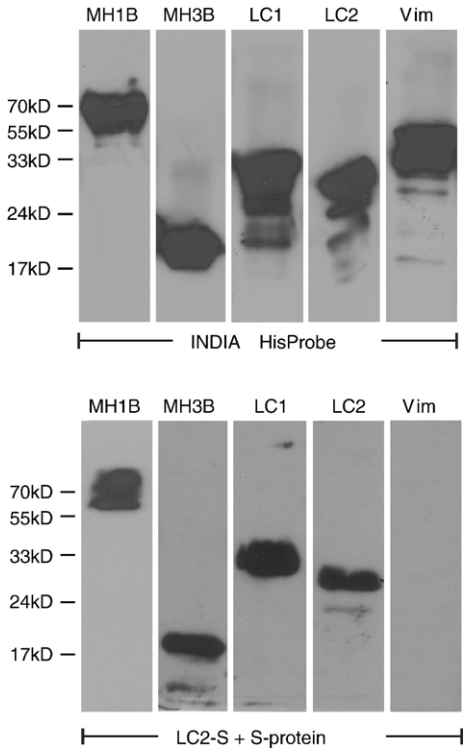

Fig. 3. Self-interaction of LC2 and interaction with conserved domains of MAP1B. 3–6 µg (depending on protein size, to achieve equal molarity) of the indicated recombinant proteins (*Vim*, vimentin as negative control) were purified and fractionated by SDS-PAGE. Replica blots were probed with INDIA HisProbe to verify protein content on the blot, with S-protein alone to demonstrate lack of interaction with His-tagged proteins (not shown), and finally with purified recombinant S-tagged LC2 (*LC2-S*) followed by S-protein as indicated. The latter revealed that LC2 can interact with MH1B, MH3B, LC1, and with itself, but not with vimentin.

demonstrate that LC2 can bind to HC1 due to interaction of the conserved MH1 and MH3 domains.

3.2. The light chains of MAP1A and MAP1B can form heterodimers through interaction of their conserved MH3 domains

We have previously demonstrated that the light chain of MAP1B can form homodimers or oligomers through interaction of the MH3 domains of individual LC1 molecules [12]. In addition, MAP1 complexes were found to consist of heavy and light chains at a ratio of 1:2 [1,23]. Thus, heterotypic MAP1 complexes such as HC2/LC1/LC2 which were detected in the brain [1] could be formed by binding of a light chain heterodimer consisting of LC1 and LC2 to a heavy chain molecule. We tested the potential direct interaction of LC2 with LC1 using recombinant proteins in blot overlay experiments (Fig. 3). In addition to binding to MH1B, the LC2 probe bound to MH3B, to LC1, and to LC2. His-tagged vimentin was used as negative control, to exclude unspecific interaction of the probe with the His-tag.

To confirm these results in vivo, we co-expressed HA-tagged MH3B and myc-tagged LC1 or LC2 in PtK2 cells. MH3B co-localized with either light chain on microtubules (Fig. 4, compare A–C and D–F). Since MH3B did not bind to microtubules on its own ([12] and Fig. 4G and H), its localization on microtubules must be mediated by binding to the respective light chain which in turn bound to microtubules through its N-terminal microtubule binding domain [13]. Association of MH3B with actin fibers in the absence of ectopically expressed light chain (Fig. 4G and H) has been observed previously [12], is due to a direct interaction with actin [13], and might be responsible for the documented actin binding activity of MAP1B [14–16]. Additional nuclear localization of MH3B (Fig. 4H) presumably is due to the small size of the protein, allowing it to diffuse through nuclear pores. Together, our results showed that LC1/LC2 heterodimers could form in vivo and in vitro through interaction of their conserved MH3 domains.

4. Discussion

For a better understanding of MAP1A and MAP1B function it is important to note that in addition to homotypic complexes consisting of HC2/LC2 and HC1/LC1, respectively, heterotypic complexes consisting of HC1/LC2 or HC2/LC1 can be found at certain times in development, when both proteins are co-expressed in the brain (Fig. 1B and C; [1,18,19]). Here we show, that heterotypic complexes can form through interactions between the MH1 and MH3 domains which are conserved between MAP1A and MAP1B.

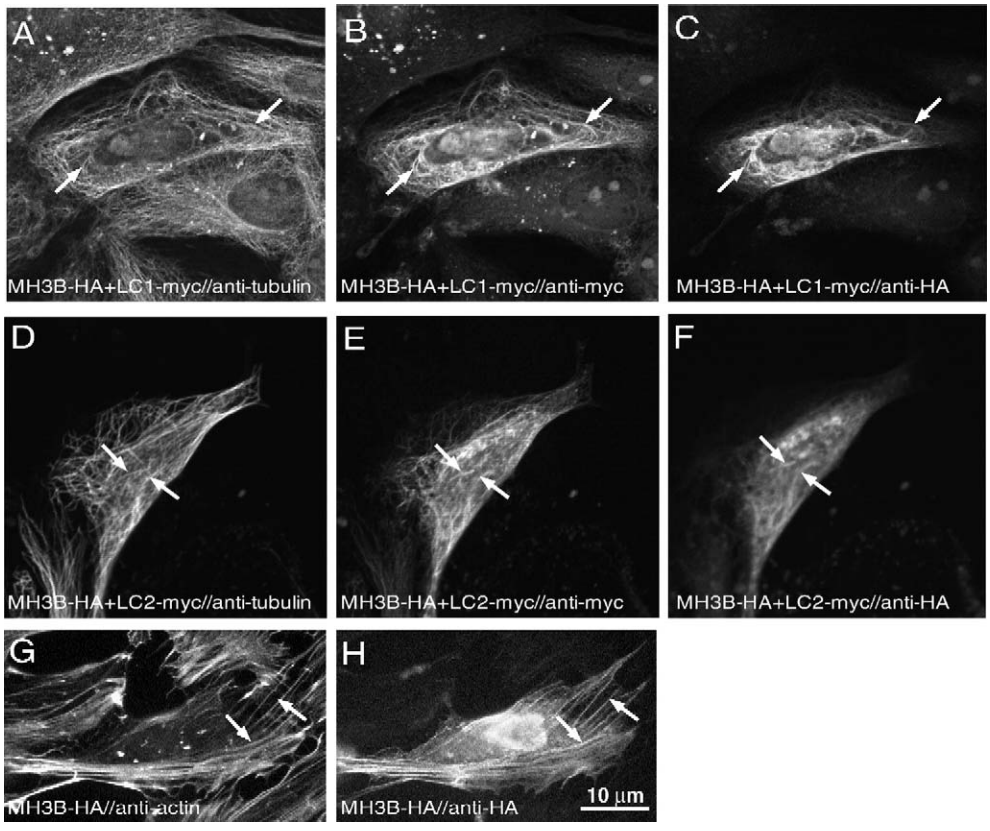

Fig. 4. LC2 can bind to the MH3 domain of MAP1B in vivo. (A–C), PtK2 cells co-expressing HA-tagged MH3B (*MH3B-HA*) and myc-tagged LC1 (*LC1-myc*) were analyzed by triple immunofluorescence microscopy using antibodies against tubulin (*anti-tubulin*) the myc-tag (*anti-myc*), and against the HA-tag (*anti-HA*). (D–F), same analysis carried out in PtK2 cells co-expressing HA-tagged MH3B (*MH3B-HA*) and myc-tagged LC2 (*LC2-myc*). In the presence of LC2 or LC1, the MH3 domain of MAP1B was found to co-localize with the respective light chain on microtubules (arrows). (G and H), cells expressing MH3B only showed binding of this domain to actin stress fibers as observed previously [12,13].

Using the MH3A domain of LC2 and various fragments of the MH1B domain of MAP1B we show not only that these conserved domains mediate heterotypic complex formation, but also that the light chain binding site on the heavy chain is contained in the C-terminal half of MH1 consisting of amino acids 211–508, thus defining a functional subdomain of MH1. Previous studies have shown that MAP1A as well as MAP1B complexes consist of one heavy and two light chains [1,23]. Two mechanisms are conceivable for the formation of such complexes: either two light chains bind individually to separate biding sites located in the conserved MH1 domains of the heavy chains or the light chains form homo- or heterodimers and bind as such to a single site on the heavy chain. For the first mechanism to be operating one might expect to find two binding sites of similar sequence in MH1 of which at least one should be located between amino acids 211–508, identified as light chain binding domain in the present study. However, no such repeated sequences were found in MH1. On the other hand, we have shown that light chains can form homodimers [12] and heterodimers (this study), consistent with the possibility that the second mechanism might underlie MAP1 complex formation.

We show here that one consequence of the conservation of MH1 and MH3 domains in MAP1A and MAP1B is the capacity of the two proteins to form heterotypic HC1/LC2 complexes and LC1/LC2 heterodimers in addition to the respective homotypic complexes and homodimers. This capacity to form homo — as well as heterotypic complexes might endow neurons with the possibility to fine tune the regulation of microtubules and actin filaments [12,13] during a critical transition period where neurons shift from axon extension to axon stabilization and synaptogenesis.

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