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Evidence against structural and functional identity of microtubule-associated protein 1B and proteoglycan claustrin

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Abstract Recently, the concept of microtubule-associated protein 1B as an intracellular 2460 amino acid protein was challenged by the suggestion that only the N-terminal 1022 codons are utilized and encode the core protein of the extracellular proteoglycan claustrin (Burg and Cole (1994) J. Neurobiol. 25, 1–22). We expressed this N-terminal MAP1B fragment in tissue culture cells and found that it bound to microtubules and was not localized in the extracellular matrix. In addition, epitope mapping demonstrated that MAP1B consisted of more than 1022 amino acids and that the reported cDNA of claustrin is incomplete.

Key words: Microtubule-associated protein 1B; Keratan sulfate proteoglycan; Claustrin; MAP5; Brain; Rat

1. Introduction

Microtubule-associated protein 1B (MAP1B) is a 280 to 300 kDa fibrous protein which is predominantly expressed in the developing nervous system [1,2]. The protein has originally been identified by its capacity to bind to and copurify with brain microtubules during repeated cycles of temperature dependent microtubule polymerization and depolymerization. Analysis of cDNA clones coding for human, rat and mouse MAP1B revealed a highly conserved sequence of about 2460 amino acids in all three species [3–6]. The mature protein consists of a heavy and a light chain which are generated by posttranslational proteolytic processing of the 2460 amino acid protein [7].

In marked contrast to these results, an independent study on the molecular characterization of claustrin, an extracellular chicken brain keratan sulfate proteoglycan (KSPG), has led to the suggestion that MAP1B is highly related if not identical to claustrin [8]. According to this report claustrin/MAP1B contains a core polypeptide of only 1038 amino acids comprising the N-terminus of the previously identified 2460 MAP1B protein. Claustrin does not bind to microtubules and is instead an extracellular KSPG [8]. These conclusions contradicted a wealth of previously published evidence that MAP1B is an intracellular microtubule-associated protein of 2460 amino acids [2] and were based mainly on three findings: (i) Screening of chicken brain cDNA libraries with antibodies to claustrin yielded clones with an open reading frame of 1038 codons highly homologous to the N-terminal part of previously cloned MAP1B cDNAs. (ii) Treatment of claustrin/MAP1B with keratanase reduced the apparent molecular weight to 117 kDa. (iii) A monoclonal antibody (mAb) raised against MAP1B, mAb MAP5 [9], reacted with purified claustrin on immunoblots.

We have constructed a rat claustrin homolog, an N-terminal fragment of rat MAP1B which corresponds in length and sequence to chicken claustrin. This fragment was expressed in rat kangaroo PtK2 cells, mouse NIH3T3 fibroblasts and rat glioma C_6 cells and the intra- and extracellular localization of this protein and its reactivity with the mAb MAP5 were analyzed. We found no evidence that this rat claustrin homolog is associated with the extracellular matrix. Instead, the protein bound to microtubules through its previously identified microtubule binding domain [3,4]. In addition, we found that the epitope for the MAP5 antibody is not present in this protein but instead in a region of full-length MAP1B located further towards the C-terminus. Our results demonstrate that (i) a protein fragment of MAP1B proposed to be homologous to the core protein of the extracellular KSPG claustrin can bind to microtubules in vivo, (ii) that MAP1B consists of more than the 1038 amino acids proposed by Burg and Cole [8] and (iii) that the reported cDNA of claustrin is incomplete. Thus, our results are in agreement with previous reports identifying MAP1B as a 2460 amino acid intracellular microtubule-associated protein [2] and provide evidence against the conclusions drawn by Burg and Cole that MAP1B is a 1038 amino acid extracellular KSPG [8].

2. Materials and methods

2.1. Constructs

The full-length rat MAP1B cDNA (MAP1B:1–2459) was obtained by joining a cDNA fragment isolated from a C_6 cell cDNA library containing exons 1–3 (isolated using a corresponding mouse probe generously provided by N. Cowan) at a unique *Aat*2 restriction site to the existing MAP1B cDNA [4]. A C-terminal in-frame myc-tag was attached and the sequence flanking the ATG initiator codon was modified to optimize expression using PCR based strategies. The correct sequence of segments derived by PCR was confirmed. MAP1B:1–508, MAP1B:1–1022 and MAP1B:1046–2459 (all with C-terminal in-frame myc-tag) were derived from MAP1B:1–2459 using convenient restriction sites and adaptor oligonucleotides. All constructs were cloned into the Tet-off expression vector pUHD10-3 [10].

2.2. Antibodies

An anti-myc peptide antiserum was raised against the synthetic peptide CTLAEQKLISEEDN (PiChem, Graz, Austria), affinity purified as described [11] and used at a concentration of 1μ g/ml for immunofluorescence microscopy and 5 μg/ml on immunoblots. An anti-myc mAb (clone 9E10 hybridoma supernatant), a guinea pig anti-myc antiserum (generously provided by Yuh Nung Jan), an α-tubulin mAb (Sigma, clone B-5-1-2), an affinity purified polyclonal rabbit anti-fibronectin antibody (Sigma), and the anti-MAP1B mAb MAP5 (clone AA6, Boehringer Mannheim) were used at dilutions of 1:2, 1:2000, 1:2000, 1:200, and 1:500, respectively. Secondary antibodies: FITC-conjugated anti-rabbit IgG antibody pre-adsorbed

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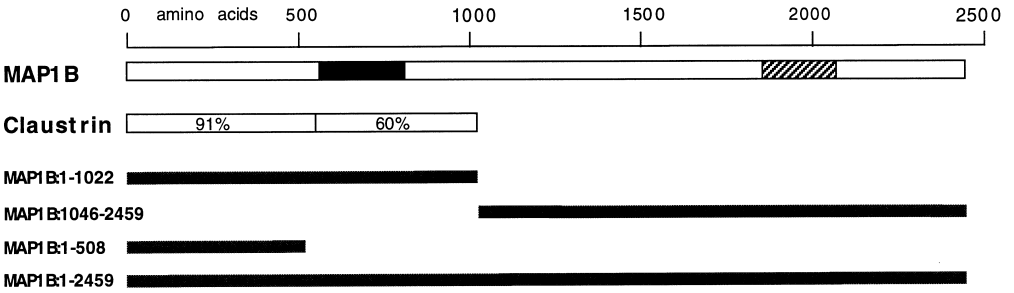

Fig. 1. Schematic representation of MAP1B, claustrin and rat MAP1B constructs. Full-length MAP1B is depicted with the N-terminal microtubule binding domain (filled box) and the imperfect 15-mer repeats (hatched box) [3,4]. Claustrin (depicted as an open box) is aligned to the MAP1B sequence. The numbers denote the percent sequence identity of the respective claustrin segments to the rat MAP1B sequence depicted above. Solid lines indicate the proteins encoded by the rat MAP1B cDNA constructs used. The numbers in the names of these constructs indicate the first and last amino acids according to the numbering of full-length rat MAP1B [4,6]. All MAP1B proteins used in this study carried a C-terminal c-myc epitope tag.

against mouse IgG, Texas-red-conjugated anti-mouse IgG antibody pre-adsorbed against rabbit IgG, Texas-red-conjugated donkey anti-guinea pig antibody (all Jackson, West Baltimore Pike, USA, dilutions 1:50); alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (Promega, dilutions 1:7500).

2.3. Immunofluorescence microscopy and immunoblotting analysis

NIH3T3, C_6 and PtK2 cells were grown in high glucose DMEM supplemented with 10% fetal calf serum in an atmosphere containing 7.5% CO_2, seeded onto coverslips or 35 mm diameter plates, transfected with lipofectamin (GIBCO BRL) according to the manufacturer's protocol and harvested after 24 h (C_6 and NIH3T3) or 48 h (PtK2). For immunofluorescence PtK2 cells were washed in PBS, fixed in methanol (−20°C, 10 min), equilibrated in PBS, blocked with 3% BSA, incubated with the primary antibodies in 1% BSA for 1 h, washed extensively in PBS, incubated for 1 h with the secondary antibody and again washed with PBS. C_6 cells were fixed using 4% paraformaldehyde (37°C), washed with PBS and blocked with 3% BSA. To stain for extracellular recombinant MAP1B proteins, cells were then incubated with the guinea pig anti-myc antiserum, washed extensively in PBS and incubated for 1 h with secondary antibody. Following further washing in PBS the cells were solubilized with 0.1% Triton X-100, blocked in 3% BSA and stained for intracellular MAP1B proteins using the mouse anti-myc mAb. Control cells were stained for fibronectin following the protocol employed for extracellular MAP1B. Specimens were analyzed by confocal microscopy (MRC600, BioRad). For immunoblot analysis [12], cells were washed with PBS, lysed in a buffer A, containing 8 M urea, 4% SDS, 0.125 M Tris-HCl pH 6.8, 12 mM EDTA, 0.3% DTT, 10 μM benzamidine, 1 mM PMSF, 2 μM pepstatin, 2 μM aprotinin, 2 μM leupeptin and bromphenol blue, sonicated, incubated at 65°C for 10 min and subjected to SDS/PAGE. The samples for Fig. 4, panel a were precipitated with methanol/chloroform [13] and resuspended again in buffer A before fractionation by SDS/PAGE.

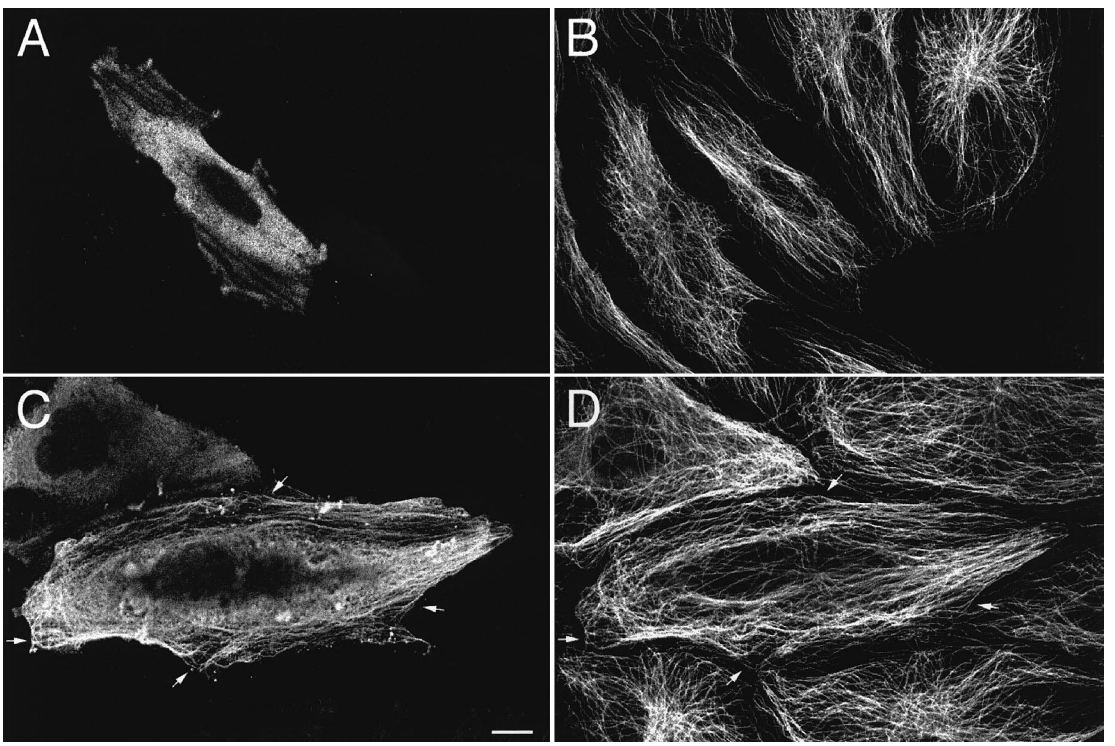

Fig. 2. Transient expression of MAP1B proteins in PtK2 cells. PtK2 cells were transfected with MAP1B:1–508 (A and B) or MAP1B:1–1022 (C and D) and analyzed by double immunofluorescence microscopy using the polyclonal rabbit anti-myc antibody (A and C) and the α-tubulin mAb (B and D). Note the diffuse intracellular localization of MAP1B:1–508 (A) and the diffuse as well as fibrillar localization of MAP1B:1–1022 (C). Fibrillar staining colocalized with microtubules (arrows). The bar indicates 10 μm.

3. Results

The reported amino acid sequence of chicken claustrin [8] comprises 1038 amino acids which correspond to amino acids 1–1022 of mouse, rat and human MAP1B [3–6]. In this region, claustrin and rat MAP1B share an overall sequence identity of 77%. The N-terminal 550 amino acids of the proteins are 91% identical, the remaining 488 amino acids of claustrin are 60% identical to the corresponding region in MAP1B (Fig. 1). This pattern of sequence homology, a highly related N-terminus of 550 amino acids with a drop in homology in the subsequent 500 amino acids, is reminiscent of the pattern of sequence conservation of mouse, rat, and human MAP1B. Taken together, these observations suggest that the cDNA reported for claustrin [8] might indeed be an N-terminal cDNA fragment of chicken MAP1B.

To directly test the relationship between MAP1B and claustrin we investigated subcellular localization, microtubule binding, and reactivity with monoclonal antibodies of a rat MAP1B fragment corresponding to claustrin. We generated a construct encoding rat MAP1B amino acids 1–1022 fused to a short c-myc peptide epitope tag for convenient immunological detection (MAP1B:1–1022; Fig. 1). For control purposes, similar myc-tagged cDNA fragments coding for amino acids 1–508 (MAP1B:1–508), 1–2459 (MAP1B:1–2459), and 1046–2459 (MAP1B:1046–2459, Fig. 1) were obtained. Expression of all constructs was put under the control of the tetracyclin responsive CMV promoter (Tet-off expression system; [10]) which proved to be a strong promoter in PtK2 and C_6 cells in the presence of the appropriate transactivator. All experiments were carried out in the absence of tetracyclin to ensure high level expression during the first 24–48 h after transfection.

The MAP1B:1–1022 protein was detected in transfected PtK2 cells by immunocytochemistry using a polyclonal rabbit antibody to the myc-tag (Fig. 2C). The protein appeared to be expressed in the cytoplasm, both in diffuse and fibrillar manner. Double labelling with an anti-tubulin mAb revealed colocalization of fibrillar MAP1B:1–1022 and microtubules (Fig. 2C and D). In contrast, MAP1B:1–508, an N-terminal fragment of rat MAP1B lacking the previously identified microtubule binding domain [3,4] did not colocalize with microtubules (Fig. 2A and B). Thus, binding of MAP1B:1–1022 to microtubules is dependent on its microtubule binding domain. These results demonstrated that MAP1B:1–1022 is an intracellular protein which binds to microtubules in vivo.

We next expressed MAP1B:1–1022 in C_6 glioma cells. We chose this cell line, because unlike PtK2 cells, C_6 cells express high levels of endogenous rat MAP1B [4]. If MAP1B/claustrin was indeed an extracellular protein as postulated [8], a putative transport mechanism necessary to transport MAP1B:1–1022 into the extracellular domain should be operating in C_6 cells. However, immunocytochemistry of C_6 cells transfected with MAP1B:1–1022 showed a staining pattern typical for intracellular proteins and did not reveal extracellular localization of MAP1B:1–1022 (Fig. 3A and B), despite the fact that C_6 cells were competent in exporting fibronectin and forming an extracellular matrix as revealed by staining with the anti-fibronectin antibody (Fig. 3C).

Finally, we tested the reactivity of MAP1B:1–1022, the rat homolog of claustrin, with the mAb MAP5, because the reactivity of this antibody with claustrin has been pivotal for the conclusion that MAP1B and claustrin might be identical [8]. Constructs encoding MAP1B:1–1022, full-length rat MAP1B (MAP1B:1–2459) or MAP1B:1046–2459 were transfected into NIH3T3 cells and whole cell lysates of transfected cells were analyzed by immunoblotting using the mAb MAP5 (Fig. 4). We found that full-length MAP1B was expressed and detectable with the MAP5 antibody. The ectopically expressed pro-

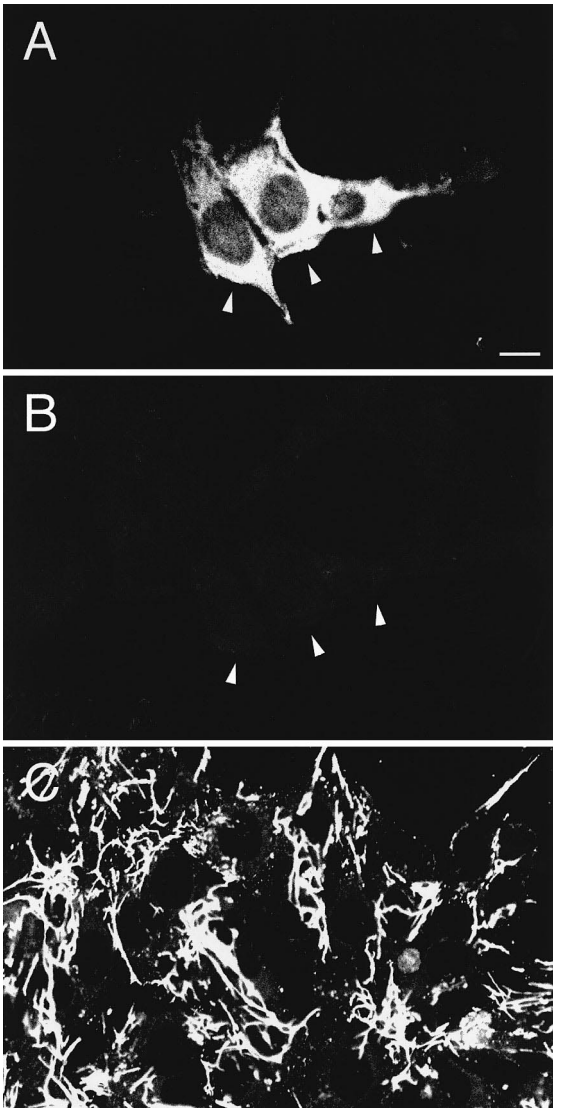

Fig. 3. Transient expression of MAP1B:1–1022 in rat glioma C_6 cells. C_6 cells were transfected with the construct encoding MAP1B:1–1022. Intracellular expression of the protein was detected with the mouse anti-myc mAb (A, arrows indicating three transfected cells). The same cells had before been stained for extracellular expression of MAP1B:1–1022 (see Section 2) using the guinea pig anti-myc antiserum (B, arrows indicating the position of the three transfected cells). Note the diffuse intracellular localization of MAP1B:1–1022 in transfected cells (A). Due to paraformaldehyde fixation, the localization of microtubules cannot be detected in this experiment. There is no evidence for the presence of MAP1B:1–1022 in the extracellular matrix. This result is not due to a failure of the guinea pig anti-myc antiserum to detect extracellular MAP1B:1–1022 since identical results were obtained using the mouse anti-myc mAb for extracellular and the guinea pig anti-myc antiserum (not shown). Control cells were stained for the presence of fibronectin in the extracellular matrix (C). The bar indicates 10 μm.

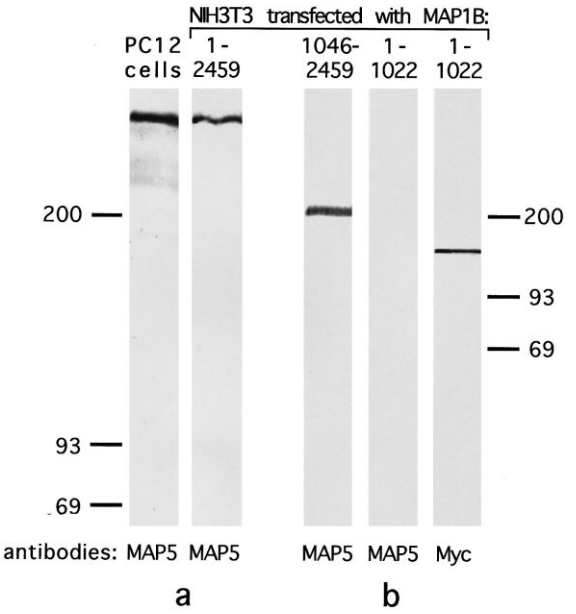

Fig. 4. Immunoblot analysis of NIH3T3 cells expressing MAP1B constructs. Whole cell lysates of NIH3T3 cells transiently transfected with the indicated constructs were analyzed by immunoblotting using the anti-MAP1B mAb MAP5 or the polyclonal rabbit anti-myc antibody as indicated. A whole cell lysate of rat pheochromocytoma PC12 cells which express endogenous MAP1B was used as positive control. The band detected with the polyclonal rabbit anti-myc antibody was specific for cells transfected with MAP1B:1–1022 and was not seen in control cells (not shown). Panels a and b are immunoblots obtained from separate gels.

tein comigrated on SDS-polyacrylamide gels with endogenous PC12 cell MAP1B. This result established that NIH3T3 cells could be used to express MAP1B and that the expressed protein was indistinguishable from endogenous MAP1B found in PC12 cells. MAP1B:1046–2459, the MAP1B fragment comprising the region of MAP1B not corresponding to claustrin was also detected by mAb MAP5. In contrast, MAP1B:1–1022 was not detected. This was not due to lack of expression since the protein was detected by the polyclonal antibody to its myc-tag. The apparent size of MAP1B:1–1022 was larger than the expected 115 kDa indicating posttranslational modification. However, it was clearly not as large as endogenous MAP1B (PC12 cells, Fig. 4).

These results refined the previously reported localization of the MAP5 epitope on MAP1B. In combination with published observations [4] the position of the MAP5 epitope can now be narrowed down to a region between amino acids 1046 (the beginning of MAP1B:1046–2459) and 1107 (the end of clone 14I; [4]). This region of MAP1B is C-terminal to the region corresponding to claustrin and therefore has no counterpart in claustrin.

The previous localization of the MAP5 epitope was carried out using defined MAP1B protein fragments expressed in *E. coli* [4], ruling out the possibility that MAP5 recognizes a putative keratan sulfate moiety of MAP1B. To exclude that due to sequence variation the epitope of the mAb MAP5 localized in rat MAP1B between amino acids 1046–1107 is present in chicken claustrin (amino acids 1–1038) we searched for sequences of at least 5 consecutive amino acids identical in claustrin 1–1038 and MAP1B 1046–1107. No such identical 5-amino acid stretches were found. Thus, the MAP5 epitope did not appear to be present in claustrin and conclusions concerning the identity of claustrin and MAP1B based on results obtained with this antibody [8] appear to be unfounded.

4. Discussion

In a recent report, Burg and Cole (1994) describe the molecular cloning of the cDNA for claustrin, an extracellular KSPG expressed in chicken brain. The cDNA was isolated using antibodies raised against purified claustrin. Sequencing revealed an open reading frame of 1038 amino acids with a sequence identity of 77% to the first 1022 amino acids of rat MAP1B. This finding together with the observed crossreactivity of purified claustrin with the anti-MAP1B mAb MAP5 as well as the apparent sensitivity of claustrin/MAP1B to treatment with keratanase prompted the authors to suggest that claustrin might be identical to MAP1B and that MAP1B was an extracellular KSPG rather than an intracellular microtubule-associated protein [8]. These suggestions were made despite the previous identification of MAP1B cDNAs with contiguous open reading frames of 2464, 2459 and 2468 amino acids in mouse, rat and human [3–6], only the first 1022 of which are homologous to claustrin.

In the present study we thought to test the hypothesis that claustrin is identical to MAP1B by analyzing subcellular localization and antibody reactivity of appropriately designed recombinant MAP1B fragments. Given the high degree of sequence conservation between chicken claustrin and the N-terminal 1022 amino acids of mammalian MAP1B, we assumed in accordance with Burg and Cole [8] that results found for chicken claustrin/MAP1B should be reproducible in mammalian systems. We have therefore transiently expressed the N-terminal rat MAP1B fragment of 1022 amino acids, corresponding in length and sequence to claustrin, in PtK2, NIH3T3 and C_6 cells and analyzed its subcellular localization and its reactivity with the anti-MAP1B mAb MAP5. Neither with PtK2 cells nor with C_6 glioma cells did we find evidence for extracellular localization of this protein (Figs. 2 and 3). In contrast, we were able to show that the protein binds to microtubules in vivo and that microtubule binding is dependent on the presence of the microtubule binding domain previously assigned to the region between amino acids 525 to 805 [3,4].

A finding pivotal for the suggestion that claustrin is identical to MAP1B was the observation that purified claustrin reacts with the anti-MAP1B mAb MAP5 [8]. The epitope of this antibody has previously been mapped to a region between amino acids 693 to 1107 [4]. This epitope mapping was carried out with MAP1B fragments expressed in *E. coli*, indicating that MAP5 recognized a peptide epitope and not, as suggested by Burg and Cole, a putative keratan sulfate moiety on MAP1B/claustrin [8]. By expressing recombinant MAP1B constructs in NIH3T3 cells and analysing the reactivity of the expressed proteins by immunoblots we were able to limit the epitope-containing region of MAP1B to amino acids 1046 to 1107 (Fig. 4). This region is C-terminal to the part of MAP1B (amino acids 1–1022) which was reported to be homologous to claustrin [8]. Our results demonstrate that MAP1B must comprise a larger portion of the previously identified 2459 amino acid open reading frame than amino acids 1–1022. Furthermore, we show that MAP1B:1046–2459 is significantly smaller than MAP1B:1–2459 or endoge-

nous MAP1B whereas all three proteins react with MAP5, indicating that a portion of the MAP1B open reading frame containing amino acids 1–1022 contiguous with additional amino acids is translated to yield high molecular mass MAP1B. This conclusion is supported by the finding that at least two additional mAbs to MAP1B react with epitopes located further to the C-terminus of the MAP1B open reading frame between amino acids 1639 to 2080 [4]. Taken together, these results provide strong evidence for the prevailing concept of MAP1B being translated as a 2459 amino acid protein and contradict the suggestion that MAP1B contains a core protein of only 1038 amino acids whereas the rest of the protein mass is made up by posttranslational modifications [8]. At the same time our results show that the reported claustrin cDNA has insufficient coding capacity to code for a protein reactive with the anti-MAP1B mAb MAP5. Thus, if claustrin was related to MAP1B as suggested by Burg and Cole based on the reactivity with the MAP5 antibody [8] it must comprise a larger region of homology with MAP1B than the reported 1022 amino acids.

Our results do not exclude the possibility that MAP1B and claustrin are immunologically related. In fact, extracellular proteins immunologically related to MAP1 and/or MAP2 have been reported elsewhere [14]. However, the suggested strict identity [8] of the two proteins appears to be unlikely in the light of the results presented here which argue against the redefinition of MAP1B as an extracellular KSPG.

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