MSAP Is a Novel MIR-interacting Protein That Enhances Neurite Outgrowth and Increases Myosin Regulatory Light Chain*

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Dynamic interactions between the actin cytoskeleton and specific proteins are crucial for changes in cell shape and motility. Here we describe a novel protein MSAP (MIR-interacting saposin-like protein) that is a positive regulator of neurite outgrowth. MSAP is expressed in different tissues, including brain, and has an apparent molecular weight of 21 kDa. MSAP interacts with the ezrin-radixin-moesin (ERM)-like myosin regulatory light chain-interacting protein (MIR), and the two proteins are co-localized in cell lines and in primary neurons. Overexpression of MSAP enhances neurite outgrowth in neuroblastoma and PC12 cells, whereas down-regulation of MSAP using RNA silencing led to inhibition of neurite formation. The stimulation of neurite outgrowth by MSAP was abrogated by the overexpression of MIR, which induced a decrease in the levels of myosin regulatory light chain (MRLC). This reduction in MRLC by MIR was inhibited by blocking the activity of proteasome and by overexpression of MSAP, suggesting an effect on protein stability. Evidence was obtained that MIR decreases MRLC by inducing its ubiquitination and that the effect of MIR on MRLC is counteracted in the presence of MSAP. MSAP can stabilize MRLC and thus bring about an increase in neurite outgrowth.

Interactions between the cell membrane and the cytoskeleton play a major role in different aspects of cell differentiation, such as cell motility, cell division, and establishment of cellular architecture. Of particular importance in this context is the association of cortical actin with the cell membrane. The ERM proteins are members of the large 4.1 protein family and are involved in membrane-cytoskeleton interactions. They link the actin cytoskeleton to membrane-bound proteins located at different membrane sites, i.e. microvilli, membrane ruffles, and cell-cell contacts (1–3). This is accomplished by binding of the C-terminal part of the ERM proteins to actin and of the N-terminal FERM domain to specific membrane proteins (4). FERM domains have been found in many different proteins and are thought to be modules for protein-protein and protein-membrane interactions (5). The ERM proteins are involved in cell adhesion and signal transduction events through phosphorylation (6, 7) and interaction with phosphoinositides (8). The ERM proteins play an important role in the activation of Rho family proteins and can interact both downstream (9) and upstream (10) of Rho.

In addition, the ERM proteins are involved in membrane dynamics (11). When ezrin, radixin, and moesin were simultaneously inactivated by antisense treatment in epithelial cells, cell-cell and cell-substratum adhesion was altered (12). Double suppression of radixin and moesin, but not ezrin and radixin or moesin, alters growth cone motility, inhibiting neurite extension (13). In contrast, overexpression of ezrin in insect cells leads to enhanced cell adhesion (14). The mechanisms behind these effects are, however, not well understood, but ERM proteins are known to interact with various proteins such as CD44 and ICAM-1, -2, and -3, which helps in establishing membrane specializations (for review, see Ref. 7). The identification and characterization of further binding partners for ERM proteins can give new insights into the function of these proteins.

We have recently identified a novel ERM family protein, MIR, which has an ERM domain at the N terminus and lacks actin binding, instead possessing a RING domain in the C-terminal region (15). Overexpression of MIR abrogated neurite outgrowth in PC12 cells, an effect that may be brought about by its interaction with the myosin regulatory light chain (MRGC). We describe here a novel protein interacting with MIR, called MIR-interacting saposin-like protein (MSAP), which stimulates neurite outgrowth. Sequence comparisons showed that MSAP contains a saposin domain, which is found, among others, in the sphingolipid activator proteins, the saposins (16), and in some saposin-like proteins with a similar structure (17). The effects of MSAP on neurite outgrowth were reduced by MIR. MIR was shown to induce a decrease in the levels of MRLC, which could be blocked by overexpression of MSAP or by inhibition of proteasome activity. Evidence was obtained that the decrease in MRLC levels by MIR involves ubiquitination of MRLC. MSAP overexpression was found to increase the level of MRLC, suggesting an effect on protein stability. MSAP may define a novel class of saposin-like proteins influencing MRLC protein levels and stimulating neurite outgrowth.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—The full-length coding sequence of MIR was fused to the GAL4 DNA-binding domain in the pYTH6 bait vector and used for screening a cDNA library of HeLa cells (Clontech) (15, 18). Clones positive for interaction were sequenced, and twelve were identified as MSAP. Deletions of MIR and MSAP were subcloned into the pAS 2 vector using PCR. The constructs were co-transfected into yeast together with either full-length MSAP or MIR in pGAD-GH vector and studied for protein interaction using the β-galactosidase filter assay.

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Cloning of MSAP and Expression Constructs—MSAP DNA was amplified by PCR and used for screening of a library (Clontech) (15). Positive clones were subcloned into the Blue/White selection vector pBluescript KS (Stratagene) and sequenced using an automated DNA sequencer (Applied Biosystems). The nucleotide sequence encodes a protein with an open reading frame of 182 amino acids and was named MSAP. The sequence was compared with those in the database, and all constructs were verified by sequencing.

Northern Blot Analysis—Multiple Tissue Northern (MTNTM) blots (Clontech) were prehybridized for 30 min at 68 °C using ULTRAhyb (Ambion) and hybridized at 68 °C overnight using 10^5 cpm/ml of the MSAP cRNA probe (15). Filters were washed, and the levels of MSAP mRNA were analyzed using a PhosphorImager (Amersham Biosciences) and compared with those of β-actin.

In Vitro Binding Assay—Glutathione S-transferase (GST, Amersham Biosciences) and GST-tagged MIR were expressed, purified, and immobilized onto glutathione beads according to standard methodology. 

MSAP Antibody and Immunocytochemistry—The antipeptide antibody to MSAP (amino acids 88–104, DRMKEYGEQIDPSTHRK) was raised in rabbits using standard procedures. This antiserum recognized MSAP in Western blots. For immunocytochemistry, primary hippocampal neurons and COS7 cells were cultured, fixed, and stained as described (19). Anti-MSAP antibody (diluted 1:200) or anti-MIR antibody (1:200) was added and incubated overnight at 4 °C. The signals were visualized with secondary Cy-3-tagged anti-rabbit antibodies and analyzed using a Zeiss Axioplan2 microscope and axiovision deconvolution software.

Ubiquitination Assay—N2-A cells were transfected with expression vectors for His-tagged ubiquitin (20) and GFP, GFP-MIR, pDS-MSAP, or pDS-MSAP and GFP-MIR. After 24 h, 5 μM MG132 was added for 6 h, and cells were lysed in ice-cold buffer (as described above) supplemented with protease inhibitors. Immunoprecipitation was carried out with anti-MRLC antibodies (1:250, Santa Cruz Biotechnology) and protein G-Sepharose (Amersham Biosciences). The beads were washed three times, and the pellet was boiled in 1× SDS-PAGE buffer and boiled, and the proteins were analyzed by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was then incubated with anti-MSAP, followed by goat anti-rabbit IgG conjugated to horseshad peroxidase (1:1000). Signals were detected by enhanced chemiluminescence using the SuperSignal substrate from Pierce.

Neurite Outgrowth—N2-A neuroblastoma cells were transfected with the following expression vectors: pDS-MSAP, pDS, GFP-MIR, and pDS-MSAP together with GFP-MIR. After 40 h, the number of cells bearing neurites longer than twice the cell diameter was scored, and the number of neurites/cell was counted. In each experiment more than 100 cells were counted, and data was expressed as mean ± S.E. For statistical analyses Student’s t test was used.

Pheochromocytoma PC12 cells were kept in 5% bovine serum, 5% fetal calf serum, Dulbecco’s modified Eagle’s medium, and were transfected with expression vector pDS-MSAP or pDS. After 24 h, half of the cells received 50 ng/ml NGF (nerve growth factor), and the other half was left untreated. The number of neurites was assayed after 48 h as described above, and data is shown as mean ± S.E.

For RNA silencing studies the pS1 silencer kit (Ambion) was used as described by Sui et al. (21). The following oligonucleotides were cloned into the vector: MSAP, 5′-CCTCCATCGCAGAGGAGC-TGACGCTGACCCTAGTATTTTT-3′ and 5′-ATTAAAAATCCGATGAGCGACGTCACTTTTGAATGTCATGCGCCACATCTCGAGGC-CC-3′; and scrambled, 5′-CCAGGTTCAGGAGGCTTTCTGAGAATAGAGTGAGAAGAATAGGCTTGAGGCGCG-3′ and 5′-CCAGGCTTCAGGGGTCTTCTGAGAATAGAGTGAGAAGAATAGGCTTGAGGCGCG-3′. The constructs were confirmed by sequencing. N2-A cells were transfected with the silenced constructs together with the GFP vector (ratio 10:1). After 3 days in culture the cells were stimulated by removing the serum for 16 h. Then, the cells were fixed and stained for MSAP. Cells were analyzed for the GFP signal and the presence or absence of MSAP and scored for neurites.

| A | MIR constructs | β-galactosidase activity |
|---|----------------|-------------------------|
| MIR | 1-465 | + |
| MIR | 1-406 | - |
| MIR | 1-295 | - |
| MIR | 296-465 | - |
| MIR | 296-406 | - |
| pAS2 | - | - |

| B | MSAP constructs | β-galactosidase activity |
|---|-----------------|-------------------------|
| MSAP | 1-182 | +++ |
| MSAP | 21-182 | - |
| MSAP | 21-161 | - |
| MSAP | 21-141 | - |
| MSAP | 21-83 | - |

pGAD-GH -
For MRLC analysis, N2-A cells were transfected with pDS-MSAP, EGFP, GFP-MIR, or both pDS-MSAP and GFP-MIR. After 30 h the cells were stimulated with 5 μM MG132 or vehicle for 4 h. Then the cells were lysed in 50 mM HEPES, 100 mM NaCl, 0.4% Nonidet P-40, and protease inhibitors. Equal amounts of proteins were subjected to SDS-PAGE and Western blotting as described above. Signals were visualized with polyclonal anti-MRLC (1:500, Santa Cruz Biotechnology) and antiactin (1:3000, Sigma) antibodies.

RESULTS

Identification of MSAP as a Novel MIR-interacting Protein—In previous studies we identified MIR, which is a novel ERM-like protein that binds myosin light chain (13). To search for additional proteins interacting with MIR, we used the yeast two-hybrid system (15, 18). When a HeLa cell cDNA library was screened with full-length MIR as bait, we identified a novel protein called MSAP as a potent interactant (Fig. 1). Using different deletion constructs, we observed that the interaction required the whole molecule of MIR and MSAP. Neither the ERM-like domain nor the RING domain in MIR was sufficient for binding to MSAP (Fig. 1A). Controls did not result in any binding activity. Deletion constructs of MSAP were not able to bind full-length MIR (Fig. 1B). To confirm the interaction, we performed a binding assay with GST-linked MIR and radiolabeled MSAP, generated in vitro. MSAP bound specifically to MIR-GST in this assay. In addition, MIR bound to MIR-GST (Fig. 2A), indicating that there is self-association, a feature that MIR shares with other ERM proteins (2–5). As a control, luciferase and the ERM protein ezrin (3) were applied to the beads, which resulted in no binding to MIR (Fig. 2A). Neither of these proteins bound to GST alone (data not shown). These results confirm the specific interaction between MIR and MSAP, which was also evident in co-immunoprecipitation experiments using COS7 cell lysates (Fig. 2B). MSAP was detected in anti-MIR immune complexes, whereas no signal was present in control experiments using non-immune antibodies. MSAP belongs to the family of saposin-like proteins and is expressed in various human tissues—MSAP has an open reading frame of 182 amino acids (Fig. 3A). A search in the database revealed the existence of a sequence with a high homology to MSAP (GenBankTM/EBI accession number AB 05631). This cDNA was cloned as a putative transmembrane type II protein with an unknown function and having a membrane signal anchor (22). The first 3–20 amino acids of MSAP constitute the signal anchor region, but the rest of the MSAP sequence shows similarities to a saposin domain found in the large family of saposin-like proteins (Fig. 2B). This family consists of the saposins, SAP-A, -B, -C, and -D, in addition to other related proteins such as the surfactant protein and natural killer cell (NK) lysin (16, 17). The alignment shows that MSAP has a saposin domain interrupted by two stretches of 30 and 40 amino acids (Fig. 2B). MSAP mRNA was present in all human tissues examined, in fetal as well as in adult tissue (Fig. 4). The highest levels were detected in adult placenta, liver, and pancreas (Fig. 4B). Western blot showed one major band of MSAP at 21 kDa (Fig. 4C).

MSAP and MIR Co-localize in Cells—Previous studies have shown the localization of MIR in the cytosol and at membranous structures in overexpressing cells (15, 19). To study the subcellular localization of MSAP, COS7 cells and primary hippocampal neurons were cultured and stained with anti-MIR- and anti-MSAP-specific antibodies. Endogenous MIR and MSAP exhibited a similar staining pattern with both proteins present in the cytoplasm and in particular structures of the cell (Fig. 5, a–d). In primary neurons immunofluorescence was detected around the nucleus and extended into the neurites. In COS7 cells, both proteins exhibited a punctate staining pattern that was most prominent around the nucleus. Double labeling experiments with anti-MIR antibodies (Fig. 5e) and GFP-MSAP (Fig. 5f) largely localized MIR and MSAP to the same cellular compartments (Fig. 5g).

MSAP Stimulates Neurite Outgrowth That Is Inhibited by
MIR—To study the function of MSAP, N2-A and PC12 cells were transfected with the MSAP expression plasmid. MSAP-expressing neuroblastoma cells had a significantly greater number of cells with neurites compared with controls (Fig. 6 A). This was also observed using PC12 cells cultured in the absence of NGF (Fig. 6 B). A closer analysis revealed that the number of neuroblastoma cells having two or more neurites was increased by MSAP (38.3% ± 0.7, n = 300) compared with controls (22.4% ± 2.5, n = 300). These results show that MSAP has a stimulatory effect on the outgrowth of neurites in these two neuronal systems.

In keeping with data obtained with PC12 cells (15), expression of MIR alone inhibited neurite outgrowth of N2-A cells (Fig. 6 A). To study the interplay between MSAP and MIR in neurite outgrowth, the proteins were co-expressed in neuroblastoma cells. Data showed that MIR reduced the stimulatory effect of MSAP on neurite outgrowth (Fig. 6 A). This demonstrates a functional interaction between MSAP and MIR in the cells.

To study the influence of MSAP on neurite outgrowth induced by external stimuli, transfected PC12 cells were stimulated by NGF. Data showed that MSAP enhanced neurite outgrowth (Fig. 6 B). The corresponding number in controls was about 63%, increasing to more than 90% at later time points. This shows that MSAP stimulated neurite outgrowth...
both in the presence and absence of NGF.

**Down-regulation of MSAP Inhibits Neurite Outgrowth**—To investigate the role of MSAP in neurite outgrowth in more detail, we used the method of RNA silencing. Down-regulation of MSAP in neuroblastoma cells transfected with the MSAP-silencing construct led to a 65% decrease in the number of cells having neurites compared with controls (Fig. 7).

**MIR and MSAP Affect the Level of MRLC**—The activity of the actomyosin complex is a major determinant for alterations in cell contractility (23), which in neuronal cells is observed as alterations in neurite outgrowth (24, 25). To study the mechanism by which MIR and MSAP can regulate neurite outgrowth, we analyzed their effects on MRLC, which has a major influence on actomyosin activity. The results showed that MRLC was down-regulated by about 70% after MIR overexpression compared with controls (Fig. 8). In contrast, the levels of MRLC increased about 2-fold after MSAP, suggesting an effect on protein stability (Fig. 8). Most notably, the decrease in MRLC mediated by MIR was partly counteracted by the presence of MSAP, indicating that the two proteins functionally interact in the cells (Fig. 8).

**MRLC Is Ubiquitinated in the Presence of MIR**—The down-regulation in MRLC by MIR prompted us to study the degradation of MRLC in the cells. Addition of the proteasome inhibitor MG132 restored the levels of MRLC, suggesting that the decrease in MRLC involves proteasomes (Fig. 8). MIR has a RING finger domain in its C-terminal region (15), which in other proteins is involved in ubiquitination of target proteins (26, 27). To study whether MIR can directly affect the degradation of MRLC we analyzed the ubiquitination of MRLC in cells. The results showed that MRLC became ubiquitinated in the presence of MIR (Fig. 9). The presence of MSAP was able to counteract MRLC ubiquitination (Fig. 9). This indicates that MRLC can undergo ubiquitination in the cell that is promoted by MIR and that leads to its down-regulation. MSAP, which increases MRLC levels, counteracted the decrease in MRLC observed in the presence of MIR. These effects on MRLC levels could explain the antagonistic effects of the two proteins on neurite outgrowth.

**DISCUSSION**

In this work, we describe MSAP, a novel protein that interacts with MIR and contains a saposin-like sequence. Expres-
The expression of MSAP in neuronal cells led to enhanced neurite outgrowth that was counteracted by MIR. The molecular basis for the interaction of MSAP and MIR was shown to involve regulation of the levels of MRLC. The effect of MIR on the down-regulation of MRLC was shown to be dependent on the activity of proteasomes, and MRLC was ubiquitinated through MIR. The results demonstrate that the steady-state levels of MRLC are tightly regulated in cells by protein degradation involving an interplay between the antagonistic proteins MIR and MSAP. In this study MSAP was found to be the positive regulator increasing MRLC levels and stimulating neurite outgrowth.

MSAP, as cloned here, was similar to the sequence found in the data base under the name TMEM4. However, so far no function had been assigned to this putative protein (22). In the present study, the protein was found to interact with MIR, and we propose therefore the name MSAP, MIR-interacting saposin-like protein.

MSAP contains a saposin domain with the conservation of six cysteine residues and adjacent hydrophobic amino acids.

**Fig. 6.** MSAP stimulates neurite outgrowth inhibited by MIR. A, MSAP was transfected into N2-A neuroblastoma cells alone or in combination with MIR. The EGFP-expressing vector served as control. The number of cells bearing neurites was determined after 24 h. Experiments were done in triplicates, and the values represent mean ± S.E. **, p ≤ 0.01 for control versus MSAP-expressing cells and for MSAP-expressing versus MIR-expressing cells. *, p ≤ 0.05 for MSAP- and MIR-expressing versus MIR-expressing cells. B, MSAP was transfected into PC12 cells cultured in the absence or presence of NGF. The number of cells bearing neurites was determined after 48 h. Experiments were done in triplicates, and the values represent mean ± S.E. **, p ≤ 0.01 for control versus MSAP-expressing cells.
with highest overall homology to SAP-B. The saposin domain is present in SAP-A, -B, -C, and -D saposins (16) and in other proteins with a similar primary structure, such as NK lysin (17). The crystal structure of NK lysin shows a globular structure of the protein formed by three disulfide bridges and two central hydrophobic pockets. MSAP contains the saposin domain interrupted by two stretches of 30 and 40 amino acid residues, respectively, which probably are situated in the loop regions, as defined by the crystal structure of NK lysin (17). Therefore, it is reasonable to assume that MSAP may have a structure similar to NK lysin and may exhibit the saposin fold.

Prosaposin plays a role in glycosphingolipid metabolism but can also influence death of cultured glial cells (28) and cerebellar granule neurons (29). In PC12 and neuroblastoma cell lines, prosaposin and SAP-C have been shown to induce neurite outgrowth and act as neurotrophic factors (30). The neurotrophic activity in the molecules was localized to a stretch of around 10 amino acids (30, 31). Interestingly, MSAP lacks this stretch of amino acids. The mechanisms for the stimulation of neurite outgrowth by MSAP are therefore different from the saposins and can account for its interaction with MIR and the regulation of MRLC levels.

The findings in the present work that MSAP represents an important factor involved in regulation of neurite outgrowth not only were based upon data from overexpression studies but also were substantiated by experiments using RNA silencing of MSAP. In these experiments there was a significant 65% reduction in neurite outgrowth after the down-regulation of MSAP. This data shows that the levels of MSAP are probably tightly regulated in neuronal cells so as to allow neurite outgrowth and its inhibition. In addition, MIR may also be regulated by different stimuli in the cells or during cell motility. These aspects need to be addressed in more detail in the future.

Neurite outgrowth is driven by different cellular signals converging on the actomyosin complex. MRLC directly controls myosin II activity and the overall contractile force in the cell.
The activity of myosin itself is mainly influenced by protein phosphorylation through the opposing activities of myosin light chain kinases and phosphatases (32). Among the protein kinases, RhoA causes changes in myosin phosphorylation and alters the assembly of stress fibers and focal adhesions (33). In neuronal cells, enhanced activity of RhoA and the downstream kinase, ROCK, affects growth cone morphology and induces neurite retraction (24, 25). Although much is known about the roles played by different kinases in myosin activation, the significance of protein-protein interactions that may influence MRLC or its level is not fully understood. In this study we observed that the levels of MRLC were regulated by MSAP and MIR, which acted in an antagonistic manner with regard to the protein. The results showed that MRLC is regulated not only through phosphorylation but also at the level of protein degradation. These two modes for control of MRLC, however, may interact, and it is important to study whether MSAP can also affect the phosphorylation status of MRLC.

Previous studies have shown that different myosin isoforms are present in brain including myosin IIA and IIB (34). Myosin II is involved in neurite outgrowth, and myosin IIB gene-deleted mice showed changes in growth cone motility (35). Hyperactivity of myosin induced by constitutively active myosin light chain kinase altered axon guidance in Drosophila (36), and both myosin IIA and -B can form bipolar filament networks in growth cones (37). Apart from phosphorylation events affecting the activity of myosin, the motor properties of the molecule may also be regulated directly by MRLC (38). In addition, expression levels of MRLC have been shown to vary in pancreatic cancer cells (39) and in cardiac myocytes (40). However, changes in the levels of MRLC in neuronal and other cells have so far not been studied. The present results suggest that MRLC levels are tightly regulated in the cell through the interplay between the molecules MSAP and MIR, which are linked to changes in neurite outgrowth. In view of the importance of MRLC in the regulation of the actomyosin complex and cytoskeletal activities, it is probable that the observed mode of regulation of MRLC levels by MSAP and MIR has profound effects in cell motility in other systems, too. We are currently studying these issues using cell types other than neurons.

In the present work we also studied the possible mechanism by which MRLC is regulated under the influence of MSAP and MIR. MSAP increased the levels of MRLC probably by stabilizing the protein and counteracting the effects of MIR. MIR was found to down-regulate MRLC in a process that was largely blocked by the inhibition of proteasome activity. MRLC was also ubiquitinated in the presence of MIR, indicating that MRLC becomes a target for ubiquitination by MIR. MIR was found previously to bind MRLC, which led to the inhibition of neurite outgrowth (15). The present results suggest a mechanism for this effect of MIR through its induction of MRLC degradation via ubiquitination. Besides the ERM domain, MIR has a RING finger motif in the C-terminal region (15). It has been shown that such domains in other proteins act as ubiquitin ligases and are involved in the ubiquitination of target proteins leading to their degradation (26, 27). The present results indicate that the RING finger in MIR may also possess such a ubiquitin ligase activity, and we show here that MRLC becomes ubiquitinated in the presence of MIR. We are currently studying the exact mechanism and regions in MIR responsible for the ubiquitination of MRLC and their interplay with MSAP.

In conclusion, we have identified the novel human protein MSAP, which is involved in the regulation of MRLC levels and in the stimulation of neurite outgrowth. MSAP acts in an antagonistic manner to MIR, which can down-regulate MRLC in a process involving ubiquitination and degradation of the molecule. The exact pathways influenced by MIR and MSAP in controlling the stability and degradation of MRLC remain to be studied in more detail. In view of the observed effect of MSAP on cell motility and neurite outgrowth, it will also be interesting to study the role played by MSAP in different human disorders.

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