A 45 amino acid residue domain necessary and sufficient for proteolytic cleavage of the MAP1B polyprotein precursor

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Abstract The microtubule-associated proteins 1B and 1A are synthesized as polyprotein precursors which are rapidly cleaved to give rise to heavy and light chains constituting the respective microtubule-associated protein 1B or microtubule-associated protein 1A complex. To identify domains necessary for precursor processing, we expressed microtubule-associated protein 1B deletion mutants in fibroblasts and monitored proteolytic cleavage of the precursor proteins by immunoblot analysis. We found that a novel hydrophilic, proline-rich 45 amino acid domain containing the cleavage site is necessary and sufficient for processing. This domain is conserved in microtubule-associated protein 1A. Additional sequences in the N-terminal half of the heavy chain contribute to the efficiency of cleavage.

Key words: Microtubule-associated protein 1B; Polyprotein precursor; Proteolytic cleavage; Domain structure; Enhanced green fluorescent protein/β-galactosidase fusion protein

1. Introduction

The microtubule-associated protein (MAP) 1B is a high molecular mass protein complex consisting of a 280 kDa heavy chain and at least one light chain of 32 kDa [1,2]. MAP1B is expressed predominantly in the central nervous system and recent gene targeting studies revealed that it plays a role during neuronal differentiation [3,4]. Concomitantly, MAP1B levels are high during development and decline in the adult brain, whereas the related protein MAP1A is expressed in a reciprocal pattern [2]. MAP1B and MAP1A complexes are related in several aspects [5]. They are similar in size, share three major and several minor regions of sequence homology and each consists of heavy and light chains. Moreover, both protein complexes are synthesized as polyprotein precursors which are rapidly cleaved to give rise to the respective heavy and light chains. The site of proteolytic cleavage of the MAP1B precursor has been narrowed down to a region in the proximity of amino acid 2210 by sequencing the N-terminus of the light chain [6] and by combined electrophoretic mobility and epitope mapping analyses [7]. The corresponding site for rat MAP1A cleavage was determined by amino acid sequencing to be in the proximity of amino acid 2554 [5].

Consistent with the finding that MAP1B expression can be detected in a number of adult mouse tissues apart from brain [6,8–10], we [7] and others [6] have demonstrated that correct proteolytic processing of the MAP1B polyprotein precursor is not restricted to neural cells. Here, we exploited the fact that the MAP1B precursor is cleaved efficiently in NIH3T3 fibroblasts to identify and analyze domains necessary for proteolytic processing.

2. Materials and methods

All MAP1B constructs were derived from full length rat MAP1B cDNA with a C-terminal myc-tag [11] and were cloned into the Tet-Off expression vector pUHD10-3 [12]. The construct encoding the enhanced green fluorescent protein (GFP)/β-Gal fusion protein was obtained by inserting the *Bam*HI fragment of plasmid pGNA containing the lacZ gene without its first two codons [13] into the pEGFP-C1 vector (CLONTECH) using an appropriate oligonucleotide linker which was designed to facilitate subsequent insertion of a PCR fragment derived from rat MAP1B cDNA encoding amino acids 2183–2227. The correct sequence of this PCR insert, the junctions in all fusion proteins, as well as the sequence flanking the deletion in construct FLΔCS were confirmed by sequencing.

NIH3T3 cells were grown, transfected with lipofectamine (Gibco BRL) and harvested after 24 h as described [11]. For immunoblot analysis [14], cells were washed with PBS, lysed in a buffer 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 bromophenol blue and sonicated. The lysates were incubated for 10 min at 65°C, subjected to SDS-PAGE and transferred to nitrocellulose. For immunological detection, we used an affinity-purified polyclonal rabbit anti-myc peptide antibody at a concentration of 5 μg/ml [11] and the anti-MAP1B mAb MAP5 (Clone AA6, Boehringer Mannheim) recognizing an epitope located between amino acids 1046–1107 [11,15] at a dilution of 1:500. A polyclonal antibody to GFP (Molecular Probes) and a monoclonal antibody (mAb) to β-Gal (Boehringer Mannheim) were used at dilutions of 1:1000. Secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (Promega, dilutions 1:7500).

3. Results and discussion

To identify domains of the MAP1B polyprotein precursor necessary for its correct proteolytic cleavage, we transiently expressed various MAP1B deletion mutants in NIH3T3 cells and subsequently analyzed cell lysates for specific cleavage products by immunoblotting. We first determined the effect of increasing N-terminal deletions (N1-N4, GFP/N5: Fig. 1). Surprisingly, all precursor proteins were cleaved and the light chains generated from these N-terminally truncated precursors co-migrated with light chain generated from full length MAP1B (FL) and with a protein representing the bonafide light chain synthesized from a construct encoding amino acids 2210–2459 (LC: Fig. 2A, compare lanes FL and LC to lanes N1–N4, GFP/N5). This indicated that amino acids 1–2182 (lacking in the largest of the N-terminal deletions, GFP/N5)

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Abbreviations: MAP1B and MAP1A, microtubule-associated proteins 1B and 1A; GFP, enhanced green fluorescent protein; β-Gal, β-galactosidase

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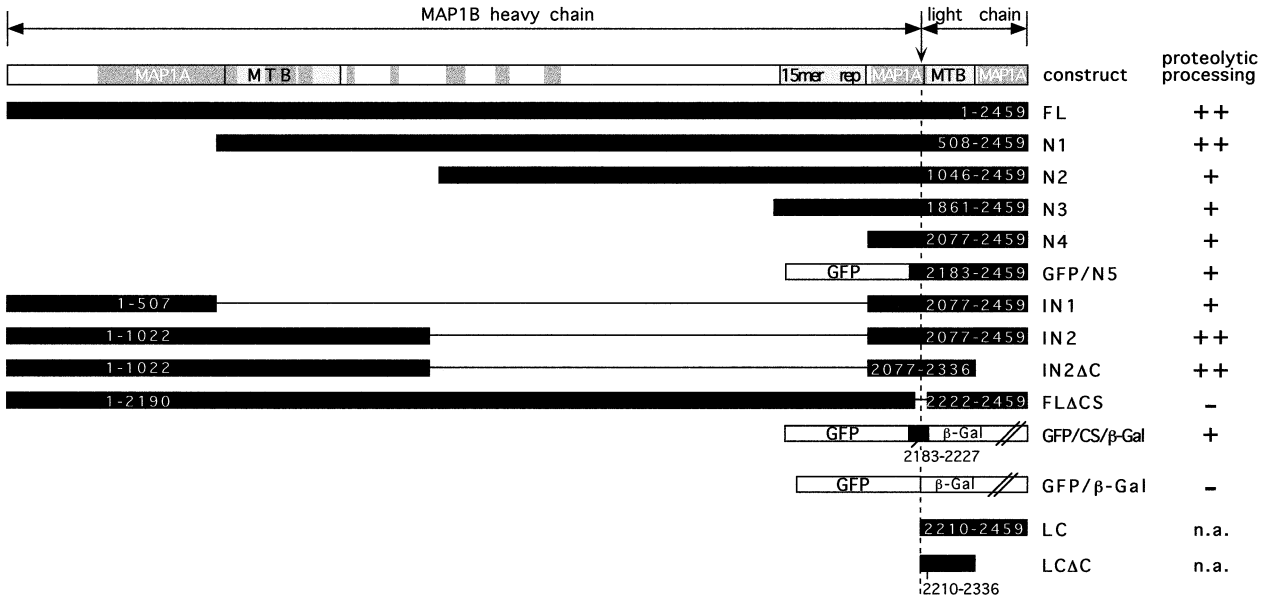

Fig. 1. MAP1B mutant proteins and their proteolytic processing. The MAP1B polyprotein precursor is schematically outlined on top. The positions of the microtubule binding domains (MTB) and the 15-mer repeat (15mer rep) are indicated by lightly shaded boxes. The three major (labelled MAP1A) and seven minor regions of sequence homology with MAP1A are indicated by dark shading. The site of cleavage of the precursor into heavy and light chain in the proximity of amino acid 2210 is marked by the vertical arrow. cDNA constructs used in this study encoding full length (FL) or mutant MAP1B proteins are described with respect to the amino acid residues present in the proteins. All proteins except the GFP/β-Gal fusion proteins carried C-terminal myc-tags. GFP was fused to the N-terminal deletion mutant N5 to be able to detect the N-terminal cleavage product. The MAP1B light chain (LC) or a light chain lacking its C-terminus (LCΔC) and authentic GFP and β-Gal were expressed to obtain size markers for specific cleavage products. The efficiency of proteolytic processing in NIH3T3 cells was estimated from immunoblot analysis of lysates from cells expressing the respective proteins.

were dispensable for correct precursor cleavage. However, we observed a marked decrease in the efficiency of cleavage as a result of deleting 1045 or more N-terminal amino acids. For example, we compared the efficiency of proteolytic processing of full length MAP1B (FL) and deletion mutant N2 (lacking amino acid residues 1–1045) by comparing the amount of uncleaved precursor relative to the amount of heavy chain fragment generated by cleavage, exploiting the fact that precursors as well as heavy chain fragments can be detected by mAb MAP5 (Fig. 2B). Full length MAP1B was processed efficiently with only a small fraction of precursor remaining uncleaved (Fig. 2B, lane FL). In contrast, less than 50% of mutant N2 was cleaved (Fig. 2B, lane N2).

These results were confirmed and extended using internal deletion mutants IN1, IN2 and IN2ΔC (Fig. 1). Mutants IN1 and IN2 were cleaved to give rise to light chains of a correct size (Fig. 2C, compare lanes LC, IN1 and IN2). Likewise, mutant IN2ΔC was cleaved to give rise to a C-terminally truncated version of the light chain. As expected, this truncated light chain co-migrated with a protein comprising amino acid residues 2210–2336 (Fig. 2C, compare lanes IN2ΔC and LCΔC) confirming the cleavage site in the close proximity of amino acid 2210. Mutants IN2 and IN2ΔC were cleaved efficiently, demonstrating that amino acid residues 1023–2076 (lacking in IN2 and IN2ΔC) and 2337–2459 (lacking in IN2ΔC) were dispensable for correct and efficient processing. In contrast, a considerable fraction of mutant IN1 remained uncleaved (compare Fig. 2C, lanes IN1 and IN2), indicating that amino acids 508–1022 which were absent in IN1 but present in IN2, although not essential, were necessary for efficient processing of the precursor. This region harbors four small stretches of homology to MAP1A (Fig. 1, compare the N-termini of mutants IN1 and IN2 with the schematic on top). Since MAP1A is also synthesized by proteolytic cleavage of a polyprotein precursor [5], we suggest that one or more of these small homology regions might be involved. In contrast, our results showed that the long MAP1A homologous regions in the highly conserved MAP1B N-terminus (amino acids 1–507) and C-terminus (amino acids 2337–2459) do not play a role in processing, because mutants lacking the N-terminus (N1) or C-terminus (IN2ΔC) were cleaved efficiently. This is of particular interest, since we have shown that these two long MAP1A homology domains mediate heavy chain-light chain interaction in the MAP1B complex [7]. Thus, our present results suggest that this interaction is not a prerequisite for proteolytic cleavage of the polyprotein precursor.

To determine the role of sequences flanking the proteolytic cleavage site of the polyprotein precursor, we expressed MAP1B mutant FLΔCS which lacked 31 amino acid residues (2191–2221) flanking the cleavage site in the proximity of amino acid 2210 (Fig. 1). Cell lysates were analyzed by immunoblotting using mAb MAP5 and anti-myc antibody and were compared to lysates from cells expressing wild-type MAP1B (FL: Fig. 3A and B). As expected, most of the wild-type precursor was cleaved to give rise to heavy chain (Fig. 3A, lane FL, detected with MAP5) and light chain (Fig. 3B, lane FL, detected with anti-myc). In contrast, the FLΔCS precursor did not yield a light chain of the expected size (Fig. 3B, lane FLΔCS), but remained uncleaved and was detected with both the MAP5 and the anti-myc antibody at a size slightly larger than the genuine heavy chain (Fig. 3A, compare lanes FL and FLΔCS). This demonstrated that the 31 amino acid residues surrounding the cleavage site were essential for correct processing of the MAP1B precursor. To test whether

these sequences were also sufficient for processing, we generated a cDNA construct encoding a GFP/β-Gal fusion protein and inserted MAP1B cDNA sequences corresponding to amino acid residues 2183–2227 between the GFP and β-Gal moieties (GFP/CS/β-Gal, Fig. 1). In contrast to the GFP/β-Gal fusion protein lacking the MAP1B cleavage site (Fig. 3C and D, lanes GFP/β-Gal), GFP/CS/β-Gal was cleaved, albeit at a low efficiency, to give rise to a GFP fragment (Fig. 3C, lane GFP/CS/β-Gal) and a β-Gal fragment (Fig. 3D, compare lanes GFP/CS/β-Gal and β-Gal), each of the appropriate size. This demonstrated that MAP1B amino acid residues 2183–2227 are sufficient to confer and direct proteolytic cleavage.

Assuming that the mechanism of proteolytic processing is the same for MAP1B and MAP1A, we expect that crucial amino acid residues should be conserved at the respective cleavage sites. Alignment of the corresponding regions of all available MAP1B [15–17] and MAP1A [5,18] sequences revealed a hydrophilic proline-rich sequence with eight prolines and a number of aspartate, glutamate, arginine and lysine residues at conserved positions (Fig. 4). This sequence did not match any known protein sequence motifs or protease recognition and cleavage sites suggesting that it contained a

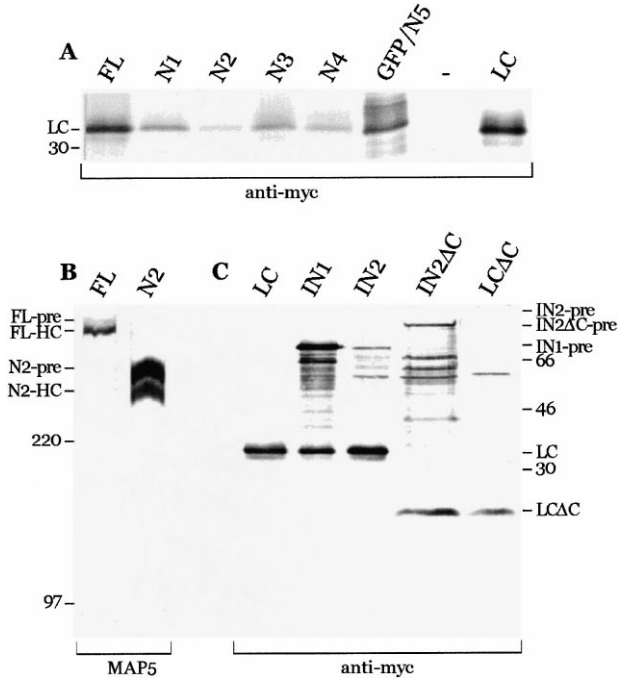

Fig. 2. Large parts of the MAP1B polyprotein precursor are dispensable for correct proteolytic processing. Cells were transiently transfected with the indicated constructs and cell lysates were analyzed by immunoblotting using the indicated antibodies. The positions of the respective precursor proteins (pre) and heavy (HC) and light chain (LC) fragments resulting from specific proteolytic cleavage of the precursors as well as positions of size markers (in kDa) are marked. (A) Light chains cleaved off the indicated precursors are detected by an antibody to their C-terminal myc-tag. No proteins are detected in lysates from cells transfected with empty expression vector (−). (B) The position of the uncleaved full length precursor (FL-pre) was determined using cells transfected with FLΔCS (which is not cleaved: see Fig. 3). (C) The bands in lanes IN1, IN2 and IN2ΔC corresponding neither to the respective uncleaved precursor nor to the light chain generated by cleavage are due to non-specific protein degradation.

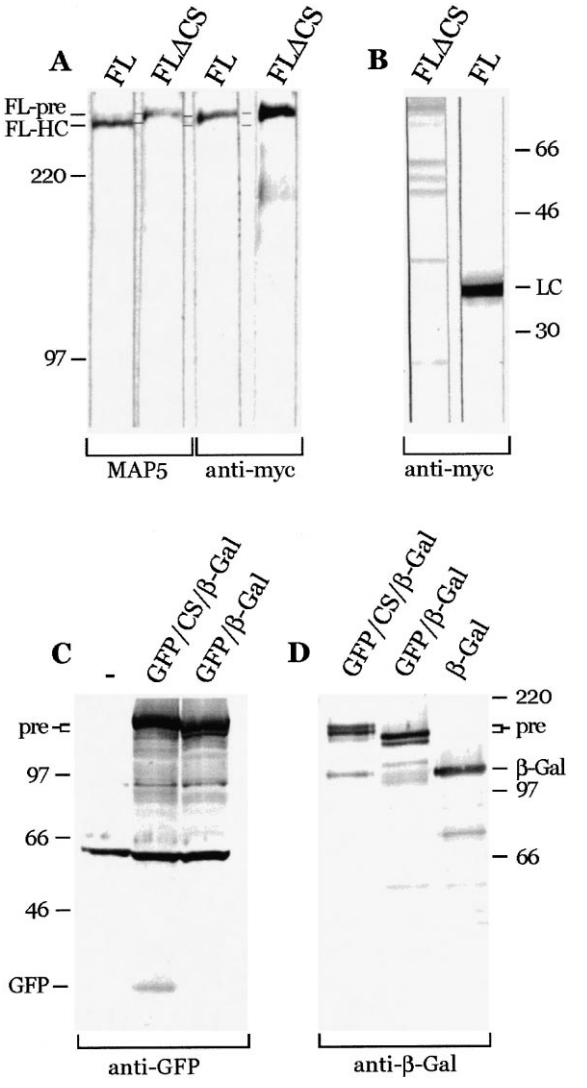

Fig. 3. A 45 amino acid long sequence around the MAP1B cleavage site is necessary and sufficient for correct proteolytic processing. Cells were transiently transfected with the indicated constructs and cell lysates were analyzed by immunoblotting using the indicated antibodies. The positions of the respective precursor proteins (pre) and heavy (HC) and light chain (LC) fragments resulting from specific proteolytic cleavage of the precursors as well as positions of size markers (in kDa) are marked. (A) Analysis of FLΔCS cleavage on a 6% gel. Note the slightly larger size of the protein reacting with MAP5 in FLΔCS lysates compared to FL lysates. A band comigrating with this FLΔCS protein was immunoreactive with the anti-myc antibody, demonstrating that this was the uncleaved precursor protein. A small amount of uncleaved precursor could also be detected in lysates from cells expressing FL, immunoreactive with both the MAP5 and the anti-myc antibodies (lanes FL). (B) Analysis of the same extracts as in A on a 10% gel. (C) Analysis of GFP/CS/β-Gal cleavage on a 10% gel. The position of the GFP moiety of the GFP/β-Gal fusion protein was determined using cells transfected with a construct encoding GFP only (not shown). Note that the strongly immunoreactive band at ~60 kDa was stained non-specifically, as it was also detected in non-transfected cells (−). (D) Analysis of GFP/CS/β-Gal cleavage on a 6% gel. The faint bands detected in GFP/β-Gal lysates that were similar in size to β-Gal were most likely due to non-specific protein degradation as they, in contrast to the β-Gal band in GFP/CS/β-Gal lysates, did not exactly match the expected size of authentic β-Gal.

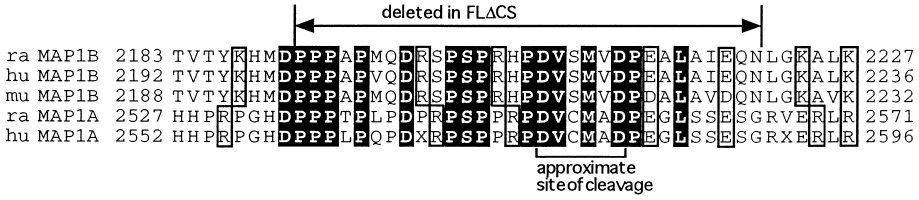

Fig. 4. Conservation of amino acid sequence at the cleavage sites of MAP1B and MAP1A of rat (ra), human (hu) and mouse (mu). The rat MAP1B sequence shown represents the amino acid residues inserted between the GFP and β-Gal moieties in GFP/CS/β-Gal. Residues deleted in FLΔCS are indicated. Identical amino acid residues are highlighted in black and charged amino acid residues at identical or similar positions are boxed. The approximate position of proteolytic cleavage as inferred from the N-terminus of the light chain is indicated.

novel recognition and/or cleavage site for an as yet unidentified protease. In addition, this stretch might constitute a readily accessible hydrophilic hinge region between the heavy and light chain. Owing to the large number of prolines, it has probably a poorly defined secondary structure in solution. The short stretches of homology of MAP1B and MAP1A located between amino acids 508 and 1022 which are necessary to increase the efficiency of processing might stabilize the secondary structure of the hinge region for optimal proteolytic cleavage.

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