VAMP3, syntaxin-13 and SNAP23 are involved in secretion of matrix metalloproteinases, degradation of the extracellular matrix and cell invasion

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Summary

Cellular remodeling of the extracellular matrix (ECM), an essential component of many physiological and pathological processes, is dependent on the trafficking and secretion of matrix metalloproteinases (MMPs). Soluble NSF attachment protein receptor (SNARE)-mediated membrane traffic has documented roles in cell-ECM interactions and the present study specifically examines SNARE function in the trafficking of MMPs during ECM degradation. Using the invasive human fibrosarcoma cell line HT-1080, we demonstrate that a plasma membrane SNARE, SNAP23, and an endosomal v-SNARE, VAMP3 (also known as cellubrevin), partly colocalize with MMP2 and MMP9, and that inhibition of these SNAREs using dominant-negative SNARE mutants impaired secretion of the MMPs. Inhibition of VAMP3, SNAP23 or syntaxin-13 using dominant-negative SNARES, RNA interference or tetanus toxin impaired trafficking of membrane type 1 MMP to the cell surface. Consistent with these observations, we found that blocking the function of these SNAREs reduced the ability of HT-1080 cells to degrade a gelatin substrate in situ and impaired invasion of HT-1080 cells in vitro. The results reveal the importance of VAMP3, syntaxin-13 and SNAP23 in the trafficking of MMP during degradation of ECM substrates and subsequent cellular invasion.

Supplementary material available online at
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Key words: Cellular invasion, ECM degradation, Matrix metalloproteinase, MMP, SNARE

Introduction

Cellular remodeling of the extracellular matrix (ECM) is essential to physiological processes such as embryogenesis and wound healing, and to pathological conditions such as arthritis and cancer. Invasion of the ECM by tumor cells is required for the growth of primary tumors and for the ability of tumors to metastasize. Invasion through an ECM barrier is a complex, stepwise process involving cell adhesion, ECM proteolysis by secreted matrix metalloproteinases (MMPs), and migration of the tumor cell (Stetler-Stevenson et al., 1993). The intracellular mechanisms that regulate these activities are currently subjects of intensive study, and evidence is emerging that trafficking and secretion of MMPs is central to the control of ECM degradation and cellular invasion (Miyata et al., 2004; Schnaeker et al., 2004).

MMPs are a family of zinc-dependent proteolytic enzymes that degrade the ECM. Most MMPs are soluble and are secreted from the cell, but a subset of MMPs are integral membrane proteinases that stay anchored to the cell membrane as they degrade the ECM and activate other MMPs. Consistent with their known involvement in ECM degradation, MMPs have been shown to play important roles in embryogenesis and wound healing, and also to contribute to the progression of cancer (Murray, 2001). Studies indicate that increased expression of MMP2 and MMP9 (also known as gelatinases) and the membrane-bound membrane type 1 MMP (MT1-MMP) correlates with aggressive forms of colorectal cancer, breast cancer and melanoma (Mendes et al., 2005; Seftor et al., 2001; Zhang et al., 2005; Zucker et al., 1999). Understanding of the functional importance of MMPs is also growing. For example, recent findings suggest that MT1-MMP is a crucial factor during invasion of ovarian tumor cells (Sodek et al., 2007) and breast carcinoma cells (Hotary et al., 2006), and that hypoxia-induced invasion of breast carcinoma cells is dependent on the activity of MT1-MMP and MMP2 (Munoz-Najar et al., 2006). Indeed, the evidence revealing an important role for secretion of MMPs in many types of cancer has led to consideration of these enzymes as prognostic markers for cancer progression (Li et al., 2004; Sakata et al., 2004).

Evidence is emerging that vesicular trafficking of MMPs is a crucial factor in the regulation of ECM remodeling and tumor-cell invasion. In melanoma cells, MMP2- and MMP9-containing vesicles are stored and trafficked along microtubules (Schnaeker et al., 2004). MT1-MMP is recycled from internal compartments to the cell surface, where it activates MMP2 through cleavage of the pro-domain in MMP2 (Strongin et al., 1995). Evidence also suggests that MT1-MMP trafficking facilitates activation of cell surface αV integrin in support of cell motility (Deryugina et al., 2004). In addition, studies are beginning to delineate the molecular mechanisms that control MMP trafficking. Delivery of MT1-MMP to the plasma membrane has been shown to involve a Rab8-dependent pathway (Bravo-Cordero et al., 2007). Internalization of MT1-MMP appears to occur through a combination of clathrin-dependent and clathrin-independent pathways (Annabi et al., 2001; Jiang et al., 2001; Remacle et al., 2003) that deliver MT1-MMP to endosomal compartments marked by EEA1, Rab4 and Rab11 (Jiang et al., 2001; Remacle et al., 2005; Wang et al., 2004).

Membrane traffic between intracellular compartments is dependent on SNAREs [soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) attachment protein receptors] and there is some

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evidence that SNAREs contribute to trafficking of MMPs. Syntaxin-4 (Miyata et al., 2004) and Ti-VAMP (tetanus-insensitive vesicle-associated membrane protein, also known as VAMP7) (Steffen et al., 2008) have been shown to be involved in cellular invasion and trafficking of MT1-MMP. SNAP25 (synaptosome-associated protein of 25 kDa) has been found to partly colocalize with MMP7 in epithelial cells (Gorodeski, 2007). There is also recent evidence that directly links SNARE-mediated intracellular membrane traffic to cell-ECM interactions (Al-Awar et al., 2000; Roberts et al., 2001). We (Gonon et al., 2005; Skalski and Coppolino, 2005; Tayeb et al., 2005), along with others (Proux-Gillardeaux et al., 2005), have reported that SNARE-mediated trafficking, including that mediated by the plasma membrane SNARE SNAP23 and the endosomal SNARE VAMP3, is required for cell adhesion and migration in cultured mammalian cell systems.

In the present study, we examined the roles that SNAP23, VAMP3 and syntaxin-13 play in the secretion of MMPs and the degradation of ECM by HT-1080 fibrosarcoma cells. We report that SNAP23 and VAMP3 are required for secretion of MMP2 and MMP9, and that these SNAREs and syntaxin-13 are involved in trafficking of the membrane-bound MT1-MMP to the cell surface. Inhibiting the function of SNAP23, VAMP3 or syntaxin-13 using dominant-negative mutants of the SNAREs, RNA interference (RNAi) or tetanus toxin impaired the in situ degradation of gelatin by the cells. We present data that are the first to show that VAMP3, syntaxin-13 and SNAP23 are required for efficient invasion by HT-1080 cells in vitro. The impaired ECM degradation and reduced invasive capacity observed in cells as a consequence of inhibiting SNARE function suggests an important role for SNARE-mediated traffic in tumor progression.

Results

GFP-tagged SNAREs partly overlap with endogenous MMP2 and MMP9 in HT-1080 cells

MMP2 and MMP9 are secreted proteinases that have been shown to contribute to tumor-cell invasion and metastasis. Having identified roles for SNAP23 (Gonon et al., 2005) and VAMP3 (Proux-Gillardeaux et al., 2005; Skalski and Coppolino, 2005; Tayeb et al., 2005) in the non-invasive motility of cells, we sought to investigate the involvement of these SNAREs in the trafficking and secretion of MMPs during cell invasion. SNAP23 is a plasma membrane SNARE found in many cell types, and VAMP3 is a known binding partner of SNAP23 (Hepp et al., 1999). To study MMP activity during cell invasion, we used the human cell line HT-1080, which is derived from a highly invasive fibrosarcoma and is known to express MMP2 and MMP9 along with MT1-MMP (Ginestra et al., 1997; Stanton et al., 1998). The subcellular distributions of endogenous MMP2 and MMP9 were compared with those of GFP-SNAP23 and GFP-VAMP3, and it was observed that SNAP23 (Fig. 1A) and VAMP3 (Fig. 1B) partly colocalized with both MMP2 and MMP9 at the periphery of HT-1080 cells in response to treatment of the cells with the tumor promoter PMA (phorbol 12-myristate 13-acetate). PMA was added to stimulate trafficking of MMPs, as has been previously reported (Foda et al., 1996; Zucker et al., 2002). Fig. 1C shows the distributions of GFP-VAMP3 and MMP2 in cells that were not treated with PMA. Little overlap at the cell periphery was observed and similar results were obtained with GFP-SNAP23, as well as when MMP9 was monitored without PMA treatment (not shown).

Intracellular distributions of GFP-tagged SNAREs were consistent in cells expressing low levels of the GFP constructs and correlated well with endogenous SNAREs (data not shown). The specificity of the MMP antibodies was confirmed by western blot analysis of HT-1080 cell lysate (supplementary material Fig. S1). Species cross-reactivity of the MMP and SNARE antibodies used here did not permit immunofluorescence microscopy of both endogenous MMPs and endogenous SNAREs in the same cell; however, we did observe partial colocalization of GFP-tagged MMP2 or GFP-tagged MMP9 with endogenous SNAP23 (Fig. 1D). Similar distributions were observed for GFP-MMP2, GFP-MMP9 and endogenous VAMP3 (data not shown). Furthermore, the PMA-stimulated colocalization of MMP2 and MMP9 with GFP-SNAP23 and GFP-VAMP3 at the edge of HT-1080 cells was specific for these SNAREs and was not observed in cells transfected with a GFP-tagged form of the Golgi SNARE GS15 (GFP-GS15) (Fig. 1E).

SNARE constructs bind specifically to endogenous SNAREs in HT-1080 cells

To investigate the functional role of SNAREs in MMP secretion, we utilized mutant forms of SNARE proteins that can be expressed in cells to disrupt the function of endogenous SNAREs. SNAP23cΔ9 is a dominant-negative form of SNAP23 that lacks nine residues at the C-terminus. SNAP23cΔ9 forms non-functional complexes with SNAP23-binding partners and has been shown to inhibit SNAP23-mediated exocytosis (Huang et al., 2001), transferrin recycling (Scott et al., 2003) and cell adhesion (Gonon et al., 2005). We also generated soluble mutant forms of VAMP3 and syntaxin-13. VAMP3 was targeted because of its observed colocalization with MMP2 and MMP9 (Fig. 1); syntaxin-13 (also known as syntaxin-12) was targeted because it is known to interact with SNAP23 (Tang et al., 1998) and with SNAP25, a neuronal homolog of SNAP 23 (Aikawa et al., 2006). We generated mutant forms of these SNAREs lacking transmembrane domains (VAMP3cyto and Syn13cyto). These mutant forms of the SNAREs are soluble and are predicted to compete with endogenous counterparts in the formation of SNARE complexes, thus inhibiting membrane fusion events mediated by the endogenous SNAREs.

The ability of the mutant SNARE proteins to form complexes with cognate SNAREs was assessed by immunoprecipitating SNARE complexes from transiently transfected populations of cells. In syntaxin-13 immunoprecipitate of HT-1080 cells transfected with GFP-tagged SNAP23cΔ9 or GFP-tagged full-length SNAP23 (SNAP23FL), the transfected GFP-tagged SNARE constructs were detected in SNAP23 western blots (supplementary material Fig. S2A). Immunoprecipitation of syntaxin-13 from HT-1080 cells transfected with GFP-tagged soluble VAMP3 (VAMP3cyto) or GFP-tagged full-length VAMP3 (VAMP3FL) revealed that syntaxin-13 also formed complexes with these SNARE constructs as detected in VAMP3 western blots (supplementary material Fig. S2B). Additional bands seen in syntaxin-13 immunoprecipitate are possibly breakdown products of transfected SNARE constructs. Cells transfected with GFP-tagged soluble syntaxin-13 (Syn13cyto) or GFP-tagged full-length syntaxin-13 (Syn13FL) were also analyzed and it was found that SNAP23 immunoprecipitates from these cells contained the indicated GFP-tagged syntaxin-13 proteins (supplementary material Fig. S2C).

These experiments were carried out in transiently transfected populations of cells with transfection efficiencies approaching 50%. Immunoprecipitate of endogenous SNAREs from such mixed populations of cells probably contain both transfected GFP-tagged SNAREs and endogenous cognate binding partners. In accordance with this, we detected endogenous SNAP23

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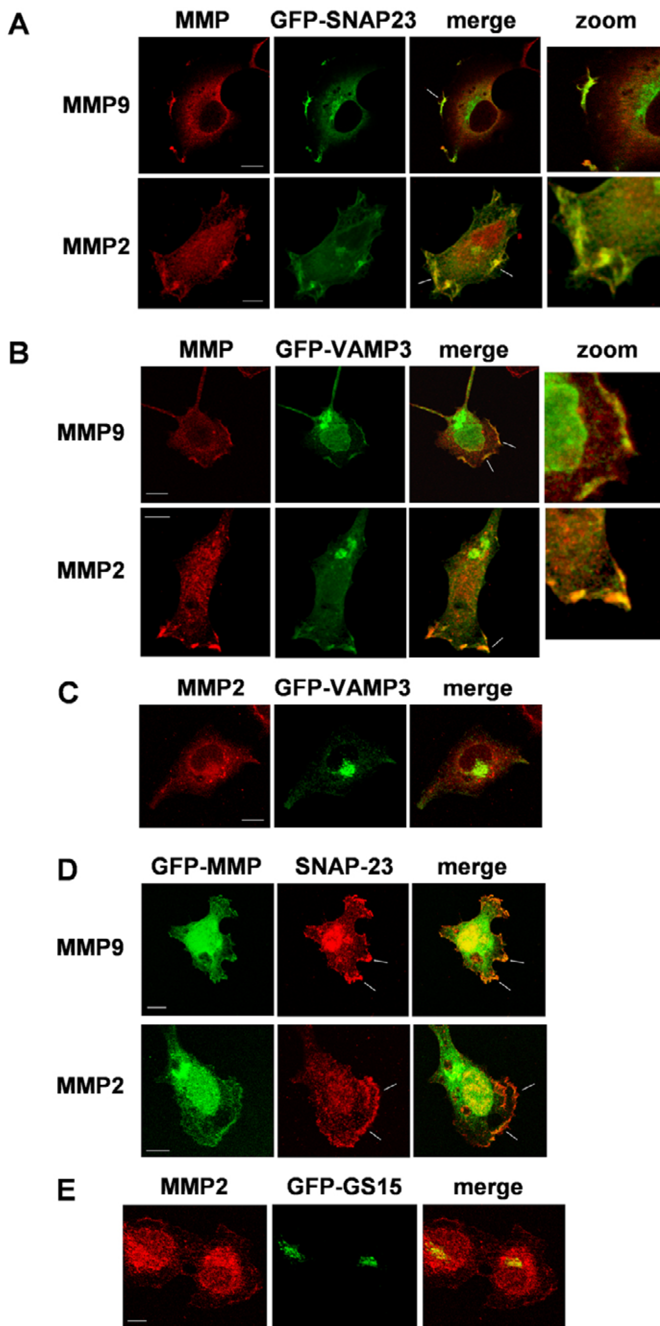

Fig. 1. MMP2 and MMP9 colocalize with SNAP23 and VAMP3. HT-1080 cells were transfected with (A) GFP-SNAP23, (B,C) GFP-VAMP3, (D) GFP-MMP2 or GFP-MMP9 or (E) GFP-GS15 overnight, serum-starved for 2 hours and stimulated with 500 nM PMA for 3.5 hours (A,B,D,E). The cells were then fixed, permeabilized, stained with antibodies to MMP2, MMP9 (A,B,C,E) or SNAP23 (D) and imaged using confocal microscopy. Zoom in A and B shows digital magnification of merged images. Arrows in A and B indicate sites where MMP and SNARE colocalize. (C) Control cells were transfected with GFP-VAMP3, but not treated with PMA. (D) Cells were transfected with GFP-MMP9 (top row) or GFP-MMP2 (bottom row), treated with PMA and stained with anti-SNAP23. (E) Cells transfected with GFP-GS15 and treated with PMA. Scale bars: 10 µm.

(supplementary material Fig. S2A) and VAMP3 (supplementary material Fig. S2B) in syntaxin-13 immunoprecipitate, and endogenous syntaxin-13 in SNAP-23 immunoprecipitate (supplementary material Fig. S2C). Although we cannot formally exclude the possibility that some SNAREs interact after cell lysis, the experiments were carried out at 4°C in the presence of detergent to minimize the likelihood of this. These experiments therefore suggest that the endogenous SNAREs we have targeted form interactions within HT-1080 cells and can also bind to the mutant constructs that we have generated.

To assess the specificity with which soluble SNARE constructs bind to endogenous SNAREs, we examined GS15, a Golgi SNARE that has not been shown to interact with syntaxin-13. As in experiments above, GFP-tagged constructs containing full-length GS15 (GS15FL) or its cytoplasmic domain (GS15cyto) were transfected into HT-1080 cells. Unlike VAMP3 or SNAP23, GS15 (transfected construct or endogenous protein) was not detected in GS15 western blots of syntaxin-13 immunoprecipitate (supplementary material Fig. S2D). In the case of GFP-SNAP23cΔ9, we subsequently compared the effects of its expression in HT-1080 with those described elsewhere (Scott et al., 2003). Consistent with previously published results describing its function as an inhibitor of SNAP23, we found that expression of GFP-SNAP23cΔ9 impaired the recycling of transferrin in HT-1080 cells (data not shown).

During analysis of the effects of SNARE construct expression on MMP activity, we wanted to ensure that inhibitory SNARE complexes were expressed at similar levels to full-length control constructs. We found that truncated SNARE constructs were expressed more efficiently than full-length constructs in transient transfections, so expression times in all experiments were adjusted to compensate for this. Comparable levels of GFP-SNAP23cΔ9 and GFP-SNAP23FL were detected in cell lysates after 12 and 36 hours of expression, respectively (supplementary material Fig. S2E). Comparable levels of GFP-VAMP3cyto and GFP-VAMP3FL (supplementary material Fig. S2F), and GFP-Syn13cyto and GFP-Syn13FL (supplementary material Fig. S2G) were also observed after 12 hours of expression of the soluble form and 36 hours of expression of the full-length protein. This allowed all experiments described herein to be conducted using cells with levels of mutant SNARE expression that were similar to those of full-length controls.

To assess the specificity of the interactions formed by SNARE constructs within cells, lysates from cells transfected with GFP-tagged SNAP23 were subjected to immunoprecipitation with antibody to GFP. These immunoprecipitates were then analyzed by western blot for the presence of a predicted binding partner (VAMP3), a known Golgi SNARE (GS15) and another coiled-coil-containing protein (EEA1). Western blots for VAMP3 and GS15 (of the same anti-GFP immunoprecipitate) indicate that GFP-tagged SNAP23, full-length or truncated, forms complexes with VAMP3 but not GS15 in HT-1080 cells (supplementary material Fig. S3A,B). Similarly, western blots for VAMP3 and EEA1 (of the same anti-GFP immunoprecipitate) indicate that full-length or truncated forms of GFP-tagged SNAP23 interact with VAMP3 but not EEA1 in HT-1080 cells (supplementary material Fig. S3C,D). Together, these results suggest that the GFP-tagged SNARE constructs do not form interactions indiscriminately when expressed in these cells.

Mutant SNARE constructs impair secretion of MMP2 and MMP9

The observation that SNAP23 and VAMP3 can partly colocalize with MMP2 and MMP9 (Fig. 1), led us to hypothesize that these SNAREs might be involved in secretion of the MMPs. Furthermore,

because syntaxin-13 has recently been found to be involved in recycling of the cell-surface form of the chemokine CX3CL1 (Liu et al., 2005) and can bind SNAP23 and VAMP3 in HT-1080 cells (supplementary material Fig. S2), we speculated that syntaxin-13 might also play a role in MMP secretion in HT-1080 cells. Gelatin zymography was used to test the levels of MMP2 and MMP9 secreted into the medium by HT-1080 cells that had been transiently transfected with SNAP23cΔ9 or SNAP23FL, VAMP3cyto or VAMP3FL, and Syn13cyto or Syn13FL. Secreted MMP9 was not detectable in samples without PMA treatment, and mock transfection or transfection with GFP alone did not alter MMP secretion (data not shown). Expression of SNAP23cΔ9 clearly reduced PMA-induced secretion of MMP2 and MMP9, as decreased levels of these MMPs were detected in the medium collected from SNAP23cΔ9-expressing cells (Fig. 2A). Expression of VAMP3cyto also decreased secretion of these MMPs, though less dramatically than SNAP23cΔ9, and expression of Syn13cyto had no obvious effect on MMP2 or MMP9 secretion (Fig. 2A). The extent of gelatin degradation in the zymographs was quantified from three independent experiments using ImageJ software (Fig. 2C). These results demonstrate that SNAP23cΔ9 impaired secretion of MMP2 by 56% and secretion of MMP9 by 49%. VAMP3cyto impaired secretion of MMP2 by 39% and of MMP9 by 29% ($P<0.05$, Student's *t*-test). It is important to note that these experiments were carried out using populations of transiently transfected cells (with transfection rates approaching 50%) and that some of the MMP2 and MMP9 activity seen in the zymographs of SNAP23cΔ9 and VAMP3cyto samples is the result of MMP secretion by non-transfected cells. These results are thus likely to underestimate the effects that inhibition of SNAP23 or VAMP3 have on MMP secretion. Decreases in secreted MMP activity were not due to decreases in total cellular levels of MMP expression as determined by zymography of whole-cell extracts (Fig. 2B).

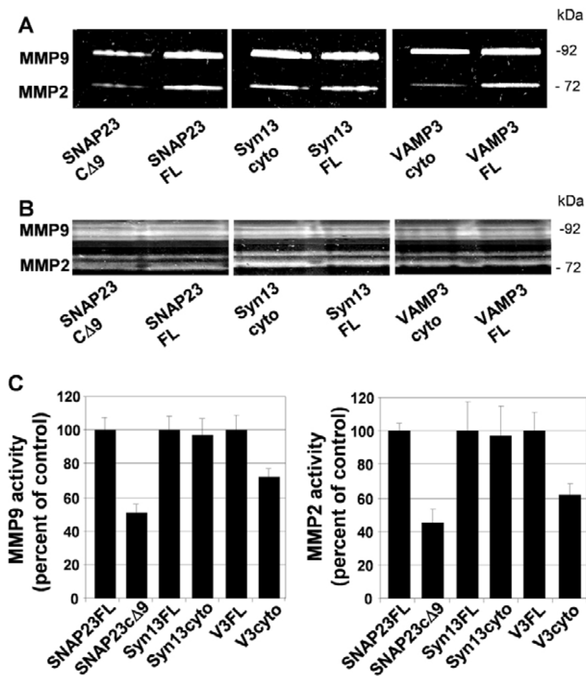

Fig. 2. Expression of SNAP23cΔ9 or VAMP3cyto impairs secretion of MMPs. HT-1080 cells were transiently transfected with mutant or full-length constructs of SNAP23, syntaxin-13 or VAMP3. After 14 hours, cells were plated on Matrigel in 10% FBS for 3.5 hours, after which the medium was replaced with serum-free medium containing 500 nM PMA. After 6.5 hours, medium alone was collected (A) or cells were extracted (B) and gelatin zymography was performed to detect MMP2 and MMP9 activity. (C) Bands of gelatin degradation in zymographs, due to MMP2 and MMP9 activity, were measured using ImageJ. Values are presented as a percentage of GFP-transfected control cells. The means ± s.e.m. of three independent experiments are shown.

VAMP3, syntaxin-13 and SNAP23 are required for trafficking of MT1-MMP

The modest effect that inhibiting VAMP3 and the apparent lack of effect that inhibiting syntaxin-13 had on the secretion of MMP2 and MMP9 does not rule out the possibility that these SNAREs regulate MMP activity through other mechanisms. One possibility is that these SNAREs are involved in the trafficking of membrane-anchored MT1-MMP to the surface of HT-1080 cells. PMA-

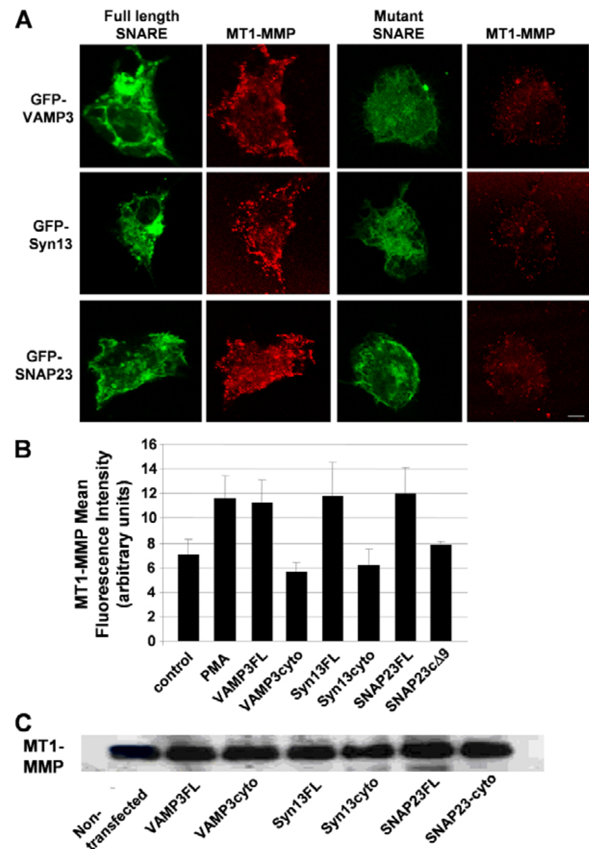

Fig. 3. Inhibition of VAMP3, syntaxin-13 or SNAP23 impairs trafficking of MT1-MMP. (A) HT-1080 cells were transfected with GFP-tagged mutant or full-length VAMP3, syntaxin-13 or SNAP23 constructs. Cells were serum-starved and then 500 nM PMA was added to induce trafficking of MT1-MMP. Cells were stained with MT1-MMP antibody at 4°C and then fixed with 4% PFA. GFP and cell-surface MT1-MMP were imaged using confocal microscopy. Scale bar: 10 µm. (B) Quantification of cell-surface MT1-MMP levels by flow cytometry. Cells were transfected as in A, treated with PMA, or vehicle control (DMSO), and then stained at 4°C for surface MT1-MMP using anti-MT1-MMP antibody and Alexa-Fuor-647-conjugated secondary antibody. Fluorescence in GFP-positive cells was then measured. Means ± s.e.m. are from three independent experiments in which 10,000 cells per sample per experiment were measured. (C) Cells transfected with the indicated SNARE constructs were lysed and equal amounts of protein were analyzed by SDS-PAGE and western blot for expression of MT1-MMP.

induced delivery of MT1-MMP to the cell surface was monitored by antibody staining of non-permeabilized cells. In cells transfected with VAMP3cyto, Syn13cyto or SNAP23cΔ9, little MT1-MMP was detected on the surface of cells after stimulation with PMA (Fig. 3A). By contrast, cell-surface MT1-MMP was obvious in cells transfected with full-length constructs (Fig. 3A), suggesting that delivery of MT1-MMP to the plasma membrane is dependent on the activities of VAMP3, syntaxin-13 and SNAP23. The cause of the clustered appearance of cell-surface MT1-MMP staining is not known. This pattern is consistent with previous reports (Bravo-Cordero et al., 2007; Ispanovic et al., 2008; Miyata et al., 2004; Shinozaki et al., 2008) and might result from targeted exocytosis. The conditions for the experiments in Fig. 3A prevented internalization of externally applied material, including MT1-MMP antibody, as confirmed by monitoring rhodamine-labeled transferrin endocytosis in samples. All labeled transferrin that could be detected was removable by briefly rinsing the cells in an acid wash buffer (dissociating the transferrin from its receptor), indicating that it had not been endocytically internalized (supplementary material Fig. S4).

To quantitatively assess the delivery of MT1-MMP to the cell surface, surface levels of MT1-MMP were measured using flow cytometry. Quantification of mean fluorescence intensities indicated that the amount of MT1-MMP on the surface of HT-1080 cells, after treatment with PMA, was strongly decreased by expression of VAMP3cyto, Syn13cyto and SNAP23cΔ9, but not their wild-type counterparts (Fig. 3B). In the flow cytometry experiments, only GFP-positive cells were measured, avoiding collection of data from non-transfected cells. Mock transfection or transfection of GFP alone did not affect cell-surface levels of MT1-MMP (not shown). For the data in Fig. 3B, Student's *t*-tests indicated significant differences between mutant SNARE constructs and the corresponding full-length control (VAMP3cyto, P=0.043; Syn13cyto, P=0.049; SNAP23cΔ9, P=0.015). Expression of the mutant forms of the SNAREs did not alter total cellular levels of MT1-MMP, as assessed by western blotting of cell lysates (Fig. 3C).

Mutant SNARE constructs inhibit degradation of a gelatin matrix

The requirements for SNAP23, syntaxin-13 and VAMP3 function in degradation of an extracellular matrix were examined using an established gelatin degradation assay (Hoover et al., 2005; Itoh et al., 2001; Tague et al., 2004). For these studies, HT-1080 cells were transfected with the indicated constructs, incubated for appropriate periods, plated on coverslips coated with fluorescently labeled (Texas red) gelatin, and incubated for 24 hours. The coverslips were then examined to determine the extent of gelatin degradation. Expression of SNAP23cΔ9 significantly impaired the capacity of the cells to degrade a gelatin matrix (Fig. 4A,C). Inhibition of either syntaxin-13 or VAMP3 using the indicated soluble SNARE constructs also impaired degradation of the gelatin matrix, although to a lesser extent (Fig. 4A,C). Expression of GFP-tagged wild-type SNARE constructs did not alter degradation of gelatin relative to non-transfected cells.

As another approach to probe SNARE function in HT-1080 cells, we used a cDNA encoding the catalytic chain of tetanus toxin (TeTx). This toxin proteolytically cleaves VAMP1, VAMP2 and VAMP3 and has been used extensively to experimentally inhibit these SNAREs in cellular studies (Fields et al., 2007; Gaisano et al., 1994; Tayeb et al., 2005). Expression of the catalytic chain of TeTx impeded gelatin degradation to an extent similar to that found for treatment with VAMP3cyto (Fig. 4B,C). Cells transfected with TeTx and GFP were compared to cells transfected with GFP alone (which did not differ from non-transfected controls). For the data in Fig. 4C, Student's *t*-tests indicated significant differences between SNARE inhibitors and the corresponding full-length or GFP controls (VAMP3cyto, P=0.0027; TeTx, P=0.0068; Syn13cyto, P=0.0442; SNAP23cΔ9, P=0.0007).

RNAi-mediated downregulation of syntaxin-13 and SNAP23 impairs matrix degradation

To confirm the involvement of syntaxin-13 and SNAP23 in the degradation of the gelatin matrix by HT-1080 cells, these SNAREs

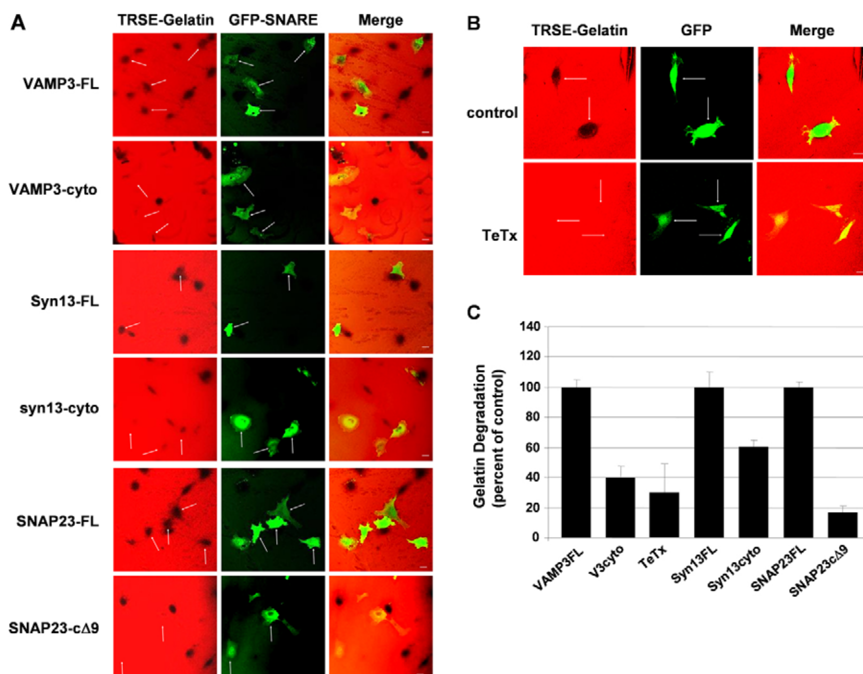

Fig. 4. Expression of VAMP3cyto, Syn13cyto, SNAP23cΔ9 or TeTx decreases gelatin degradation in HT-1080 cells. Cells were transfected with GFP-tagged mutant or full-length VAMP3, syntaxin-13 or SNAP23, or with the catalytic chain of TeTx and then plated on Texas-red-labeled gelatin and incubated for 24 hours. The number of transfected cells able to degrade the gelatin was counted. (A) Images of cells expressing the indicated SNARE constructs on Texas-red-labeled gelatin. (B) Images of cells expressing GFP alone (control) or GFP + TeTx on Texas-red-labeled gelatin. Scale bars: 10 μm. (C) Gelatin degradation was quantified by counting the number of cells able to degrade the gelatin in each sample. Values are presented as a percentage of GFP-transfected control. Means ± s.e.m. are from three independent experiments using 50 cells per sample per experiment.

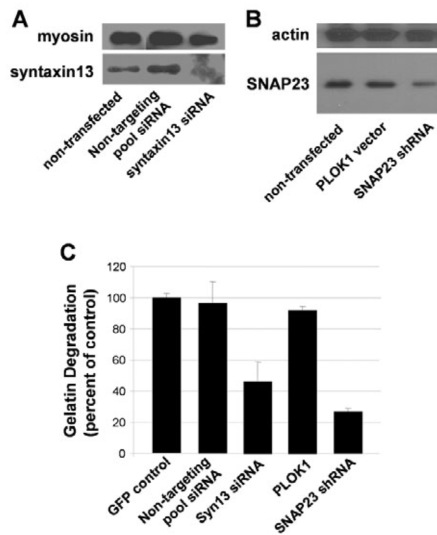

Fig. 5. RNAi-mediated downregulation of syntaxin-13 or SNAP23 decreases gelatin degradation. HT-1080 cells were co-transfected with (A) pEGFP-N1 and siRNA targeting syntaxin-13 or a non-targeting pool siRNA, or (B) pEGFP-N1 and shRNA against SNAP-23 or PLOK1 shRNA control. After 72 hours, cells were either lysed and analyzed by western blotting for syntaxin-13 and myosin (A) or SNAP23 and actin (B), or plated on Texas-red-labeled gelatin and incubated for 24 hours. (C) Gelatin degradation was quantified by counting the number of cells able to degrade the gelatin in each sample. Values are presented as a percentage of GFP-transfected control. Means ± s.e.m. are from three independent experiments using 50 cells per sample per experiment.

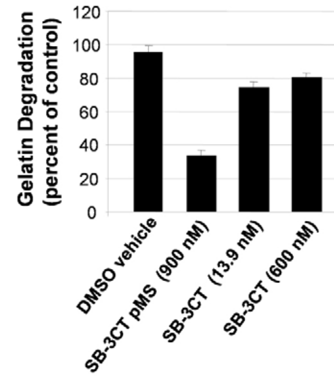

Fig. 6. Gelatin degradation by HT-1080 cells is mediated primarily through MT1-MMP. HT-1080 cells were plated on Texas-red-labeled gelatin in the presence of the indicated inhibitor or DMSO control vehicle and incubated for 24 hours. Gelatin degradation was then quantified by counting the number of cells able to degrade the gelatin in each sample. Values are presented as a percentage of GFP-transfected control. Means ± s.e.m. are from three independent experiments using 50 cells per sample per experiment.

were targeted using RNAi. HT-1080 cells were transfected with a combination of GFP and syntaxin-13 siRNA or SNAP23 shRNA for 72 hours. Knockdown of syntaxin-13 (Fig. 5A) and SNAP23 (Fig. 5B) was assessed by western blotting. Degradation and actin of a Texas-red-labeled gelatin matrix was quantified as in Fig. 4 and knockdown of either syntaxin-13 or SNAP23 resulted in significant reduction in the degradation of the gelatin (Fig. 5C). These experiments were carried out using transient transfection; thus, the gelatin degradation assays, based on fluorescent micrographs, reflect analyses of only transfected cells, whereas the western blots represent mixed populations of transfected and non-transfected cells.

Gelatin degradation by HT-1080 cells is mediated primarily through MT1-MMP

To assess the contributions that the different MMPs were making to the degradation of the gelatin matrix, we tested the capacity of the HT-1080 cells to degrade the matrix in the presence of specific chemical inhibitors of MMP activity, SB-3CT and SB-3CT pMS (Ikejiri et al., 2005; Kruger et al., 2005). SB-3CT was applied to the cells at concentrations reported to inhibit MMP2 and MMP9 (13.9 nM for MMP2; 600 nM for MMP9). A sulfonamido analog of SB-3CT, SB-3CT pMS, was applied at 900 nM, a concentration that inhibits MMP2, MMP9 and MT1-MMP. Quantification of gelatin degradation revealed that inhibition of MMP2 or MMP9 had modest effects on gelatin breakdown, but that inhibition of MT1-MMP produced a strong reduction in gelatin degradation (Fig. 6). Student's *t*-tests indicated significant differences between MMP inhibitors compared to control vector (MMP9, P=0.017; MMP2, P=0.025; MT1-MMP P=0.004).

VAMP3, syntaxin-13 and SNAP23 are required for invasion in HT-1080 cells

Having observed decreased MMP secretion and gelatin degradation caused by inhibition of SNARE function, we next tested whether blocking SNARE function would lead to decreased cell invasion in HT-1080 cells. The ability of HT-1080 cells expressing mutant or wild-type SNARE constructs to invade was investigated using modified Boyden chambers (Shaw, 2005) containing membranes coated with a collagen-derived extracellular matrix barrier (Matrigel) and using fetal bovine serum (FBS) as a chemo-attractant. Compared to control cells expressing wild-type SNAREs, invasion by cells expressing VAMP3cyto, Syn13cyto or SNAP23cΔ9 was decreased by approximately 70% (Fig. 7A). Expression of wild-type SNARE constructs did not alter cell invasion compared with non-transfected samples or samples transfected with GFP alone (data not shown). Transfection of cells with TeTx caused a similar impairment of cell invasion to that caused by expression of VAMP3cyto (Fig. 7A). Transfection of cells with siRNA against syntaxin-13, or shRNA against SNAP23, also impaired invasion (Fig. 7C) to an extent that was comparable to that resulting from expression of mutant forms of these SNAREs.

Tumor-cell invasion is a multistep process involving cell adhesion, MMP secretion and cell migration. We determined that the observed deficit in cell invasion resulting from inhibition of SNAREs was most probably attributable to decreased MMP-mediated degradation of the ECM because expression of VAMP3cyto, Syn13cyto or SNAP23cΔ9 had no effect on the spreading of HT-1080 cells on collagen (data not shown) or cell migration as measured by Transwell migration assays (Fig. 7B). Furthermore, expression of SNAP23 shRNA and syntaxin-13 siRNA had no effect on Transwell cell migration compared to cells transfected with control shRNA (PLOK1) and siRNA (non-targeting siRNA) (Fig. 7D).

Discussion

In this study, we report that the functions of VAMP3, syntaxin-13 and SNAP23 are required for ECM remodeling and invasion by HT-1080 cells. Blocking the function of these SNAREs did not

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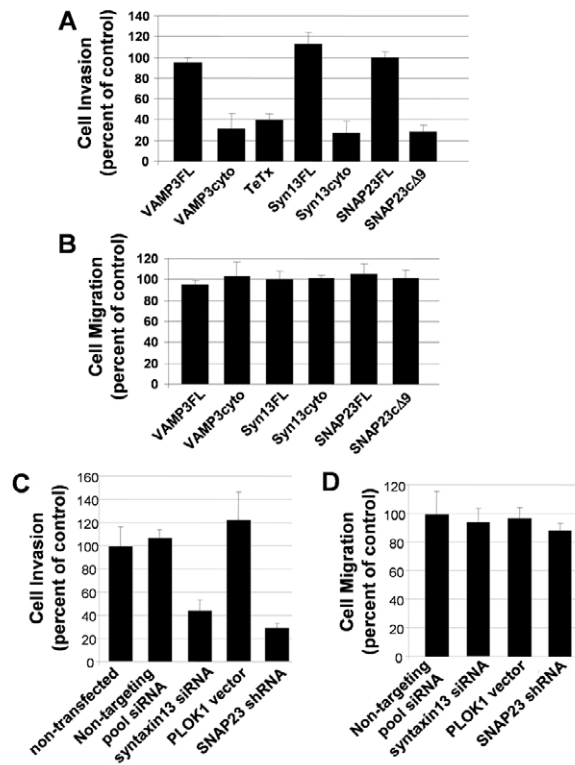

Fig. 7. Inhibition of VAMP3, syntaxin-13 or SNAP23 impairs HT-1080 cell invasion. HT-1080 cells were transiently transfected with VAMP3cyto, Syn13cyto, GFP-SNAP23cΔ9, the corresponding GFP-tagged full-length wild-type SNARE or the catalytic chain of TeTx. (A) 10 hours after transfection, cells were harvested and Transwell invasion assays were performed. Cells invaded through Matrigel towards 10% FBS for 24 hours and were then fixed and counted. (B) After transfection, cells were collected and Transwell migration assays were performed. Transfected cells that migrated to the underside of the membrane after 2.5 hours were counted. (C,D) Cells were co-transfected for 72 hours with pEGFP-N1 and siRNA constructs targeting syntaxin-13 or a non-targeting pool siRNA or with pEGFP-N1 and shRNA vector targeting SNAP23 or PLOK1 control. Cells were then subjected to invasion assays (C) or migration assays (D) as above. In all graphs, values are presented as a percentage of GFP-transfected control; means ± s.e.m. from more than three independent experiments are shown.

impair migration in two-dimensional migration assays; however, their function was required for in situ degradation of a gelatin matrix. Consistent with these findings are the observations that VAMP3 and SNAP23 were required for secretion of the matrix metalloproteinases MMP2 and MMP9, whereas both these SNAREs as well as syntaxin-13 were necessary for efficient delivery of MT1-MMP to the cell surface. Secretion of MMPs by tumor cells leads to the breakdown of the ECM and enables cells to invade surrounding tissue and gain access to the circulation, facilitating the metastatic spread of tumors (Ballin et al., 1988; Kawashima et al., 1994; Tester et al., 2000). Trafficking of MT1-MMP to the cell surface is important in tumor progression, not only for its ability to degrade the ECM but also for its ability to activate secreted MMP2 (Hofmann et al., 2000). In the HT-1080 cells used here, we found that MT1-MMP played a more significant role in the degradation of gelatin than did MMP2 or MMP9. These observations are consistent with current models describing MT1-MMP as a central mediator of ECM proteolysis (Hotary et al., 2006; Sabeh et al., 2009; Sabeh et al., 2004). With clearly measurable influence on the delivery of MT1-MMP to the cell surface, the functions of the SNAREs described in this study might make important contributions to the invasive capacity of tumor cells in vivo.

The observed colocalization of SNAREs with MMP2 and MMP9 in response to PMA treatment (Fig. 1) indicates that these proteins can be found in membrane ruffles, and this is consistent with the notion that the MMPs are present at sites of membrane remodeling. The biological nature of this colocalization is not clear at this point. It is possible that vesicles containing MMP2 and MMP9 accumulate at ruffles prior to exocytosis. Furthermore, extracellular MMP, which has not yet dissipated from the cell surface, might also be detected. In either case, the membrane ruffles, which contain both the indicated SNAREs and MMPs, might mark the site of exocytosis of the MMPs.

Evidence shown in supplementary material Fig. 2 indicates that syntaxin-13 can associate with SNAP23 and VAMP3 in HT-1080 cells. It is thus plausible that these SNAREs interact, though not necessarily in a single complex, to facilitate the vesicular transport of MMPs. The differential effects that inhibition of SNARE function had on the secretion of MMP2 and MMP9 suggests that different SNAREs are involved in the trafficking of these MMPs. MMP2 is constitutively secreted in HT-1080 cells, whereas MMP9 secretion must be induced (Williger et al., 1999). While MMP2 and MMP9 partly colocalize with both SNAP23 and VAMP3, inhibition studies indicate that interfering with SNAP23 had a more potent effect on the secretion of the MMPs than did inhibition of VAMP3. Furthermore, blocking VAMP3 function seemed to preferentially impede MMP2 secretion relative to MMP9 (on the basis of the observed band intensities in Fig. 4A). It is possible that the pathways for secretion of MMP2 and MMP9 are different, with that of MMP2 being more dependent on VAMP3 than that of MMP9. This possibility is consistent with previous reports of separate populations of cytoplasmic vesicles containing MMP2 and MMP9 (Schnaeker et al., 2004). Although we have not defined vesicle populations containing MMPs in the present study, we are currently working to do so. At this point, it is reasonable to propose that the MMPs studied here are newly synthesized and subsequently transported in vesicles through compartments containing VAMP3 (e.g. recycling endosomes) to the plasma membrane. Our evidence does suggest that the constitutively secreted MMP2 is more dependent on VAMP3-mediated traffic than is MMP9. As mentioned above, SNAP23 is a plasma membrane SNARE that might participate in many membrane trafficking pathways; the relatively potent effects of its inhibition observed here are consistent with this notion.

Inhibition of syntaxin-13 reduced the in situ degradation of a gelatin matrix by HT-1080 cells, although we did not observe significant effects of blocking this SNARE on MMP2 or MMP9 secretion. Importantly, syntaxin-13 function was required for efficient trafficking of MT1-MMP to the cell surface. Thus, the effects of blocking syntaxin-13 function on degradation of gelatin in situ might have been mediated through impaired trafficking of MT1-MMP and, as a consequence, reduced activation of MMP2 near the cell surface. This explanation is in agreement with the well-described importance of MT1-MMP in tumor-cell invasion (Hotary et al., 2003; Sabeh et al., 2004).

The results of the experiments herein are important to consider in developing a model for the involvement of vesicle-mediated membrane trafficking in tumor-cell invasion. Several studies have revealed the roles that proteins involved in membrane trafficking, such as caveolin-1, play in regulating MMP2 and MMP9 activity,

tumor cell invasion in vivo and MT1-MMP-dependent cell migration (Labrecque et al., 2004; Williams et al., 2004). In addition, recent work defines a central role for the v-SNARE Ti-VAMP in MT1-MMP-dependent invasion in MDA-MB-231 human breast cancer cells (Steffen et al., 2008). Interestingly, the observed effects of TeTx suggest that this enzyme could be used to target SNAREs and impede tumor-cell invasion in vivo. In some species, SNAP23 is a target for *Clostridium botulinum* toxin A or E (Banerjee et al., 2001; Leung et al., 1998) and this fact affords speculation that a related or modified form of this toxin might be used to modulate tumor cell invasion in humans. Here, we have specifically targeted SNAP23 (using the SNAP23cΔ9 construct) in a manner that mimics cleavage by *C. botulinum* toxin A and observed dramatic effects on both ECM degradation and cell invasion. Collectively, the findings lend support to the suggestion that these *C. botulinum* toxins warrant further study as inhibitors of tumor-cell invasion.

In conclusion, we have demonstrated that the functions of the plasma membrane SNARE SNAP23, and the endosomal SNAREs VAMP3 and syntaxin-13, are necessary for the efficient trafficking of MT1-MMP to the surface of HT-1080 cells. SNAP23 and VAMP3 functions were also required for the normal secretion of MMP2 and MMP9. Furthermore, blocking the functions of these SNAREs impaired the proteolytic degradation of a gelatin matrix in situ and cell invasion in vitro. Collectively, these data are consistent with a model of invasion in which SNARE-mediated secretion of MMPs, and MT1-MMP in particular, is required for ECM degradation, which in turn facilitates movement of the cells. It remains to be determined whether additional SNARE proteins are involved in the trafficking of MMPs in this system. Future studies will be directed at characterizing the function of other SNAREs in this context to further elucidate the molecular mechanisms that control cell invasion.

Materials and Methods

Reagents and cDNA constructs

All chemicals were purchased from Sigma (St Louis, MO) or Fisher Scientific (Nepean, ON) unless otherwise indicated. Antibodies to the following proteins were obtained from the indicated suppliers: SNAP23 (Abcam, ab4114-200), syntaxin-13 (Stressgen, VAM-SV026E), VAMP3 (ABR, PA1-767), MT1-MMP (Abcam, ab3644-500), MMP2 (Abcam, ab2462-1) and MMP9 (Neomarkers, GE-213). All secondary antibodies and Texas red were purchased from Molecular Probes. Inhibitors of MMPs (SB-3CT and SB-3CT pMS) were purchased from EMD Chemicals (Gibbstown, NJ). cDNAs for VAMP3FL, SNAP23-FL, SNAP23cΔ9 and tetanus toxin in pcDNA3.1 were generous gifts from William S. Trimble (Hospital for Sick Children, Toronto, ON). GFP-MMP9 was a generous gift from Rene Harrison (University of Toronto, Toronto, ON). GFP-SNAP23FL and GFP-SNAP23cΔ9 were created by insertion of the genes into pEGFP-C1 (BD Biosciences) at *Xho*I and *Eco*RI sites, VAMP3FL was sub-cloned into a pEGFP-N1 (BD Biosciences) vector. GFP-MMP2 was created by insertion of PCR-amplified MMP2 (Open Biosystems, Huntsville, AL) into pEGFP-N1 (BD Biosciences) vector at *Xho*I and *Sac*II sites. The C-terminal enhanced green fluorescent protein (EGFP) constructs encoding Syn13FL, Syn13cyto and VAMP3cyto were PCR-amplified from a HeLa cell cDNA library and cloned into pEGFP-N1. The following oligonucleotides were used as primers: MMP2FORWARD (5′-TTAATTCTCGAGACGATGGAGGCGCTAATGG-3′), MMP2REVERSE (5′-TATAAACCGCGGGCAGCCTAGCCAGTCGGA-3′), Syn13FORWARD (5′-CTAGCTCGA-GATGTATCGGAATCCCGGG-3′), Syn13REVERSE-FL (5′-CTAG-GAATTCCTTCGTTTTATAAACT-AGCCAGA-3′), Syn13REVERSEcyto (5′-CTAGGAATTCGGACAAAGCACGAGGATACACA-3′), V3FORWARD (5′-CTAGCTCGAGATGTCTACAGGGGTGCCTTC-3′) and V3REVERSEcyto (5′-CTAGGAATTCGCTTGCAGTTCTTCCACCAA-3′). SNAP-23 shRNA construct 144931 (5′-TTATCTCCCAATTAGAAGAGC-3′) was purchased from Open Biosystems. Control shRNA vector (PLOK1) was a generous gift from Ray Lu (University of Guelph, Guelph, ON). ON-TARGETplus SMARTpool siRNA against syntaxin-13 (5′-CCACAAAUCAGCUCGCCAA-3′, 5′-GAGGAUCAGUAUA-UCGGUA-3′, 5′-ACACUACAGUCUCGUAAUA-3′, 5′-GCUCAGAGGUGCA-CGUCGA-3′) and control ON-TARGETplus Non-targeting Pool were purchased from Dharmacon.

Cell culture and transfection

HT-1080 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FBS (Sigma) under 5% CO_2 at 37°C. Cells were transfected with FuGENE 6 (Roche) transfection reagent as described by the manufacturer's protocol. Full-length and cytoplasmic domains of SNARE constructs were expressed for 24-32 hours. siRNA and shRNA constructs were expressed for 96 hours total for invasion, migration, and gelatin degradation assays and for 72 hours to assess knockdown. shRNA transfections were performed twice (48 hours post-initial transfection). Co-transfections were performed using a 1:10 molar ratio of marker pEGFP-N1 plasmid to either tetanus toxin plasmid, shRNA plasmid or siRNA.

Immunofluorescence microscopy

Cells were grown on glass coverslips or plated onto fibronectin-coated (20 μg/ml) glass coverslips, serum-starved and treated with PMA where indicated, and subsequently fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS). Samples were then permeabilized with 0.1% Triton X-100 in PBS and blocked with 5% (w/v) skimmed milk powder in PBS before staining with primary and secondary antibody, followed by washing and mounting. Samples were imaged using a 40× or 63× (NA 1.4) lens on a Leica DM-IRE2 inverted microscope with a Leica TCS SP2 system (Leica, Heidelberg, Germany). Images were captured and 3D reconstructions were performed using Leica Confocal Software package.

Immunoprecipitation

Cyanogen-bromide-activated Sepharose beads (Sigma) were coated with antibody according to the manufacturer's instructions. Cells were lysed with 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100 and protease inhibitor cocktail (Sigma) in PBS. Lysate was incubated with antibody-bound beads overnight at 4°C and then washed four times with lysis buffer. Bound proteins were eluted using 2.5× SDS running buffer, heated to 100°C. Proteins were separated using SDS PAGE.

Gelatin zymography

Six-well dishes were coated with 2.5 mg/ml Matrigel in sterile water (0.4 ml per well). Matrigel was dried overnight, and rehydrated for 1 hour in DMEM prior to seeding of cells. Cells were plated on Matrigel in DMEM with 10% FBS for 3.5 hours and then washed with DMEM. PMA (200 nM) in 0.8 ml serum-free DMEM was added to cells and incubated for 6.5 hours. Media or cells were collected, centrifuged for 8 minutes at 200 ***g*** and the supernatant separated on a gelatin-containing SDS-PAGE gel. The gel was washed three times for 20 minutes in 2.5% Triton X-100 in 50 mM Tris-HCl pH 7.4 (1 hour total) and was then incubated overnight in buffer (50 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$, 0.15 M NaCl, and 0.02% sodium azide) at 37°C before staining with Coomassie blue. The gelatin degradation bands were quantified using ImageJ software (NIH, Bethesda, MD). The size of the gelatin degradation bands, due to MMP2 and MMP9, of three separate experiments were measured.

MT1-MMP trafficking

Cells were grown on glass coverslips, and serum-starved for 3 hours in DMEM. PMA (500 nM) was added to cells to induce trafficking of MT1-MMP to the cell surface. Cells were washed with ice-cold PBS and incubated with 1% BSA in PBS for 30 minutes at 4°C on ice to prevent internalization of MT1-MMP. Anti-MT1-MMP antibodies (Abcam, ab3644) were added to the cells at 8 μg/ml for 1.5 hours. Cells were rinsed with 0.2 M glycine in HCL pH 2.5, followed by successive rinses with PBS to remove any unbound antibody, and fixed with 4% paraformaldehyde. After fixation, cell were incubated with Alexa-Flour-594 secondary antibody for 1 hour. MT1-MMP surface expression was examined using the 63× (NA 1.4) lens of a Leica inverted microscope with constant gain and pin-hole parameters.

For flow cytometry of cell-surface MT1-MMP, HT-1080 cells were transfected with SNARE constructs and incubated for 21 hours. The cells were then serum-starved for 3 hours to allow internalization of MT1-MMP prior to the addition of 500 nM PMA to induce trafficking of MT1-MMP. After PMA treatment for 10 minutes, the cells were lifted in ice-cold 5 mM EDTA in PBS pH 7.4. From this point on, all manipulations were done at 4°C to prevent internalization of cell-surface proteins. The cells were labeled with rabbit anti-MT1-MMP (Abcam, ab3644; 1:50 dilution in PBS containing EDTA and 1% BSA) for one hour at 4°C, after which they were washed three times and labeled with anti-rabbit Alexa-Fluor-647 secondary antibody. The cells were analyzed using a three-laser FACSAria cell sorter from BD Bioscience. Some 10,000 cells were counted per sample per experiment.

Gelatin degradation assay

Gelatin degradation assays were performed as previously described (Hoover et al., 2005). Briefly, coverslips were coated with a thin layer of 2% gelatin in PBS and dried overnight at 4°C. The coverslips were then rinsed in PBS and fixed in PBS containing 0.5% glutaraldehyde for 30 minutes. They were then washed and stained with Texas red-X succinimidyl ester (TRSE) (0.6 μl TRSE in 12 ml of PBS containing 0.1 M $NaHCO_3$) for 30 minutes and washed. The coverslips were quenched in DMEM for 1 hour and cells were seeded on them at 30% confluency and incubated for 24 hours. Degradation areas made by transfected cells were counted and scored as the

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percentage of area degraded per cell (+1 for fully degraded, +0.5 for partially degraded, and 0 for no degradation).

Cell invasion assay

Cell culture inserts, in 24-well dishes (Costar), were prepared with and without Matrigel. The lower chamber was coated with 20 μg/ml fibronectin and the upper chamber with 0.15 mg/ml Matrigel (BD Biosciences). HT-1080 cells were transfected for 8 hours, at which point they were lifted and seeded onto the upper surface, either without Matrigel (control) or coated with Matrigel, in serum-free media (80,000 cells per well). The cells that invaded towards the chemo-attractant (10% FBS) in the lower chamber and penetrated the Matrigel were fixed with 4% paraformaldehyde, stained with DAPI and counted. Cells that did not invade were removed with a cotton swab prior to fixation of sample. Eight fields of cells per membrane were counted. The data are presented as the number of cells that invaded through the Matrigel divided by the number of cells that migrated through the control insert (setting mock-treated, GFP transfected cells at 100%.)

Cell migration assays

Transwell migration assays were performed as previously described (Tayeb et al., 2005). Cells were serum-starved for 1.5 hours, resuspended in serum-free DMEM and placed in the top well of an AP48 Transwell migration chamber (Neuroprobe, Gaithersburg, MD) at 10,000 cells per well. The lower wells were filled with DMEM containing 10% FBS and covered with a fibronectin-coated polycarbonate membrane with 8 μm pores. After 2.5 hours at 37°C, the membrane was removed and the cells were fixed, stained with DAPI and the transfected cells on the bottom and top of the membrane were counted using fluorescence microscopy. The data are presented as the number of mutant-SNARE-expressing cells that migrated as a percentage of control cells expressing wild-type SNARE.

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