

Research Article

MSAP enhances migration of C6 glioma cells through phosphorylation of the myosin regulatory light chain

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Abstract. A key regulatory mechanism in cell motility is the control of myosin activity, which in non-muscle cells is determined by phosphorylation of the myosin regulatory light chain (MRLC). Here we show that MRLC-interacting protein (MIR)-interacting saposin-like protein (MSAP) enhances cell spreading in fibroblasts and migration of rat C6 glioma cells through increases in MRLC phosphorylation. Overexpression of MSAP enhanced the motility of glioma cells measured in matrigel invasion chambers and using a scratch assay. Downregulation of

MSAP by RNA interference significantly decreased glioma cell migration and phosphorylation of MRLC. Inhibition of the corresponding MRLC kinase by ML-7 did not affect migration of MSAP-overexpressing cells. The present results show that MSAP controls glioma cell migration via enhancement of MRLC phosphorylation. This effect is independent of the activity of MRLC kinase. Thus, MSAP is a novel modulator of cell motility that influences migration of glioma cells and possibly other tumors.

Key words. MSAP; myosin regulatory light chain; phosphorylation; C6 glioma; cell motility.

Cell migration in general and tumor cell motility in particular are complex processes regulated by the interaction of specific proteins in the extracellular space with those in the cell membrane, to result in signaling to the cell cytoskeleton [1, 2]. Tumor cells, such as gliomas, exhibit a high rate of cell motility and migration that contributes to the invasiveness of the tumors [3]. The molecular mechanisms regulating tumor cell motility are, however, not fully understood. In many cells, integrins in the cell membrane interact with extracellular matrix components, leading to the activation of signaling pathways, which in turn can regulate effector molecules such as the small GTPases [4]. In addition, turnover of focal adhesions is required to facilitate cell movement [5]. The ultimate targets of these signaling events are the microtubules and the actomyosin system, the activity of which leads to cell spreading and migration.

The actomyosin complex is a key determinant for alterations in cell shape and contractility [6, 7]. Phosphorylation of the myosin regulatory light chain (MRLC) at Ser19 is the major driving force for the activity of the actomyosin complex. It is influenced mainly by the opposing activities of MRLC kinase (MRLCK) and Rho-kinase (ROCK), and the corresponding phosphatases [8–10]. Recently, ZIP kinase was shown to regulate MRLC phosphorylation in fibroblasts at both Ser19 and Thr18 residues [11]. Different proteins, including Ca²⁺/calmodulin, act on MRLCK, influencing the phosphorylation and the activity of this enzyme [9]. In fibroblasts, the activity of MRLCK was shown to be crucial for the turnover of adhesion sites at the leading edge of the cells [5].

Members of the Rho family of small GTPases, including Cdc42, Rac1 and RhoA, are major regulators of the actin cytoskeleton, having opposite effects on MRLC phosphorylation and cell contractility [2, 12, 13]. Rac and Cdc42

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inactivate MRLCK through the p21-activated kinase (PAK) [14], whereas RhoA, via ROCK, inactivates the myosin phosphatase thereby enhancing MRLC phosphorylation [10, 15, 16]. This can also be accomplished by a direct phosphorylation of MRLC by ROCK [8]. The proteins of the Rho family also interact with other proteins in the cell that can bring about changes in cell shape and motility. Among them are signaling proteins, linking the activity of Rho proteins to the activation state of the cell [2, 9], and the ezrin/radixin/moesin ERM proteins, which are involved in membrane-cytoskeleton interactions [17–20].

We have recently cloned MRLC-interacting (MIR)-interacting saposin-like protein (MSAP), a protein that positively influences neurite outgrowth [21]. The activity of MSAP was linked to its interaction with MIR, an E3-ubiquitin ligase that downregulates MRLC through the ubiquitin-dependent proteasome system [21]. In the present work, we studied the role of MSAP in cell spreading of fibroblasts and migration of C6 glioma cells, a model for tumor cell invasiveness. The results show that MSAP dramatically increases glioma cell migration through MRLC phosphorylation without affecting the protein levels.

Materials and methods

Plasmids

The following constructs were used: full-length MSAP cloned into either pcDNA3C (Invitrogen), pEGFP-C1 or pDS-N1 (Clontech). Deletion constructs encoding amino acids (aa) 1–146 and 146–182 were constructed using an *EcoRI* site in the MSAP sequence. All the deletion constructs were subcloned into pEGFP-C1 and sequenced. For controls, empty pcDNA-3C, pEGFP-C1 or pDS-N1 were used.

Cell culture

Swiss 3T3 fibroblast or rat C6 glioma cells were cultured in DMEM containing 10% fetal calf serum. Fibroblasts were transfected with either empty or His-MSAP expression vector using Lipofectamine 2000 (Invitrogen), and stable clones were selected using G418 (400 µg/ml; Life Technologies). Protein expression was determined by Western blotting (fig. 1A, left). The level of expression of His-tagged MSAP compared to control cells is clearly evident. For transient transfection in C6 glioma cells, the above-mentioned constructs were used. Again, protein expression was analyzed on Western blots (fig. 1A, right). In the upper panel, the expression level of pDS-tagged MSAP is shown, and in the lower panel, endogenous MSAP in both cell types is revealed.

Spreading assays

To study cell spreading, control and His-MSAP-overexpressing cells were plated for 1 h onto culture dishes

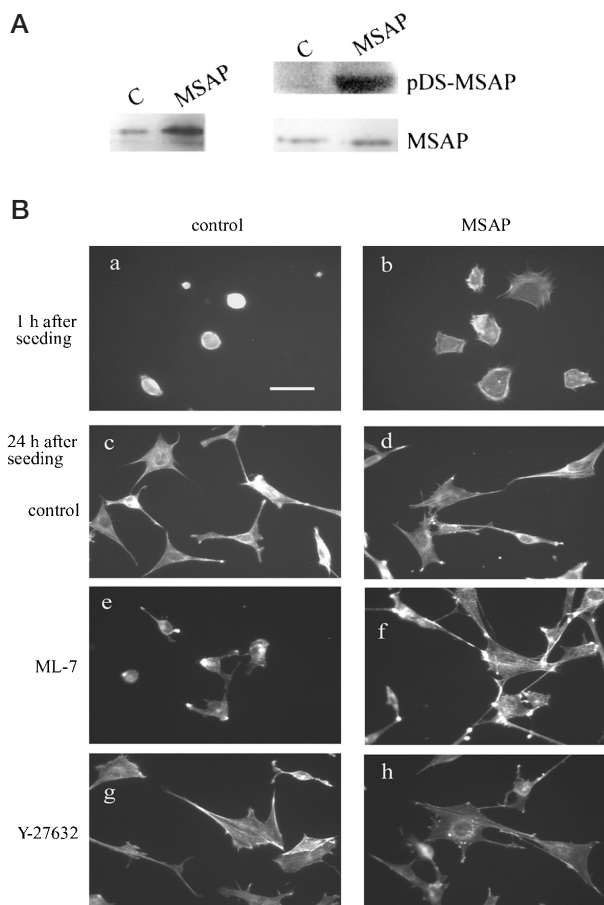


Figure 1. MSAP overexpression enhances spreading of fibroblasts (A) 3T3 (left) or C6 glioma cells (right) were stably transfected with the indicated control or MSAP expression plasmids. The expression of MSAP was then analyzed on a Western blot using anti-MSAP antibodies. (B) 3T3 fibroblasts were mock transfected (a, c, e, g) or stably transfected with MSAP (b, d, f, h) and plated onto culture dishes coated with fibronectin. Note the difference in cell spreading at 1 h after seeding. After 24 h, 10 µM ML-7 (e, f), 10 µM Y-27632 (g, h) or vehicle (c, d) were added to cells for 30 min. Cells were fixed and the stress fibers were visualized using phalloidin-rhodamine. Note the difference between control and MSAP-expressing cells after ML-7. Scale bar, 25 µm.

precoated with fibronectin (50 µg/ml), washed and fixed using 4% paraformaldehyde (PFA) in PBS. To study effects of inhibitors, the cells were incubated overnight in serum-free DMEM, and 10 µM ML-7 or 10 µM Y-27632 was added for 30 min; vehicle (ethanol) was used for the control. The cells were fixed with 4% PFA and stained with TRITC-labeled rhodamine, viewed under epifluorescence (Zeiss Axiophot 2 microscope), and photographs were taken using a CCD camera. Western blots of cell lysates were done essentially as described previously [21]. The phosphorylation of MRLC was determined using a specific anti-pMRLC antibody (No. 3671; Cell Signaling Technology). To control for equal loading of samples, the blots were stained with Ponceau S and, additionally, incubated with anti-actin antibodies (Sigma).

Migration assay for glioma cells

Rat C6 glioma cells were used for migration assays. Cells were plated on coverslips coated with buffer, BSA (1 mg/ml) or poly-L-lysine (100 µg/ml), and transiently transfected with the following constructs using lipofectamine (Invitrogen): pDS-N1, pDS-MSAP, siMSAP RNAs or scramble siRNA sequences, and grown to confluence. Using a P200 pipette tip, a scratch was made by scraping across the coverslips, and several lines were drawn at intervals perpendicularly to the scratch to facilitate orientation and to make sure that the same areas were evaluated before and after the scratch. The cells were incubated further; inhibitors or vehicle were added as above. Pictures were taken at time zero and 18 h, and the extent of migration was analyzed using the NIH image software (<http://rsb.info.nih.gov/nih-image/Default.html>).

For the transwell migration assay, BioCoat matrigel invasion chambers were used (BD Biosciences). C6 glioma cells were transfected with either pDS, pDS-MSAP or the three deletion constructs. After trypsinization, 2.5×10^4 cells were added to the chamber, and the cells were allowed to migrate for 22 h. The total number of transfected cells was assessed, non-invasive cells were removed from the upper compartment using a cell scraper, and invasive cells were analyzed by fluorescence microscopy.

All experiments were done in triplicate, and the Student's t test or the Mann-Whitney U test were used for statistical analysis.

RNA silencing

To downregulate MSAP in glioma cells we used the silencer siRNA cocktail kit (Ambion). Sense and antisense long RNA covering the whole cDNA was synthesized from T7 primers. After annealing, thereby generating long dsRNA, small dsRNAs were made by digestion with RNaseIII. MSAP-specific siRNAs were purified on columns. For the control, in vitro-transcribed scramble siRNA sequences were used (Ambion). The efficiency of silencing was assessed both by immunocytochemistry and Western blotting. Rat C6 glioma cells were transfected with either MSAP-specific siRNAs or with scramble sequences using lipofectamine at 25 nM. Two days after transfection, the cells were treated with inhibitors (vehicle, ML-7 at 10 µM, Y-27632 at 10 µM). The cells were washed with PBS and lysed in RIPA buffer, and equal amounts of proteins as determined by the DC protein assay (Bio-Rad) were run on 12% SDS-PAGE. The following primary antibodies were used in combination with horseradish peroxidase-tagged goat anti-mouse or anti-rabbit secondary antibodies: rabbit anti-MSAP [21] at 1:3000, rabbit anti-pMRLC (No. 3671; Cell Signalling Technology) at 1:1000, mouse anti-MRLC (Sigma) at 1:3000 and rabbit anti-actin (Sigma) at 1:3000. The signals were visualized with enhanced chemiluminescence (Pierce).

Immunocytochemistry

Transfected or untransfected glioma cells were fixed in 4% PFA, washed and permeabilized in 0.05% Triton X-100 in PBS/10% normal goat serum. The actin cytoskeleton was visualized with phalloidin-rhodamine.

Results

MSAP affects spreading of fibroblasts

To study the function of MSAP in Swiss 3T3 fibroblasts, we transfected the expression vector encoding His-tagged MSAP into cells that were subsequently fixed and stained for actin by phalloidin-rhodamine. Data showed that at early time points, MSAP-transfected cells spread more than control cells (fig. 1B). At 24 h after seeding, the difference between control and MSAP-expressing cells diminished, and cells displayed several long protrusions. Treatment with ML-7 for 30 min to inhibit MRLCK [22] did not change the morphology of MSAP-expressing cells, in contrast to controls (fig 1B). Staining for actin revealed an almost complete loss of stress fibers in control but not MSAP-expressing cells after ML-7 treatment. On the other hand, treatment of control and MSAP-expressing fibroblasts with Y-27632, which is a specific ROCK inhibitor, did not alter cell morphology.

MSAP affects the phosphorylation of MRLC

To study the mechanisms by which MSAP affects the cytoskeleton, we analyzed the phosphorylation of the myosin regulatory light chain MRLC that is a key regulator of cell motility [23]. MRLC was phosphorylated to a higher extent in MSAP-overexpressing fibroblasts, compared with control cells (fig. 2A). At the same time there was no change in the steady-state levels of MRLC between control and MSAP-overexpressing cells (fig. 2A). The addition of ML-7 did not affect phosphorylation of MRLC in MSAP-expressing cells, although it caused a decrease in control cells (fig. 2A). Quantification of data revealed that MRLC phosphorylation was reduced by about 70% after ML-7 in control cells, and remained virtually unchanged in those expressing MSAP (fig. 2B). In contrast, treatment of the cells with Y-27632, inhibiting ROCK, decreased MRLC phosphorylation by about 60% in both MSAP-overexpressing and control cells. This suggests that in fibroblasts, MSAP does not affect the steady-state levels of MRLC but influences the phosphorylation state of the protein through a mechanism not involving MRLCK.

MSAP enhances migration of rat C6 glioma cells

C6 glioma cells are known to exhibit a high rate of cell motility and represent a good model to study cell migration [24]. We found that MSAP is expressed in these cells, as shown using the specific antibody (fig. 3). For this

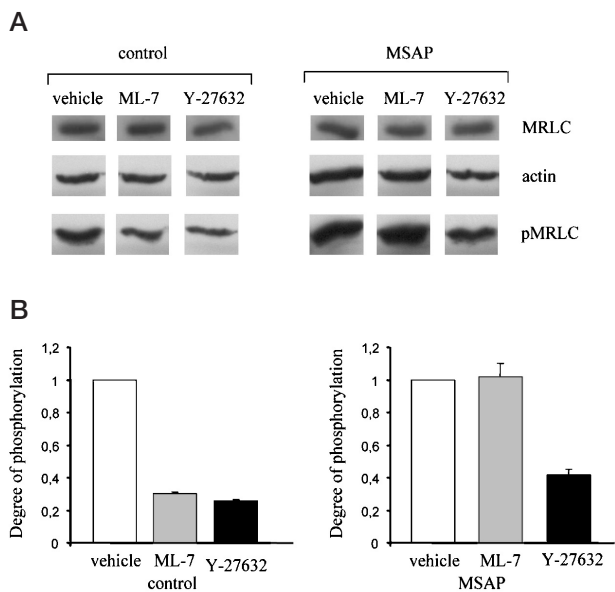


Figure 2. MSAP enhances MRLC phosphorylation. Phosphorylation of MRLC was determined in control and MSAP-expressing cells using the specific anti-pMRLC antibody. (A) MRLC phosphorylation is enhanced by MSAP overexpression. Note higher levels of MRLC phosphorylation in MSAP-expressing cells and the lack of decrease after ML-7 treatment. The protein levels of MRLC were not changed by the treatments. (B) Quantification of MRLC phosphorylation after inhibitor treatment was done using NIH image software. Data are shown as mean + SD (n = 3).

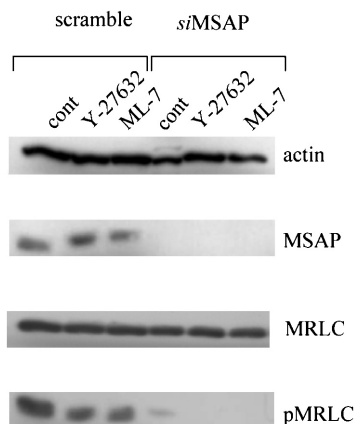


Figure 3. MSAP downregulation inhibits MRLC phosphorylation. C6 glioma cells were transfected with either scramble or MSAP-specific siRNAs and treated as indicated. The effect of MSAP silencing was assessed with immunoblotting using anti-actin, anti-MSAP, anti-MRLC and anti-pMRLC antibodies. Note the efficient downregulation of MSAP compared to the control and the concomitant downregulation of MRLC phosphorylation.

study, we employed a migration chamber and matrigel basement membrane matrix using control and MSAP-overexpressing glioma cells. The results showed that 70% of the MSAP-expressing glioma cells invaded the matrix within 18 h, whereas only 35% of the control cells did (fig. 4A). This shows that the presence of high MSAP

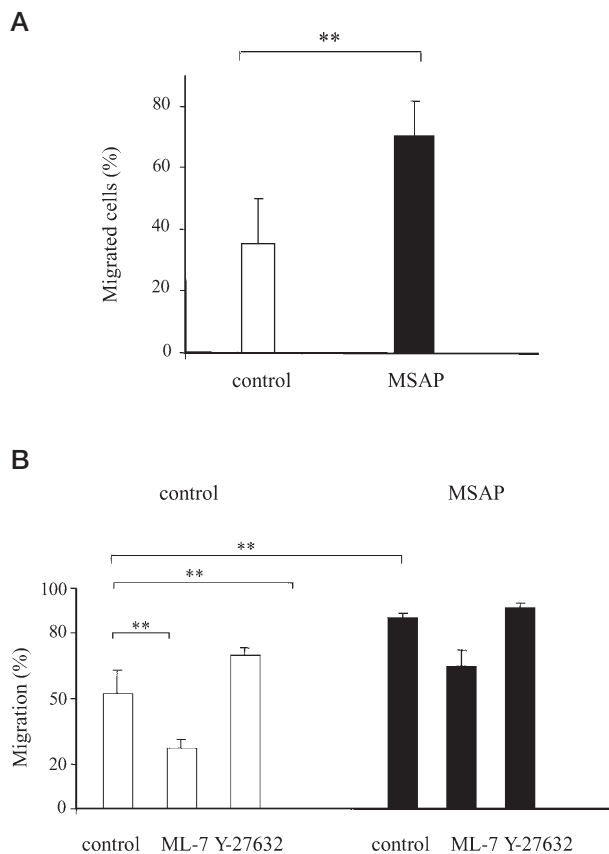


Figure 4. MSAP overexpression enhances migration of glioma cells. (A) Using matrigel invasion chambers, the migration of the cells was analyzed. Note that MSAP-overexpressing cells invade the matrix twice as efficiently as control cells. Values are given as mean + SD; **p ≤ 0.05 (n = 3). (B) Migration of rat C6 glioma cells was assessed using a scratch assay. Control and MSAP-overexpressing cells were seeded and grown to confluence. A scratch was made across the monolayer, vehicle or inhibitors (ML-7 or Y-27632 at 10 μM each) were added, and the cells were allowed to migrate. Migration was analysed using NIH image software. Note the higher migratory potential of MSAP expressing cells, which is only partially inhibited by ML-7. Data are shown as mean + SD; **p ≤ 0.05 (n = 3).

levels enhances glioma cell migration significantly. This increase in glioma cell migration required the complete MSAP protein, since none of the truncated proteins used in this study, neither the one lacking the N terminus (comprising aa 1–146) nor that without the C terminus (comprising aa 146–182), had an effect on migration, as compared with control cells.

To study this further, a scratch assay was applied, in which a scratch was made through the confluent cell layer and cells allowed to migrate. In this setup, MSAP-overexpressing glioma cells were found to bridge the gap more readily than controls (87% vs 48% control cells; see fig. 4B). These assays together with data from the invasion chamber show that overexpression of MSAP results in a higher ability of glioma cells to migrate. The enhanced migration of MSAP-overexpressing cells was observed

irrespective of different coating conditions (data not shown). Moreover, as in fibroblasts, the addition of ML-7 did not significantly affect the migration of MSAP-overexpressing glioma cells, although inhibiting that of controls by about 50% (fig. 4B). Using the compound Y-27632 to block the activity of ROCK, there was no significant effect on migration of MSAP-expressing or control glioma cells. This suggests that in the C6 cells under these conditions, the activity of MRLCK is more involved in cell migration than that of the ROCK. Most significantly in these cells, the function of MSAP was linked to neither the activity of MRLC nor that of Rho kinase.

Downregulation of MSAP using RNAi

To substantiate the role of MSAP in cell migration, MSAP was downregulated using RNA silencing in C6 cells. The efficiency was assessed by Western blotting that showed a significant decrease in MSAP in the glioma cells (fig. 3). Most importantly, using the scratch assay, MSAP-deficient cells migrated much less than controls (Fig. 5A). Results showed that in MSAP-deficient cells, the phosphorylation of MRLC was heavily decreased, whereas

the level of MRLC protein itself was not changed (fig. 3). Control experiments using scrambled siRNA sequences produced no decrease in the phosphorylation or in the levels of MRLC (fig. 3). Treatment of cells with ML-7 and Y-27632 reduced MRLC phosphorylation in both MSAP-deficient and control glioma cells (fig. 3). Likewise, inhibition of MRLCK with ML-7 reduced migration of MSAP-depleted cells to a similar extent as in control cells. The results show that glioma cell migration is significantly decreased in cells lacking MSAP, although the basic migratory responses toward inhibition of the activities of the MRLC and ROCK remain intact.

Discussion

One hallmark of cancer cell invasion is increased tumor cell spreading and migration. Cell motility is generated by the activity of, among others, myosin II, which is eventually transduced to rearrangements of focal adhesions and integrin-assembled protein complexes at the cell membrane. Crucial for an understanding of the sequence of events regulating cell movements is an elucidation of the role of the different molecules involved. We have recently identified and characterized MSAP, a protein that enhances neurite outgrowth via interaction with MIR [21]. MIR was shown to downregulate the levels of MRLC by ubiquitination and degradation. MSAP in turn counteracted the decrease in MRLC caused by MIR, showing that MRLC is a target for MSAP in neural-like cells [21]. Adding to the function of MSAP, we show here that MSAP enhances migration of rat C6 glioma cells and spreading of fibroblasts through phosphorylation of MRLC without affecting the levels of MRLC. Thus, the mechanism of action of MSAP in cell motility seems to be cell type specific.

In the present study, we observed that overexpression of MSAP enhanced migration of C6 glioma cells, while silencing of MSAP by RNAi reduced cell migration. These results show that the relative levels of MSAP contribute to the extent of migration of glioma cells, making MSAP an interesting target for intervention studies.

The mechanisms behind these effects were related to changes in the phosphorylation status of MRLC induced by MSAP, suggesting that MRLC can be a direct or indirect target for MSAP in glioma cells. We found that MSAP significantly increased the levels of MRLC phosphorylation in overexpressing cells. Enhanced MRLC phosphorylation by MSAP was related to an increase in cell migration observed in both an invasion chamber using matrigel matrix as well as the scratch assay. Blocking MRLCK activity with ML-7 did not reduce migration or MRLC phosphorylation in MSAP-expressing glioma cells in contrast to control cells. The results showed that

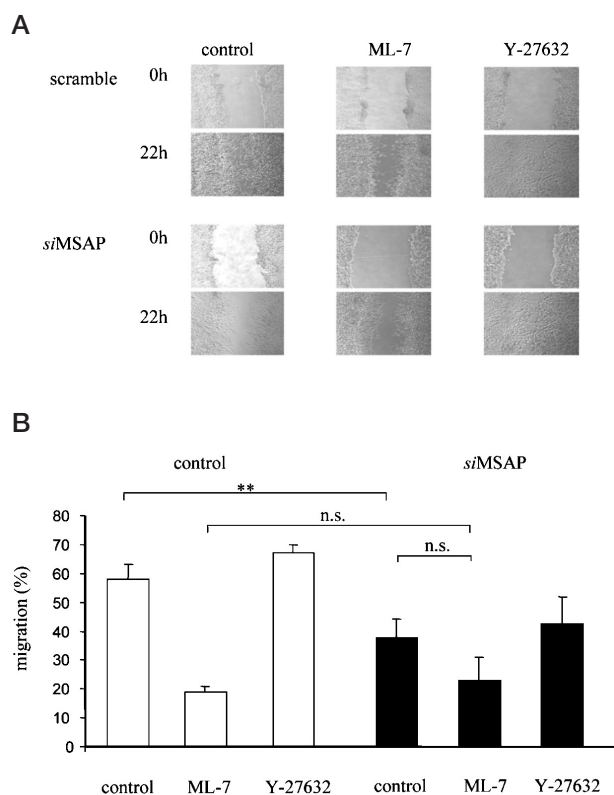


Figure 5. MSAP downregulation reduces glioma cell migration. (A) Migration of rat C6 glioma cells was analyzed in a scratch assay after downregulation of MSAP by silencing in the presence or absence of ML-7 or Y-27632 (10 μ M each). Images at 0 and 22 h after scratch are shown. Note the lower migration potential of cells lacking MSAP when compared to control cells. (B) Quantification was done as described. Data are shown as the mean + SD; ** $p \leq 0.05$; n.s., not significant ($p > 0.05$) ($n = 3$).

the effect of MSAP is largely independent of the activity of MRLCK in the glioma and 3T3 fibroblast cells. In contrast, employing Y-27632 to block the activity of ROCK reduced MRLC phosphorylation brought about by MSAP expression in the fibroblast but not in the C6 glioma cells. This suggests that whereas the effect of MSAP may be linked to the ROCK pathway in the 3T3 cells, MSAP probably uses other cellular pathways or kinases in the glioma cells.

We have earlier shown that MSAP resides at the endoplasmic reticulum (ER) in neural cells [21], and this was also observed here for glioma cells (data not shown). MSAP could be involved in ER-related responses controlling Ca^{2+} levels and thus influence the activity of protein kinases. Targeting of MSAP to the ER was found essential for its action, as truncated MSAP proteins lacking the ER-targeting sequence had no effect in the migration assay. We are currently studying the role of the ER in the MSAP response using specific blockers of calcium fluxes and specific kinase inhibitors in these cells.

Apart from MRLCK and ROCK, several other kinases are known to phosphorylate MRLC, including ERK, death-associated protein kinase (DAPK [25]), zipper-interacting protein kinase (ZIPK [26]) and PAK family members (PAK2 [27]). Future analyses will show whether MSAP could interfere with one or several of these kinases to exert its function.

Taken together, the present results show that MSAP is an essential factor influencing cell spreading of fibroblasts and migration of glioma cells. Overexpression of MSAP enhanced, whereas downregulation of the protein by RNA silencing decreased glioma cell migration. These effects of MSAP were correlated to altered levels of MRLC phosphorylation independently of the activity of the corresponding MRLCK. The exact mechanisms underlying the effect of MSAP on glioma cell migration and the nature of the kinases involved remain to be studied in more detail. In view of the present data and the observed effects of MSAP on migration of C6 glioma cells, MSAP seems an interesting target to consider for inhibition of cancer cell migration and/or invasion.

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