Oligomeric Forms of the Metastasis-related Mts1 (S100A4)
Protein Stimulate Neuronal Differentiation in Cultures
of Rat Hippocampal Neurons

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Neuronal differentiation and axonal growth are controlled by a variety of factors including neurotrophic factors, extracellular matrix components, and cell adhesion molecules. Here we describe a novel and very efficient neuritogenic factor, the metastasis-related Mts1 protein, belonging to the S100 protein family. The oligomeric but not the dimeric form of Mts1 strongly induces differentiation of cultured hippocampal neurons. A mutant with a single Y75F amino acid substitution, which stabilizes the dimeric form of Mts1, is unable to promote neurite extension. Disulfide bonds do not play an essential role in the Mts1 neuritogenic activity. Mts1-stimulated neurite outgrowth involves activation of phospholipase C and protein kinase C, depends on the intracellular level of Ca\textsuperscript{2+}, and requires activation of the extracellular signal-regulated kinases (ERKs) 1 and 2.

S100 proteins were first identified in brain and were for a long time considered to be nervous system-specific proteins (1). Later S100 proteins were discovered in a variety of other tissues and cells. In the nervous system S100 proteins are highly enriched in glial cells. The primary structure of S100 proteins is highly conserved (2). The tertiary structure of several S100 proteins has been reported, revealing a novel homodimeric fold (3). Structural studies have demonstrated the presence of intramolecular disulfide bridges in S100A7 and intermolecular disulfide bridges between two monomers in S100A10. An S100 monomer contains two EF-hand calcium binding domains (2). The calcium binding results in a conformational change with subsequent exposure of a hydrophobic patch through which S100 proteins can interact with their target (4). Intracellularly, S100 proteins have been shown to interact with numerous targets and modulate multiple cellular processes including remodeling of the cytoskeleton, cell growth, and differentiation (5, 6). Extracellularly, disulfide-linked dimers of the S100B protein have been demonstrated to stimulate neurite outgrowth in primary cultures of neurons from the cerebral cortex (7).

The \textit{mts1/S100A4} gene was isolated as a gene specifically expressed in metastatic tumor cell lines (8), and results obtained in transgenic mice strongly suggest an important role for the \textit{mts1} gene in tumor progression (9, 10). The biological functions of the Mts1 protein are only partially known. It has been shown that Mts1 is associated with actin stress fibers, and probably it is through this interaction that Mts1 affects the organization of the actin cytoskeleton and cellular motility (11). The heavy chain of non-muscle myosin (MHC)\textsuperscript{1} has been identified as an intracellular target of Mts1 (12). The role of Mts1 in metastasis has been suggested to depend on its effect on cell motility and possibly cytokinesis by controlling myosin assembly/disassembly presumably through modulation of MHC phosphorylation by protein kinase C (PKC) (13).

We here show for the first time that the Mts1 protein can function extracellularly as a very efficient neuritogenic factor. Neurite outgrowth induced by the oligomeric form of Mts1 is not dependent on the presence of intermolecular disulfide bonds. We also addressed the signal transduction mechanisms underlying Mts1-induced neuronal differentiation and showed that Mts1-stimulated neurite outgrowth involves activation of phospholipase C and protein kinase C, depends on the intracellular level of Ca\textsuperscript{2+}, and requires activation of the extracellular signal-regulated kinases (ERKs) 1 and 2.

EXPERIMENTAL PROCEDURES

Materials—A phospholipase C (PLC) inhibitor, U-73122 (1-((17b-
3-methoxyestra-1.3–4(10)-trien-17-yl)amino)hexyl)-1H-pyrrrole-2,5-
dion), two PKC inhibitors, calphostin C (UCN-10928c) and Safingol
((2S,3S)-2-amino-1,3-oxadecanediol), two inhibitors of L-type voltage-operated channels, nifedipine and verapamil, an intracellular Ca\textsuperscript{2+} antagonist, which blocks the release of Ca\textsuperscript{2+} from intracellular stores, TMB-8 (8-(1-N,N-diethylamino)octyl-3,4,5-trimethoxybenzolate), a calmodulin (CaM) kinase II inhibitor KN-62 (1-[N-(O-bis-1,5-isoquinolinesulfonyl)-N-methyl-1-tyrosyl-4-phenylpiperazone], a CaM antagonist R-cAMPS were all from Calbiochem. Two non-competitive inhibitors of mitogen-activated protein (MAP) kinase kinase, PD98059 and U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminoethyl]thiobuta
diene), were from New England Biolabs (Beverly, MA) and Promega Corp. (Madison, WI), respectively. According to the manufacturer, the \textit{IC\textsubscript{50}} values for the inhibitory activity of PD98059 and U0126 against MAP kinase 1 are \textasciitilde5–10 \mu M and 50 \mu M, respectively. p42/44 MAP kinase (Thr-202/Tyr-204), stress-activated protein kinase/c-Jun NH\textsubscript{2}-terminal kinase (Thr-183/Tyr-185) and p38 MAP kinase (Thr-180/\n
\textsuperscript{1} The abbreviations used are: MHC, myosin heavy chain; PKC and
PKA, protein kinase C and A, respectively; ERK, extracellular signal-
regulated kinase; CaM, calmodulin; MAP, mitogen-activated protein;
FGF, fibroblast growth factor; PAG, polyacrylamide gel electrophore-
sis; PBS, phosphate-buffered saline; wt, wild type; PLC, phospholipase
C.

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Tyr-182) antibody kits were from New England Biolabs. Recombinant human basic fibroblast growth factor (FGF) was from Life Technologies, Inc. Homodimeric S100 isofrom from bovine brain, composed of ββ subunits (S100B) was from Calbiochem. Recombinant rat S100A1 was cloned and produced as described (14).

**Characterization and Purification of Proteins**—For Mts1 mutagenesis, all mutations were introduced into Mts1 by the inverse polymerase chain reaction using the pQE30-Mts1 plasmid (13) as a template with the following primers: reverse primer, 5′-CCCTCGGAAGTCAACTTCTG-3′; Y57F forward primer, 5′-TTGCTCGTTCTGGTGCTCGTA-3′; Del75 forward primer, 5′-GGTTGCTCGTTCTGGTGCTCGTA-3′. Cys82 substitutions in Mts1 mutants were produced as described (12). A recombinant C-terminal peptide of human myosin heavy chain named Hmyo4–3B (1762–1961 amino acids) was isolated and purified as described (13).

**Primary Cultures of Hippocampal Neurons, Video Recording, and Image Analysis of Neurites**—Hippocampus was isolated from Wistar rat embryos at gestational day 18, and the dissociated cells were grown as described previously (15). For analysis of neurite outgrowth, 5000 cells/well were seeded on four-well LabTek coverslides and grown in the absence or presence of 5 μM Mts1 for 24 h (Fig. 1a). In dose-relevant concentrations in the range of 5–10 μM Mts1 for the indicated times. Cell extracts were obtained as described previously (17) and kept frozen at −80°C until use. Proteins (20 μg) were separated by SDS-PAGE and transferred to a polyvinylidine fluoride membrane (Millipore, Bedford, MA). After blocking in Tris-buffered saline containing 0.05% (v/v) Tween 20 and 5% (w/v) nonfat dry milk for 1 h at room temperature, membranes were incubated overnight with an anti-phospho-MAP kinase antibody (diluted 1:1000) in the blocking buffer and, after washing, incubated with a goat anti-rabbit IgG horseradish peroxidase conjugate diluted 1:2000 in Tris-buffered saline. Bound antibodies were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). To estimate the total amount of ERK1 and ERK2, membranes were stripped with 50 mM dithiothreitol, 2% SDS in 62.5 mM Tris-HCl, pH 6.8, for 30 min at 60°C and re-probed with an anti-MAP kinase antibody that detects total MAP kinase proteins. The polypeptide bands were quantified by scanning using the PRImage analysis software developed at the Protein Laboratory, Copenhagen University, Denmark, as described previously (17).

**Size Exclusion Chromatography**—A Superdex75 column (1.5 cm × 90.0 cm) (Amersham Pharmacia Biotech) was equilibrated with a buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 1 mM 1,4-dithiothreitol, pH 7.5 (TND buffer) with and without 5 μM CaCl2. The column was calibrated with standard weight determinations using gel filtration chromatography standards (Bio-Rad). 1 ml of the mixed protein standard (2 mg/ml) or 1 ml samples of Mts1 protein were loaded onto the column, and 3-ml fractions were collected and monitored with A280 readings. Dextran blue (Bio-Rad) was applied to the column to determine its void volume. The molecular weight of Mts1 was determined as described in Landar et al. (18).

**RESULTS**

**Mts1 Stimulates Neuronal Differentiation**—The ability of recombinant Mts1 to stimulate neurite outgrowth from hippocampal neurons was tested in vitro. Cultures were grown in serum-free neurobasal medium in the presence of a B27 supplement containing trophic factors necessary for survival and growth of hippocampal neurons. In non-treated cultures, hippocampal neurons did not differentiate by extending processes (Fig. 1a). Treatment of hippocampal neurons with recombinant Mts1 induced sprouting at 12 h (Fig. 1b), extension of long neurites at 24 h (Fig. 1c), and outgrowth of multiple, long, branching neuronal processes at 72 h (Fig. 1d). In dose-response experiments it was found that non-fractionated recombinant Mts1 had its maximal growth stimulation activity at concentrations in the range of 5–10 μM (Fig. 2a). When hippocampal cells were plated on Mts1-coated slides, insoluble Mts1 displayed a neurite outgrowth-promoting activity similar to that obtained by applying Mts1 in the medium (data not shown). We also determined the time of exposure to Mts1...
needed for hippocampal neurons in culture to respond by neurite extension (Fig. 2b). Cells exposed to the Mts1 protein for less than 30 min already displayed a 4-fold increase in the total length of neurites per cell when determined after 24 h of culture. The response reached its maximum, a 10-fold increase, when neurons were exposed to Mts1 for 6 h. Longer incubation times with Mts1 did not lead to a further increase of the response (Fig. 2b). When hippocampal neurons were treated for 24 h with a recombinant control protein (the COOH-terminal peptide of the non-muscle myosin heavy chain MHC), no stimulation of neurite outgrowth was observed. The presence of Mts1-specific antibodies in the culture medium completely abrogated its neuritogenic activity, indicating that the effect of Mts1 on neurite outgrowth was specific (Fig. 2c). Treatment of culture medium with a nonspecific rabbit polyclonal anti-chicken FGF receptor antibodies had no effect on Mts1-stimulated neurite outgrowth (data not shown).

We compared the neuritogenic activity of Mts1 with the

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**Fig. 1.** Confocal micrographs of low density hippocampal cell cultures grown in the absence (a) or presence of 5 μM concentrations of recombinant Mts1 (b, c, and d) for 12 h (b), 24 h (a and c), and 72 h (d). Cells were immunostained for GAP-43; bar = 10 μm.

**Fig. 2.** Effect of Mts1 on neurite outgrowth in primary cultures of dissociated hippocampal cells. a, a dose-response study, in which hippocampal cells were grown for 24 h in the presence of increasing concentrations of Mts1. b, effect of the period of treatment with 5 μM Mts1 on neurite outgrowth. Hippocampal neurons were seeded and allowed to attach for 1 h, after which recombinant Mts1/S100A4 was added (time 0). Subsequently, Mts1 was removed at various times by changing the culture medium, and neurite length per cell was measured 24 h after the addition of the protein. c, specificity of the neuritogenic activity of Mts1. Hippocampal neurons were grown for 24 h in the absence (control) or in the presence of 5 μM recombinant Mts1 (Mts1), or in 5 μM recombinant control protein (the COOH-terminal peptide of the non-muscle myosin heavy chain MHC, MHC-peptide), or in 5 μM Mts1 together with Mts1-specific polyclonal antibodies (Mts1-Ab).
Neuritogenic activity of Mts1 (S100A4)

forms a stable dimer, del75 is unable to form a dimer in the yeast two-hybrid system. Surprisingly, Y75F did not stimulate neurite outgrowth from hippocampal neurons, whereas neurons treated with del75 for 24 h displayed abundant neurites, the rate of neurite outgrowth comparable with that seen in cultures treated with wtMts1 (Fig. 3).

Only Oligomeric Forms of Mts1 Stimulate Neurite Outgrowth—To determine which structural forms of Mts1 have neuritogenic activity, size exclusion chromatography of recombinant wtMts1 and its mutants was performed. The wtMts1 protein was eluted with a distribution of molecular masses ranging from 30 (dimer) to 200 kDa (oligomers) (Fig. 4a). The elution profile of the Y75F mutant was different; 85% of the Y75F protein was eluted from the gel filtration column as a single peak with a molecular weight corresponding to a dimer (Fig. 4b). In contrast, most of the del75 protein was eluted as high molecular weight material (Fig. 4c). Addition of reducing agents or changes of ionic strength did not affect the elution profiles of the dimeric and oligomeric forms of Mts1.

Fractions of peaks I, II, and III from wtMts1, Y75F, and del75, eluted from the Superdex75 column (Fig. 4, a, b, and c) were adjusted to a protein concentration of 50 µM and analyzed by dynamic light scattering. The calculated molecular masses were found to be 28.9 kDa (peak III), 47.2 kDa (peak II), and 143–200 kDa (peak I). Fractions corresponding to peaks I, II, and III of Mts1 were further analyzed by SDS-PAGE (Fig. 4d, upper panel) and by immunoblotting using affinity-purified antibodies (Fig. 4d, lower panel). Under reducing conditions, Mts1 protein appeared as a 13-kDa band in all fractions. Immunoblotting confirmed the presence of Mts1 in the three peaks. The two mutant proteins, Y75F and del75, appeared like the wild type as a 13-kDa band in all fractions (data not shown). We also determined the relative contribution of the different forms of the Mts1 protein eluted from the column as peaks I, II, and III to the neuritogenic activity of the protein. The inserts in Fig. 4, a, b, and c, show that the high molecular weight complexes (peak I) of wtMts1 as well as the del75 mutant (peak II) stimulated neurite outgrowth. We therefore conclude that only oligomeric forms with size tetramer and higher, but not dimeric forms of Mts1, can stimulate neurite outgrowth. The concentration of the oligomeric form of Mts1 that induced the neuritogenic response (calculated per monomer) was approximately 90 nM, which is 50–100 times lower than the optimal concentration of the crude preparation of recombinant Mts1 (Fig. 2a).

Mts1-stimulated Neurite Outgrowth Does Not Require Activation of Protein Kinase A—Extracellular protein ligands such as growth factors trigger a biological response by binding and thereby activating a cell surface membrane receptor. However, the receptor of Mts1 has not yet been identified. Many receptors are coupled to the heterotrimeric G proteins, and ligand binding results in activation of adenylyl cyclase, subsequent production of cAMP and activation of protein kinase A (PKA). We therefore tested whether Mts1-stimulated neurite outgrowth requires the activation of PKA. From Fig. 5a it appears that a cAMP antagonist, Rp-cAMPS, had no effect on Mts1-stimulated neurite outgrowth when tested in a concentration range of 1–50 µM. Rp-cAMPS has been demonstrated to inhibit PKA with an inhibition constant (Ki) of 11 µM (20). We therefore conclude that the neuritogenic response induced by Mts1 does not require activation of PKA.

Mts1-stimulated Neurite Outgrowth Requires Activation of Phospholipase C and Protein Kinase C and Modulation of the Intracellular Level of Ca2+—PLC isozymes are known to be primary intracellular targets of many activated receptors. Therefore, we tested whether Mts1-stimulated neurite out-
growth depended on active PLC. From Fig. 5 it appears that an inhibitor of PLC, U-73122, already strongly inhibited Mts1-stimulated neurite outgrowth at a concentration of 0.5 μM, whereas it had little effect on control cultures. U-73122 has been demonstrated to inhibit PLC with IC50 1.0–2.1 μM in human platelets and neutrophils (21). We therefore conclude that Mts1-induced neurite outgrowth requires activation of PLC.

Activation of PLC results in hydrolysis of phosphatidylinositol 4,5-bisphosphate with subsequent release of two intracellular messengers, diacylglycerol and inositol 1,4,5-trisphosphate. Diacylglycerol is known to mediate the activation of PKC. We...
therefore tested whether Mts1-stimulated neurite outgrowth requires activation of PKC. In Fig. 5c, it can be seen that calphostin C, a potent and specific inhibitor of PKC, inhibited Mts1-stimulated neurite outgrowth with a 50% inhibition at a concentration of 0.07 μM. Safingol, another specific PKC inhibitor that competitively interacts at the regulatory phorbol binding domain and predominantly inhibits PKCα, completely abrogated Mts1-stimulated neurite outgrowth at a concentration of 8 μM (data not shown). Calphostin C and safingol have been demonstrated to inhibit PKC with IC50 values of 50 nM (22) and 37.5 μM (23), respectively. We therefore conclude that the neuromitogenic response of hippocampal neurons to Mts1 requires activation of PKC.

Diacylglycerol and inositol 1,4,5-trisphosphate, generated by hydrolysis of phosphatidylinositol 4,5-bisphosphate, both induce changes in the intracellular levels of Ca2+. Calcium influx into neurons is required for neurite outgrowth stimulated by cell adhesion molecules. To test whether Mts1-stimulated neurite outgrowth requires calcium influx or release of Ca2+ from intracellular stores, we treated hippocampal neurons with the calcium channel blockers, verapamil and nifedipine, or with TMB-8, which blocks the release of Ca2+ from intracellular stores. From Fig. 6, it appears that the L-type calcium channel blockers, verapamil and nifedipine, were effective only at high concentrations, 50% inhibition being observed at 33 and 65 μM, respectively (Fig. 6, a and b). Verapamil and nifedipine have been demonstrated to inhibit voltage-dependent L-type calcium channels at concentrations 2–3 orders of magnitude higher than those necessary to block L-type channels (24). For example, Triggle (25) reports an IC50 value for nifedipine in ileal muscle of 30 nM. This means that L-type channels may not be involved in Mts1-stimulated neuritogenic response.

In contrast, TMB-8 already strongly inhibited Mts1-stimulated neurite outgrowth, with 50% inhibition achieved at a concentration of 6.25 μM (Fig. 6c). TMB-8, one of the first agents described as an exclusive intracellular calcium antagonist, has been shown to inhibit a glutamate-stimulated increase in calcium in cultured cerebellar granule cells with IC50 more than 100 μM, thereby protecting neurons from glutamate-induced neurotoxic damage (26).

We therefore conclude that Mts1-stimulated neurite outgrowth requires modulation of the intracellular level of calcium by means of release of Ca2+ from intracellular stores or possibly by influx into neurons through channels other than L-type...
calcium channels.

Ca\(^{2+}\)-calmodulin kinase II is a major mediator of the action of Ca\(^{2+}\), and activation of Ca\(^{2+}\)-calmodulin kinase II has been shown to regulate numerous neuronal processes, including neurite outgrowth. KN-62 has been shown to bind directly to the calmodulin-binding site of Ca\(^{2+}\)-calmodulin kinase II and inhibit the phosphorylation of the myosin light chain with a \(K_i\) value of 0.9 \(\mu M\) (27). It can be seen that KN-62 already strongly inhibited neurite outgrowth stimulated by Mts1 at a concentration of 1 \(\mu M\), although the inhibition was not total, even at a dose as high as 20 \(\mu M\) (Fig. 6d). We therefore conclude that Mts1-stimulated neurite outgrowth requires activation of Ca\(^{2+}\)-calmodulin kinase II, a mediator of the action of Ca\(^{2+}\).

Mts1-stimulated Neurite Outgrowth Involves the p42/p44 Mitogen-activated Protein Kinase Cascade—Neurite outgrowth induced by growth factors has been shown to require sustained activation of the Ras-MAP kinase cascade. To examine whether the Ras-MAP kinase pathway is involved in Mts1-stimulated neurite outgrowth, hippocampal cells were treated with Mts1 and grown for 24 h in the absence or presence of various concentrations of two inhibitors of MAP kinase kinase (MEK), PD98059 (a) and U0126 (b). c, effect of Mts1 on phosphorylation of ERK1 and ERK2. Hippocampal cells were serum-starved for 16 h and incubated in PBS without or with 5 \(\mu M\) recombinant Mts1 for 10, 30, and 60 min. Cell extracts prepared in SDS-containing buffer were subjected in duplicate to SDS-PAGE and immunoblotted using polyclonal anti-phospho-MAP kinase antibodies or polyclonal anti-MAP kinase antibodies (c, upper panel and lower panel, respectively). d, quantification of ERK1 and ERK2 phosphorylation in experiments performed as shown in c. The activity is expressed relative to the control (0 min), which was set to 100%. Error bars indicate S.E. based on four independent experiments.
To further investigate the role of the MAP kinases in the neuritogenic response induced by Mts1, lysates of primary hippocampal neurons were analyzed for activation (phosphorylation) of the extracellular signal-regulated kinases ERK1 and ERK2, the p38 MAP kinase, and the c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) by means of immunoblotting. Treatment with 5 µM Mts1 induced phosphorylation of ERK1 and ERK2 in a time (Fig. 7, c and d)-dependent manner. Treatment with Mts1 had no effect on phosphorylation of p38 kinase and stress-activated protein kinase/c-Jun NH2-terminal kinase (not shown). We therefore conclude that Mts1-stimulated neurite outgrowth involves activation of the extracellular signal-regulated kinases ERK1 and ERK2.

**DISCUSSION**

The Mts1 protein is one of 18 members of the S100 family of calcium-binding proteins. We here present the first evidence that Mts1 has a function when applied extracellularly. Recombinant Mts1 added to cell culture medium strongly stimulated neurite outgrowth from hippocampal neurons. It is known that many neurotrophic factors have different effects on different type of neurons. For example, basic FGF has been demonstrated to promote neurite outgrowth of vestibulospinal neurons in vitro, but in cultures of embryonic rat hippocampal neurons, it stimulates cell proliferation. S100B has been shown to stimulate neurite outgrowth from a number of neurons including dorsal root ganglia neurons (5). To our knowledge, neuritogenic effects of S100A1 and S100B on embryonic hippocampal neurons in vitro have never previously been tested. Hippocampal neurons cultured in the presence of Mts1 displayed abundant neurites, whereas treatment with FGF, S100A1, and S100B had no effect on these cells. Incubation with specific antibodies abolished this activity of Mts1. The concentrations of recombinant Mts1 required for maximal stimulation was 5–10 µM, which is higher than that needed for other neurotrophic factors (32). However, the calculated effective concentration of Mts1 oligomers was only 90 nM.

Size exclusion chromatography followed by dynamic light scattering analysis of the individual fractions showed that recombinant Mts1 existed both as a dimer and as higher order oligomers. The Mts1 dimer did not exhibit any neuritogenic activity. Neurite outgrowth induction by Mts1 was exclusively associated with high molecular weight complexes. We have previously shown that the tyrosine 75 residue regulates the capacity of Mts1 for dimerization and that substitution of this residue with phenylalanine lead to formation of more stable dimers. The Y75F mutant of Mts1 existed predominantly as a dimer, and it exhibited a negligible neuritogenic activity. Neurite outgrowth induction from hippocampal neurons was analyzed for activation (phosphorylation) of the extracellular signal-regulated kinases ERK1 and ERK2, the p38 MAP kinase, and the c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) by means of immunoblotting. Treatment with 5 µM Mts1 induced phosphorylation of ERK1 and ERK2 in a time (Fig. 7, c and d)-dependent manner. Treatment with Mts1 had no effect on phosphorylation of p38 kinase and stress-activated protein kinase/c-Jun NH2-terminal kinase (not shown). We therefore conclude that Mts1-stimulated neurite outgrowth involves activation of the extracellular signal-regulated kinases ERK1 and ERK2.

**FIG. 8. A model of signaling pathways involved in Mts1-induced neuritogenesis.** Inhibitors are shown in red. DAG, diacylglycerol; MEK, MAP kinase kinase; IP3, inositol 1,4,5-trisphosphate.
Neuritogenic activity of Mts1 (S100A4)

...tion factors, and this activation may be mediated by receptor tyrosine kinases, G protein-coupled receptors, or cytokine receptors (38). Although the receptor for the Mts1 protein has not been identified, the demonstrated activation of ERKs in response to Mts1 treatment suggests an involvement of the Ras/Raf-signaling cascade in Mts1-stimulated neurite outgrowth. However, there are other intracellular pathways that can activate ERKs. Thus, in neurons, activation of PKC (39) or elevation of intracellular calcium also result in phosphorylation of ERKs (40). Activation of ERKs can also be mediated by the non-receptor focal adhesion kinase (FAK), which is activated in neuronal cells in response to elevated calcium (41). The Ca$^{2+}$-calmodulin-dependent protein kinase II may also be involved in activation of ERKs (42). Therefore, the fact that inhibition of the Ca$^{2+}$-calmodulin kinase II kinase in Mts1 prevented Mts1-stimulated neurite outgrowth suggests that an alternative, Ras/Raf-independent pathway may participate in the observed ERKs activation. Intracellular calcium is important for many neuronal functions including neuronal plasticity (43). The mechanism by which the Mts1 protein may increase intracellular calcium in hippocampal neurons is not understood but might occur through calcium release from intracellular stores. Interestingly, we show that Mts1 does require PLC activation and, thereby, phosphoinositide turnover to stimulate neurite outgrowth. It is commonly known that PLC activation through accumulation of diacylglycerol and inositol 1,4,5-trisphosphate results in elevation of intracellular calcium. Thus, the obtained results identify signaling cascades responsible for neuritogenesis promoted by Mts1 stimulation, as shown in Fig. 8, in which it is indicated that Mts1-induced neurite outgrowth depends on modulation of the intracellular level of Ca$^{2+}$, the subsequent activation of mediator of the action of Ca$^{2+}$, Ca$^{2+}$-calmodulin kinase II, and the activation of the PLC-PKC-MAP kinase cascade-ERK1/2 pathway.

There is evidence that S100 proteins can be secreted. Secretion of S100A8 and S100A9 proteins has been observed after activation of human monocytes by protein kinase C (44). Release of Mts1 from intact peridontal ligament cells (45) and cultured metastatic mammary carcinoma cells (46) has also been reported. Recently we have demonstrated that in vivo Mts1 is expressed in mouse embryonic macrophages (47) and in white matter astrocytes of the rat spinal cord (48). It may therefore be hypothesized that Mts1 released from astrocytes or macrophages modulates neuronal differentiation. Additional studies are needed to elucidate the functional significance of Mts1 expression in the nervous system, particularly in astrocytes, to characterize the mechanism of secretion and to identify the receptor involved in the neuritogenic activity of Mts1.

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