

Sphingosine-1-phosphate induces the association of membrane-type 1 matrix metalloproteinase with p130Cas in endothelial cells

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Abstract Membrane-type 1 matrix metalloproteinase (MT1-MMP) plays an important role in sphingosine-1-phosphate(S1P)-dependent migration of endothelial cells but the underlying mechanisms remain largely unknown. Herein, we show that S1P promotes the relocalization of MT1-MMP to peripheral actin-rich membrane ruffles that is coincident with its association with the adaptor protein p130Cas at the leading edge of migrating cells. Immunoprecipitation and confocal microscopy analyses suggest that this interaction required the tyrosine phosphorylation of p130Cas and also involves S1P-dependent phosphorylation of MT1-MMP within its cytoplasmic sequence. The interaction of MT1-MMP with p130Cas at the cell periphery suggests the existence of a close interplay between pericellular proteolysis and signaling pathways involved in EC migration.

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1. Introduction

The formation of new blood vessels by angiogenesis plays a central role in tumor growth [1]. In addition to their role in providing oxygen and nutrients to tumor cells, the high permeability of the neovessels leads to the formation of a fibrin provisional matrix [2] that promotes platelet adhesion, activation and release of several factors that promote angiogenesis [3]. Among these, the bioactive lipid sphingosine-1-phosphate (S1P) induces a variety of EC responses associated with angiogenesis [4] and accounts for the majority of the EC chemoattractive activity generated during blood clotting [5]. This potent promigratory effect of S1P is likely to be important *in vivo* since reduction of the predominant S1P receptor (S1P₁) levels abolishes angiogenesis and markedly suppresses tumor growth [6].

The mechanisms underlying the chemoattractant properties of S1P involve a number of signaling pathways (reviewed in

[4]), including Src family kinase(SFK)-dependent tyrosine phosphorylation of key signaling intermediates, such as the adaptor protein p130Cas [7]. More recently, we have shown that EC migration triggered by S1P also involves membrane type-1 matrix metalloproteinase (MT1-MMP), a transmembrane member of the MMP family that is localized at the cell surface [8,9].

MT1-MMP plays essential roles in cell locomotion and invasion by acting as a potent matrix-degrading protease that proteolyzes a broad spectrum of extracellular matrix (ECM) proteins as well as a number of cell surface-associated adhesion receptors [10,11]. In addition to its proteolytic activity, several studies have indicated that the cytoplasmic domain of MT1-MMP also play a role in these processes, possibly through activation of the extracellular signal-regulated protein kinase (ERK) signaling pathway [12,13] and the interaction of the enzyme with a number of proteins, including tyrosine phosphorylated caveolin-1 [14]. Although the mechanisms by which the cytoplasmic sequence of MT1-MMP influences these processes remain unclear, it may involve a spatiotemporal regulation of the enzyme at the cell surface through both clathrin- and caveolae-dependent endocytosis [15,16]. The LLY⁵⁷³ motif located in the cytoplasmic sequence of MT1-MMP seems to play a central role in this process [15], possibly through SFK-dependent phosphorylation of this unique cytoplasmic tyrosine residue [17].

In this study, we report that stimulation of EC with S1P induces the association of MT1-MMP with p130 Cas, suggesting that the involvement of MT1-MMP in S1P-mediated migration could involve a close cooperation of the enzyme with key intracellular signaling intermediates that regulate cell locomotion.

2. Materials and methods

2.1. Stimulation of HUVEC with S1P

Human umbilical vein endothelial cells (HUVEC) (Clonetics) grown to 90% confluence were serum-starved for 18 h in medium containing 0.5% serum, followed by incubation for different periods of time with 1 μM S1P. In some cases, cells were preincubated 6 h with 10 ng/mL pertussis toxin or 2 h with 100 nM AG1478 prior to stimulation with S1P. For treated cells were solubilized in SDS lysis buffer (10 mM Tris-HCl pH 7.4, 1% SDS, 1 mM sodium orthovanadate), followed by boiling for 5 min at 100 °C and homogenization using a 26-gauge needle.

Immunoprecipitation was carried out as described previously [14,17], using overnight incubation of lysates in the presence of 1 μg/mL of specific antibodies. Immune complexes were monitored by immunoblotting using antibodies raised against p130Cas (BD Transduction Laboratories), MT1-MMP (MAB3328, Chemicon), phospho(Y410)p130Cas (Cell Signaling Technology), or phospho(Y573)MT1-MMP [17].

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Abbreviations: EC, endothelial cell; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cell; MT-MMP, membrane-type matrix metalloproteinase; S1P, sphingosine-1-phosphate

2.2. Immunofluorescence and confocal microscopy

HUVEC were plated on cover glasses coated with 10 $\mu\text{g}/\text{mL}$ fibronectin, serum starved, and treated (or not) with 1 μM S1P for 15 min. After 5 min of incubation with 20 μM Hoechst for nuclei staining, cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min, blocked (1% BSA in Tris Buffer Saline containing 0.1% Tween) for 30 min and stained with specific primary antibodies against MT1-MMP (AB815 or MAB3328, 1/100), p130Cas (1/100), phospho(Y410)p130Cas (1/100), FAK (Cell Signaling Technology, 1/100) or phospho(Y573)MT1-MMP (1/10). Cells were then incubated for 30 min with Alexa488- or rhodamine-conjugated secondary antibodies (1/500) and slides were mounted with Immuno-Fluore Mounting Medium (MP Biomedicals). Immunostaining was visualized and photographed using a Zeiss LSM 510 Meta confocal microscope. For staining of the actin cytoskeleton, cells were incubated for 5 min with phalloidin-FITC (1/2500).

2.3. siRNA knockdown of MT1-MMP expression

siRNA specific for MT1-MMP was synthesized (Qiagen) using the published target sequence for the enzyme: 5'-AACAGCAAAGCT-GATGCAGA-3', nucleotides 228–248 [18]. Nonsilencing siRNA (5'-AATTCTCCGAACGTGTCACGT-3') was used as a negative control. Cells (70% confluency) were transfected with 100 nM of each construct using Oligofectamine (Invitrogen) and incubated for 48 h. The transfected cells were serum-starved and stimulated with S1P as described above.

3. Results

3.1. S1P induces the localization of both p130Cas and MT1-MMP to membrane ruffles

Previous studies have shown that the stimulatory effect of S1P on EC migration involves a rapid extension of actin-rich lamellipodia at the leading edge of the migrating cells [7,19]

that correlates with the relocalization of p130Cas from the cytosol to these membrane ruffles [7]. To determine a potential interaction of MT1-MMP with p130Cas, we first compared the effect of S1P on the relocalization of both proteins to peripheral actin-rich structures. Serum-starved HUVEC were plated on fibronectin-coated coverslips, stimulated 15 min with S1P and stained with anti-p130Cas or anti-MT1-MMP antibodies as well as with phalloidin-FITC to visualize the actin cytoskeleton. As shown in Fig. 1A (upper panels), p130Cas localized to the periphery of the cells and this localization was coincident with the staining of peripheral F-actin by phalloidin. Interestingly, staining of S1P-stimulated EC with anti-MT1-MMP showed that the enzyme also localized to actin-rich structures at the cell periphery (Fig. 1A, lower panels). However, we routinely observed that not all membrane ruffles contain MT1-MMP (Fig. 1A, arrowheads), suggesting a polarization of the enzyme during S1P-mediated cell migration (see below).

By contrast to its stimulatory effect on the formation of ruffles, S1P had only a marginal stimulatory effect on the formation of focal adhesions (Fig. 1B) and neither MT1-MMP nor p130Cas (not shown) associated with these structures, as reflected by the absence of colocalization of MT1-MMP with FAK (Fig. 1B) or with paxillin (data not shown). These results thus indicate that both p130Cas and MT1-MMP associated with membrane ruffles, but not focal adhesions, following stimulation of EC with S1P.

3.2. Interaction of MT1-MMP with p130Cas

In order to examine the association of MT1-MMP and p130Cas, HUVECs were stimulated with S1P and the extent

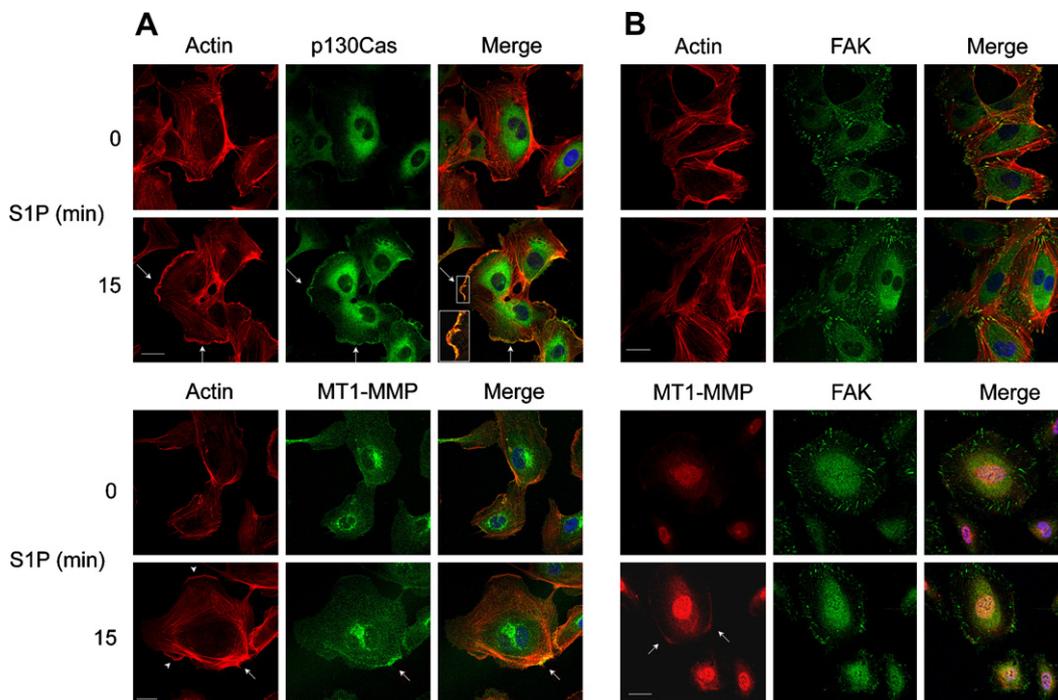


Fig. 1. S1P induces relocalization of both p130Cas and MT1-MMP to peripheral actin-rich structures. (A) HUVEC were stimulated for 15 min with 1 μM S1P and stained with polyclonal MT1-MMP or monoclonal anti-p130Cas antibodies and phalloidin-FITC. Arrows indicate areas of colocalization and arrowheads indicate ruffles showing no MT1-MMP staining. (B) Cells were stimulated with S1P as described above and stained with anti-FAK antibodies, in combination with either phalloidin-FITC (upper panels) or anti-MT1-MMP antibodies (lower panels). Arrows (lower panels) indicate the relocalization of MT1-MMP to the cell periphery. Scale bars are 10 μm .

of colocalization was monitored by staining the cells with anti-MT1-MMP and anti-p130Cas antibodies. As shown in Fig. 2A, S1P induced the relocation of both proteins to restricted areas of the cell periphery and merger of fluorescent images revealed the colocalization of the proteins in these domains (arrows).

The S1P-dependent interaction of MT1-MMP with p130Cas was next examined by co-immunoprecipitation procedures. Cells transfected with a control or with an siRNA against MT1-MMP were stimulated with 1 μ M S1P for different periods of time and the extent of association was monitored using a monoclonal anti-p130Cas antibody. As shown in Fig. 2B, S1P induced a time-dependent, bell-shaped increase in the tyrosine phosphorylation of p130Cas that correlated with the association of the protein with MT1-MMP. Addition of S1P to EC transfected with the siRNA against MT1-MMP had no effect on p130Cas phosphorylation but completely abolished its interaction with MT1-MMP, confirming that the immunoreactive material present in the immune complexes indeed correspond to MT1-MMP.

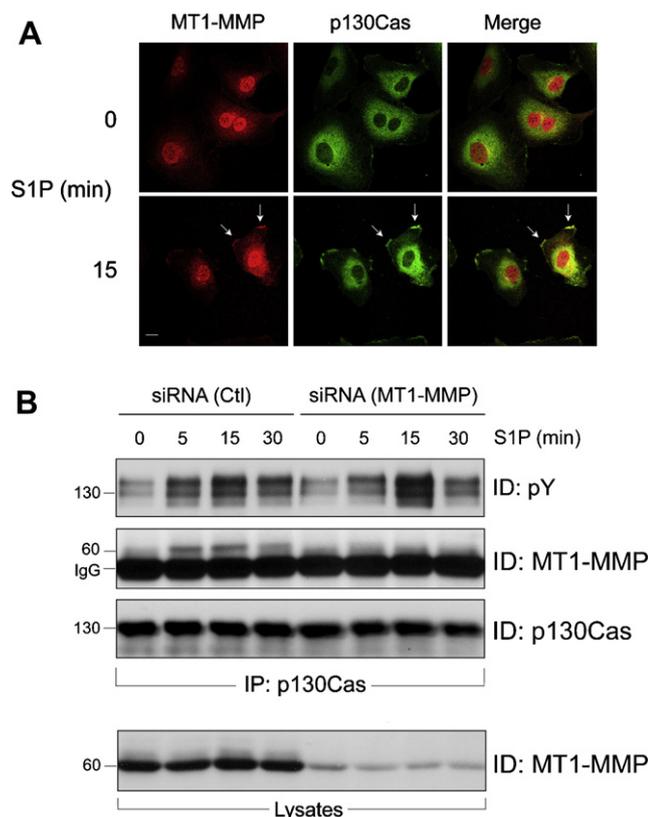


Fig. 2. S1P induces the association of MT1-MMP with p130Cas. (A) Cells plated on fibronectin-coated glasses were incubated with 1 μ M S1P for 15 min. After nuclei staining, cells were fixed, permeabilized with 0.2% Triton X-100 and stained with antibodies raised against p130Cas or MT1-MMP. Arrows indicate areas of colocalization. Scale bars are 10 μ m. (B) HUVECs were transfected with a non-silencing siRNA (control) or with an siRNA against MT1-MMP, serum-starved and then stimulated with S1P for the indicated periods of time. The extent of p130Cas phosphorylation and association with MT1-MMP was monitored by immunoprecipitation and immunoblotting. Results are representative of three distinct experiments performed on different cell preparations.

3.3. Tyrosine phosphorylation of p130Cas is important for formation of the p130Cas/MT1-MMP complex

p130Cas was originally identified as a major tyrosine-phosphorylated protein in v-crk and v-src-transformed cells [20] and subsequent studies showed that this adaptor protein contains a substrate domain consisting of several tyrosine phosphorylation sites that acts as potential SH2-binding motifs [21]. Since S1P-dependent tyrosine phosphorylation of p130Cas is involved in EC migration [7], the similarity between the kinetics of p130Cas phosphorylation and of its association with MT1-MMP (Fig. 2B) suggests that phosphorylation of p130Cas may play an important role in its association with this enzyme.

In order to explore this possibility, we monitored the interaction of MT1-MMP with tyrosine phosphorylated p130Cas, using phosphospecific antibodies raised against tyrosine 410, a residue located in the substrate domain of the protein [22]. Although these antibodies are not specific for a single tyrosine residue, they are nevertheless highly selective towards tyrosine phosphorylated p130Cas [22]. In ECs stimulated with S1P, these antibodies recognize a protein whose molecular weight and kinetics of tyrosine phosphorylation are identical to those of p130Cas and this immunoreactivity correlates with the association of p130Cas with MT1-MMP (Fig. 3A).

To monitor the impact of p130Cas phosphorylation on its interaction with MT1-MMP, we next examined the effect of pertussis toxin, a potent inhibitor of G_i -mediated signaling pathways that was previously shown to abolish S1P-dependent tyrosine phosphorylation of p130Cas in HUVECs [7]. As expected, preincubation of the cells with the toxin completely abolished the S1P-dependent tyrosine phosphorylation of p130Cas and, interestingly, abrogated its association with MT1-MMP. By contrast, preincubation of the cells with the EGF receptor inhibitor AG1478 had no effect on either p130Cas phosphorylation or on its association with MT1-MMP, suggesting that the S1P- and MT1-MMP-dependent transactivation of the EGFR previously reported [9] is not involved in the formation of the complex.

Incubation of lysates from cells stimulated with S1P with phosphospecific anti-pp130Cas antibodies resulted in the appearance of MT1-MMP in the immune complexes (Fig. 3C), further indicating that MT1-MMP interacts with phosphorylated p130Cas. In agreement with this, confocal microscopy analysis showed that anti-MT1-MMP staining at the cell periphery increased rapidly after S1P stimulation, appearing as punctate structures in a narrow band at the cell edge that colocalizes with anti-pp130Cas immunoreactivity (Fig. 3D). Overall, these results strongly suggest that the association of MT1-MMP with p130Cas requires the tyrosine phosphorylation of this protein within its substrate domain. Since tyrosine phosphorylation of p130Cas is necessary for the EC response to S1P [7], the colocalization of MT1-MMP with phosphorylated p130Cas at the cell periphery also suggests an important role of this complex in cell locomotion.

3.4. Tyrosine phosphorylated MT1-MMP interacts with p130Cas

In addition to its stimulatory effect on the tyrosine phosphorylation of p130Cas, we have recently shown that S1P also induces phosphorylation of MT1-MMP on its unique cytoplasmic tyrosine residue [17], raising the possibility that the p130Cas/MT1-MMP complex may contain, at least in part,

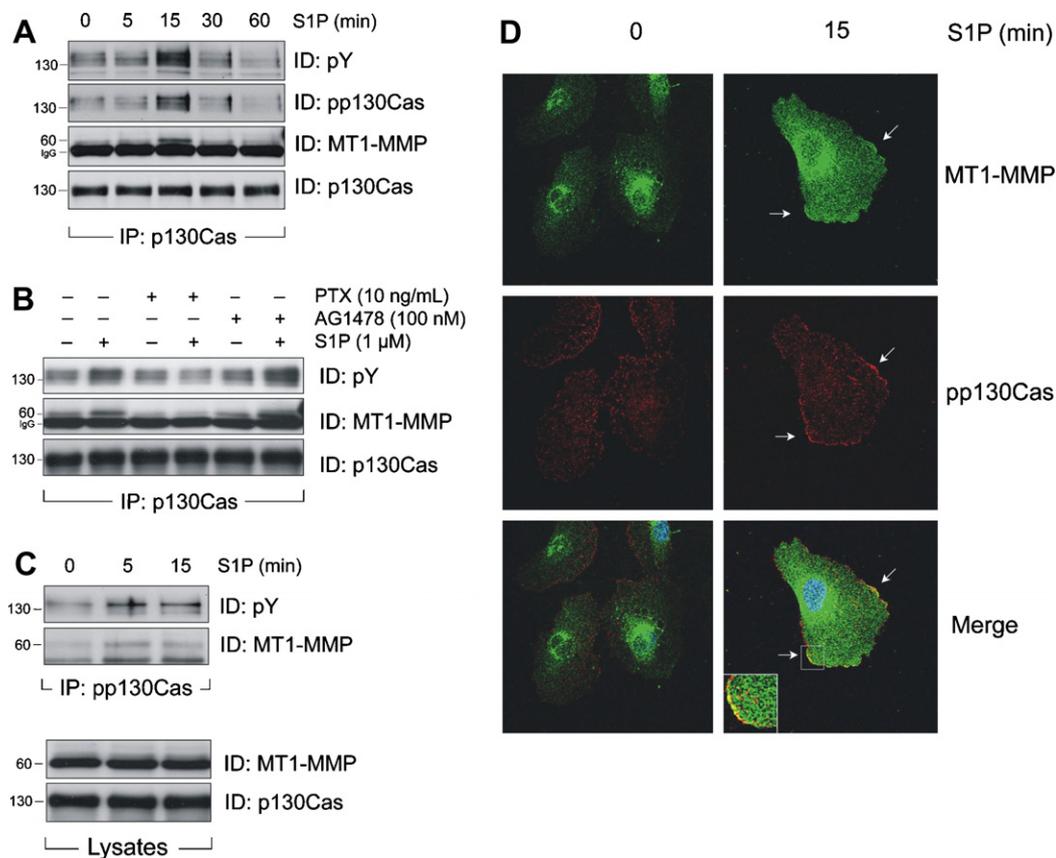


Fig. 3. MT1-MMP interacts with tyrosine phosphorylated p130Cas. (A) Serum-starved HUVEC were incubated with 1 μ M S1P for the indicated periods of time and the resulting cell lysates were immunoprecipitated with 1 μ g/mL of anti-p130Cas antibody. The immune complexes were separated by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. Results are representative of three distinct experiments performed on different cell preparations. (B) Serum-starved HUVEC were preincubated for 6 h in the presence of 10 ng/mL pertussis toxin or for 2 h with 100 nM AG1478. The extent of p130Cas phosphorylation and association with MT1-MMP was monitored as described above. (C) Serum-starved HUVEC were incubated with 1 μ M S1P for the indicated periods of time and the resulting cell lysates were immunoprecipitated with 1 μ g/mL of phosphospecific anti-p130Cas antibodies. The extent of p130Cas phosphorylation and association with MT1-MMP was monitored as described above. (D) HUVEC plated on fibronectin-coated glasses were incubated with 1 μ M S1P for 15 min and stained with antibodies raised against phosphorylated p130Cas or MT1-MMP. Arrows indicate areas of colocalization. Scale bars are 10 μ m.

the tyrosine phosphorylated form of the enzyme. Accordingly, we observed that the S1P-dependent tyrosine phosphorylation of MT1-MMP correlates with the association of the enzyme with pp130Cas (Fig. 4A). Immunoprecipitation of cell lysates using anti-pp130Cas antibodies also revealed the presence of pMT1-MMP in the immune complexes, again indicating an interaction of the tyrosine phosphorylated forms of both p130Cas and MT1-MMP.

At the cellular level, a striking characteristic of the phosphorylated MT1-MMP/p130Cas complex was its polarization at the leading edge of the EC (Fig. 4B), in a pattern very similar to that observed using antibodies recognizing association of total MT1-MMP with either p130Cas (see Fig. 2) or pp130Cas (Fig. 3). In some experiments, this colocalization of pMT1-MMP and p130Cas in cell protrusions was dramatically increased by S1P (Fig. 4B, lower panels), providing further evidence for an interaction between these proteins within specialized membrane domains.

4. Discussion

In this work, we present evidence that stimulation of EC with S1P induces major changes in the subcellular localization

of MT1-MMP, leading to its association with the adaptor protein p130Cas at the leading edge of migrating ECs. This interaction was unexpected since MT1-MMP lacks known binding motifs for either Src homology 2 (SH2) or SH3 domains that would facilitate its interaction with tyrosine kinases or tyrosine phosphorylated proteins. However, p130Cas is an important docking molecule that interacts with several signaling proteins, including FAK, Src family kinases, Crk and phosphatase PTP1B, and this network of proteins participate to the transduction of signals originating from integrin and growth factor cell surface receptors [21,23]. Although the molecular mechanisms underlying the interaction of MT1-MMP with p130Cas remain to be elucidated and likely involve additional proteins, as suggested for the binding of the enzyme to phosphorylated caveolin-1 [14], it is nevertheless tempting to speculate that the interaction of MT1-MMP with p130Cas may reflect a participation of the enzyme to signal transduction processes involved in the control of cell migration. The interaction of MT1-MMP with tyrosine phosphorylated p130Cas is of particular interest since phosphorylation of this protein has been shown to be necessary for proper cell migration triggered by integrin-mediated cell adhesion to ECM proteins as well as by various growth factors [23]. Since the importance of tyrosine phosphorylated p130Cas in cell

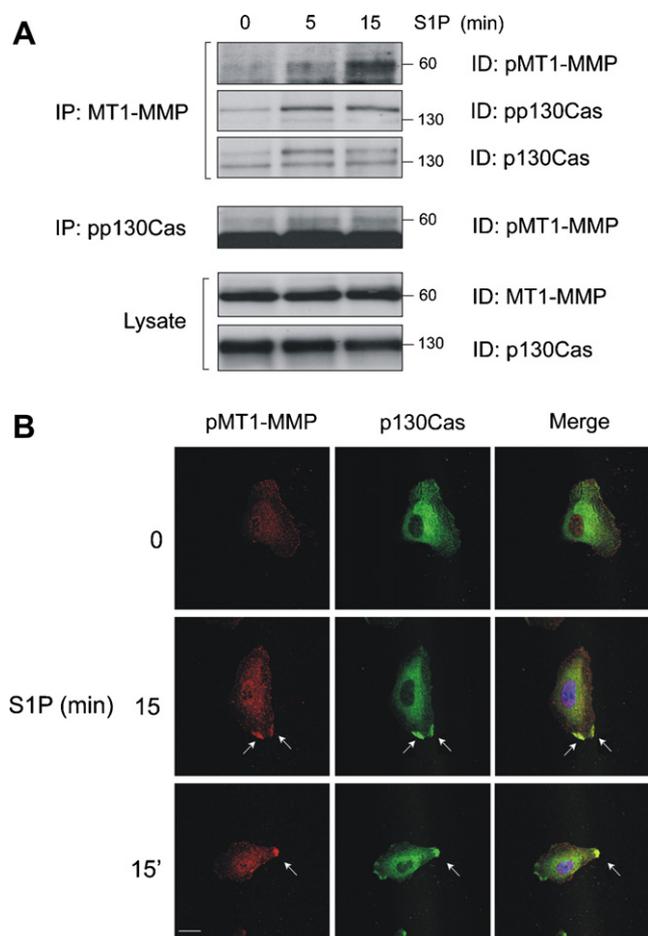


Fig. 4. Tyrosine phosphorylated MT1-MMP interacts with p130Cas. (A) HUVEC were stimulated with 1 μ M S1P for the indicated periods of time and the resulting cell lysates were immunoprecipitated with 1 μ g/mL of antibodies against pp130Cas or MT1-MMP. The immune complexes were separated by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. Results are representative of three distinct experiments performed on different cell preparations. (B) Cells plated on fibronectin-coated glasses were incubated with 1 μ M S1P for 15 min and stained with antibodies raised against p130Cas or phosphorylated MT1-MMP. Images from two different experiments of cells stimulated with S1P are shown. Arrows indicate areas of colocalization. Scale bars are 10 μ m.

motility involves its localization to the extending lamellipodia of migrating cells [23,24], the colocalization of MT1-MMP with phosphorylated p130Cas within these domains support an important role of this complex in S1P-dependent EC migration.

Our results agree with a number of studies showing that MT1-MMP is directed toward the lamellipodia at the front of migrating cells [25–27] and support a preferential localization of its proteolytic activity to restricted areas of the cell surface involved in cell migration and invasion. MT1-MMP was previously found to colocalize with caveolin-1 and α v β 3 and β 1 integrins at lamellipodia of migrating ECs [28,29], suggesting that these complexes may control the spatiotemporal proteolytic activity of the enzyme [28]. The observation of a MT1-MMP/p130Cas complex following stimulation of EC with S1P suggests that the localization of MT1-MMP to lamellipodia may also involve the interaction of the enzyme with key signaling intermediates involved in migration and thus support

a close cooperation of the enzyme with the S1P-mediated signal transduction events [8,9].

The cooperation of MT1-MMP with S1P-mediated EC migration requires the cytoplasmic domain of the enzyme [8,9], suggesting that signals originating from this domain, such as the binding to tyrosine phosphorylated caveolin-1 [14] or Src-dependent phosphorylation of the unique tyrosine residue located in this domain [17], may play important roles in this process. In this respect, we observed that the large majority of tyrosine phosphorylated MT1-MMP colocalized with p130Cas at cell protrusions following stimulation of EC with S1P, strongly suggesting a crucial role of phosphorylation of the intracellular domain of MT1-MMP for its interaction with p130Cas. Current work aimed at the identification of proteins that specifically bind to the tyrosine phosphorylated form of MT1-MMP are currently underway and should provide interesting information on the mechanisms involved in the formation of the MT1-MMP/p130Cas complex and in its localization to the leading edge of migrating cells.

There is increasing evidence that platelet-derived S1P is a potent chemoattractant for ECs and may thus play important roles in linking hemostasis to angiogenesis and tumor growth [4]. During angiogenesis, endothelial cells must overcome the physical resistance of three-dimensional ECM networks in order to migrate towards chemotactic stimuli and sustain neovessel formation [11]. The colocalization of pericellular proteolytic activities and key signaling intermediates involved in cell locomotion at the EC surface, such as the MT1-MMP/p130Cas complex reported in this study, may thus illustrate a new mechanism by which this coordinated migration of endothelial cells is achieved.

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