Membrane type I-matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface

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Summary

Membrane type 1-matrix metalloproteinase (MT1-MMP) is an integral type I transmembrane multidomain zinc-dependent endopeptidase involved in extracellular matrix remodelling in physiological as well as pathological processes. MT1-MMP participates in the regulated turnover of various extracellular matrix components as well as the activation of secreted metalloproteinases and the cleavage of various cell membrane components. MT1-MMP expression has been reported to correlate with the malignancy of various tumour types and is thought to be an important mediator of cell migration and invasion. Recently, it has been proposed that internalisation of the enzyme from the cell surface is a major short-term level of MT1-MMP regulation controlling the net amount of active enzyme present at the plasma membrane. In this paper we show that, in HT1080 fibrosarcoma cells, MT1-MMP is internalised from the cell surface and colocalises with various markers of the endocytic compartment. Interestingly, we observed that in these cells, internalisation occurs by a combination of both clathrin-mediated and -independent pathways, most probably involving caveolae. In addition, internalised MT1-MMP is recycled to the cell surface, which could, in addition to downregulation of the enzymatic activity, represent a rapid response mechanism used by the cell for relocalising active MT1-MMP at the leading edge during migration.

Key words: Caveolae, Clathrin-coated pit, Endocytosis, MT1-MMP, TIMP-2

Introduction

Matrix metalloproteinases (MMPs) constitute a large group of multidomain zinc-dependent endopeptidases that play a fundamental role in extracellular matrix (ECM) remodelling owing to their ability to degrade a broad spectrum of extracellular matrix components (for a review, see Nagase and Woessner, 1999). Most MMPs are soluble enzymes secreted in the extracellular milieu that can diffuse and target cells in trans. A subset of MMPs, the membrane type MMPs (MT-MMPs) display the common structural domains of the MMP family, but present an additional C-terminal extension that tethers them to the plasma membranes of the cells that produce them, making them important effectors of pericellular ECM degradation and proteolytic activities. MT1-, MT2-, MT3- and MT5-MMPs are integral type I transmembrane proteins with a very short intracellular domain, whereas MT4- and MT6-MMPs are anchored to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) moiety (for a review, see Hernandez-Barrantes et al., 2002).

Membrane-type 1-matrix metalloproteinase (MT1-MMP) has been widely studied since its identification and is invoked in the remodelling of the ECM in physiological (wound healing, bone growth and remodelling) as well as pathological processes (arthritis, tumour growth). This enzyme has been invoked in the regulated turnover of various ECM components such as type I, II, III and IV collagens, fibronectin, vitronectin, laminin, fibrin and proteoglycan (d'Ortho et al., 1997; Ohuchi et al., 1997) and participates in the activation of secreted MMPs such as pro-MMP-2 (Sato et al., 1994) and pro-MMP-13 (Knaüper et al., 1996), which, in turn, will cleave multiple matrix substrates (for a review, see Seiki, 2002). MT1-MMP has also been reported to process various cell membrane components – for example, cell-surface protein-glutamine γ-glutamyltransferase (tTG) (Belkin et al., 2001), pro-αv integrin subunit (Deryugina et al., 2002a) and CD44H, a major receptor for hyaluronan (Kajita et al., 2001).

MT1-MMP expression has been reported to correlate with the malignancy of multiple tumour types including lung, gastric, colon, breast, cervical carcinomas, gliomas and melanomas (for a review, see Yana and Seiki, 2002) and is thought to be an important mediator of cell migration and invasion (Hotary et al., 2000; Kajita et al., 2001). MT1-MMP has been reported to concentrate at the leading edge of various migrating cells, and its focusing at specific sites of the cell surface is thought to be determined by interaction with other membrane proteins at focal adhesions (Ellerbroek et al., 2001). CD44H (Mori et al., 2002) as well as collagen (Tam et al., 2002) have also been specifically implicated. More recently, MT1-MMP expression has been correlated with tumour growth and angiogenesis through upregulation of vascular endothelial

growth factor (VEGF) expression (Deryugina et al., 2002b; Sounni et al., 2002).

MT1-MMP proteolytic activity is highly regulated, and the absence of MT1-MMP activity contributes to abnormal development (Holmbeck et al., 1999; Zhou et al., 2000) and is a key determinant for tumour progression and cancer metastasis (for a review, see Yana and Seiki, 2002). Tissue inhibitors of metalloproteinases (TIMPs)-2, -3 and -4 (for a review, see Baker et al., 2002) as well as the GPI anchored glycoprotein RECK (reversion-inducing cysteine-rich protein with Kazal motifs) (Oh et al., 2001) have been found to inhibit MT1-MMP enzymatic activity. Homophilic oligomerisation of MT1-MMP via its hemopexin domain alone (Itoh et al., 2001) or in cooperation with the cytoplasmic and transmembrane domains (Lehti et al., 2002) promotes pro-MMP2 activation. Dimerisation of MT1-MMP molecules via a disulfide bridge between cysteine residues present in the cytoplasmic tails has also been reported at the cell surface of MT1-MMP-transfected MCF7 cells (Rozanov et al., 2001). Autocatalytic processing is also facilitated by such clustering and appears to act as a level of downregulation (Stanton et al., 1998; Itoh et al., 2001; Lehti et al., 2002).

Recently, several laboratories have proposed that a major short-term level of MT1-MMP regulation at the cell surface is by intracellular trafficking (Jiang et al., 2001; Uekita et al., 2001). MT1-MMP exocytosis is poorly understood; however, key features of this process include the export of latent pro-MT1-MMP from the endoplasmic reticulum to the Golgi apparatus, the proteolytic removal of the propeptide by furin or a related preprotein convertase (Sato et al., 1996; Yana and Weiss, 2000) probably in the trans Golgi network (TGN), the sorting of the activated enzyme from the TGN and its delivery to the cell surface. More recently, Jiang et al. (Jiang et al., 2001) and Uekita et al. (Uekita et al., 2001) have reported the internalisation of MT1-MMP from the cell surface as a means of controlling the net amount of active enzyme present at the plasma membrane. This mechanism would obviously represent a rapid response mechanism which could also be used by the cell for relocalising active MT1-MMP at the leading edge during migration (Nabeshima et al., 2000; Kajita et al., 2001).

Endocytosis is a major mechanism by which cells regulate the level of cell-surface proteins. Endocytosis occurs by clathrin-dependent, as well as clathrin-independent, mechanisms (Nichols and Lippincott-Schwartz, 2001). Clathrin-dependent endocytosis is the most well-defined process to date and is responsible for the rapid uptake of hormones, growth factors and transport molecules such as epidermal growth factors (EGF) and transferrin. The interaction of molecules involved in this process has been investigated both in vivo and in vitro, resulting in the characterisation of several important proteins including clathrin, adaptors and dynamin (Schmid, 1997). Besides clathrin-dependent endocytosis, different forms of non-clathrin-mediated internalisation have been also identified but are much less well characterised (Nichols and Lippincott-Schwartz, 2001). Caveolae are one example of a clathrin-independent and cholesterol-sensitive uptake pathway. Caveolae are flask shaped, non-coated plasma membrane invaginations present in many but not all cell types. They are abundant in fibroblasts and endothelial cells and have been recognised as centres for signalling activity at the cell surface. Despite the growing number of ligands, receptors and lipids using caveolae as a pathway for endocytosis, very little is known about the molecular mechanisms that control uptake by this pathway and where exactly this pathway leads into the cell.

The endocytosis studies conducted thus far have revealed that MT1-MMP is internalised by using a dynamin-dependent pathway in MDCK cells (Jiang et al., 2001). Additionally a clathrin-mediated uptake has been reported in CHO-K1 cells by virtue of interaction of the membrane proximal di-leucine motif localised within the enzyme cytoplasmic domain with the AP-2 adaptor complex (Uekita et al., 2001). Two further studies implicate a role for caveolae. MT1-MMP has been found in detergent-insoluble, glycolipid-rich membrane microdomains (Annabi et al., 2001) and co-sediments with caveolin-1 in U-87 glioblastoma cells, HT1080 and MT1-MMP-transfected COS-7 cells (Annabi et al., 2001). In addition, MT1-MMP has been found to colocalise with caveolin-1 at the cell surface of concanavalin A-treated microsvascular endothelial HMEC-1 cells (Puyraimond et al., 2001).

Therefore, the present study aimed to determine whether MT1-MMP uses more than one pathway for its internalisation from the cell surface. To this end, we used the $Eps^{E\Delta 95/295}$ (EGF receptor pathway substrate clone 15) dominant negative mutant which specifically blocks clathrin-mediated endocytosis, allowing the study of clathrin-independent MT1-MMP internalization. In addition, we addressed the extent to which different forms of MT1-MMP and its TIMP-2-associated complex may be endocytosed and whether the enzyme is subsequently destined for degradation or recycled to the cell surface.

Materials and Methods

All chemicals were AnalaR grade and were purchased from either BDH (Poole, UK) or Sigma Chemical Co. (Poole, UK) unless indicated otherwise.

Antibodies

Anti-EEA1 (early endosome autoantigen-1) mouse monoclonal antibody (mAb clone 14); anti-Eps15 (EGF receptor pathway substrate clone number 15) mAb (clone 17) and anti-caveolin-1 (C13630) rabbit polyclonal antibody (pAb) were purchased from BD Transduction Laboratories (Lexington, KY). Anti-TIMP-2 (67.4H11) mAb was a gift from K. Iwata (Fuji Chemical Industries, Toyama, Japan). Anti-biotin mouse mAb was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-Rab4-CT rabbit pAb was form Stressgen Biotechnologies Corporation (BC, Canada). Anti-LAMP-1 (lysosome-associated membrane protein-1) mouse mAb was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Affinity-purified rabbit anti-clathrin heavy chain immunoglobulins (IgGs) were kindly provided by Margaret S. Robinson (Cambridge Institute for Medical Research, Cambridge, UK). Anti-MT1-MMP sheep pAb IgGs (N175/6) (d'Ortho et al., 1998) were affinity purified using MT1-MMP ectodomain immobilised on a HiTrap NHS-activated HP (Amersham Pharmacia Biotech UK, Little Chalfont, UK). Bound IgGs were eluted in 100 mM glycine pH 2.5 containing 10% dioxane and the pH of the eluate was neutralised immediately by adding 0.1 volume of 1 M Tris-HCl pH 8.0. Anti-TIMP-2 sheep pAb (H225/9) was described previously (Ward et al., 1991). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and used according to the manufacturer's instructions.

Cell culture conditions, transfections and DNA constructs

All cell culture reagents were purchased from Invitrogen (Paisley, UK) unless indicated. HT1080 human fibrosarcoma and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (Hyclone Laboratories, UT), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. HT1080 stably transfected with wild-type MT1-MMP cDNA (bbHT1080 clone #2) were prepared and grown as described in Stanton et al. (Stanton et al., 1998). T47D and MCF7 cell lines were kindly donated by A. Noel (Laboratoire de Biologie des Tumeurs et du Développement, Liège, Belgium) and cultured as previously described.

Transfections were performed using FuGENE-6 reagent, according to manufacturer's instructions (Roche Diagnostics, Lewes, UK).

Wild-type dyn2aa (dyn^{wt}) and K44A (dyn^{K44A}) dynamin cDNAs in pEGFP vector were a gift from Mark McNiven (Mayo Clinic and Foundation, Rochester) (Cao et al., 2000). Vectors containing the inserts encoding EGFP-$Eps^{E\Delta 95/295}$ and EGFP-$Eps15^{wt}$ were a gift from Alexandre Benmerah (Pasteur Institute, Paris) (Benmerah et al., 1999).

Full-length MT1-MMP cDNA without 5′ and 3′ untranslated regions was inserted between the *Hin*dIII and *Eco*RI site of pCDNA3.1 Zeo^+ mammalian expression vector (Invitrogen). Cytotail truncated MT1-MMP construct (MT1ΔC) was prepared from full-length MT1-MMP by inserting a stop codon after Phe^{561} by site-directed mutagenesis.

Cell-surface biotinylation, recycling assay and immunoprecipation

Cell-surface protein biotinylation and recycling assay were performed according to Neuhaus and Soldati (Neuhaus and Soldati, 2000) with the following modifications. Wild-type HT1080 and bbHT1080 cells grown to 80-90% confluence in a six-well plate were washed twice with ice-cold SBS (Soerensen Buffer, 14.7 mM KH_2PO_4 and 2 mM Na_2HPO_4 with 120 mM Sorbitol pH 7.8) and incubated for a further 10 minutes in ice-cold SBS. Biotinylation was performed by adding 2.5 ml of freshly prepared ice-cold SBS containing 0.3 mg/ml EZ-Link NHS-SS-biotin (sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate, Perbio Science UK) per well for 15 minutes. After two washes with ice-cold SBS, the unreacted biotin was quenched for 10 minutes by incubation with ice-cold SBS containing 100 mM glycine. Quenched cells were then washed twice with ice-cold SBS and incubated at 37°C in CIM/ITS to allow the internalisation of biotinylated plasma membrane proteins. After 15 minutes, the uptake was stopped by washing the cells with ice-cold SBS and placing them on an ice bath for 10 minutes. Biotin present on cell-surface components was cleaved off by incubating the cells with 150 mM of membrane impermeant reducing agent MESNA (2-mercaptoethane sulfonic acid) in ice-cold SBS, pH 8.2 for 25 minutes. Cells were washed three times with ice-cold SBS, and then lysed in 0.2 ml/well of RIPA buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, 1% NP-40) containing proteinase inhibitor cocktail set III (Calbiochem-Novabiochem Corporation, San Diego, CA).

For recycling assay, MESNA treated cells were washed with ice-cold SBS and incubated in CIM/ITS at 37°C to allow recycling of the endocytosed biotinylated proteins. Recycling was then stopped after 15, 30 and 45 minutes by washing the cells with ice-cold SBS and placing them on an ice bath for 10 minutes. Biotin re-exposed at the cell surface was cleaved off by a second MESNA treatment (see above). Finally, the cells were washed three times with ice-cold SBS, and lysed in RIPA buffer as previously described.

RIPA protein extracts were centrifuged at 16,000 ***g*** for 10 minutes at 4°C. Supernatants were precleared with 25 μl of protein G-agarose beads for 1 hour at 4°C. After centrifugation, biotinylated proteins were immunoprecipated for 1 hour at 4°C using 25 μl protein G-agarose beads saturated with an anti-biotin mouse mAb. Immobilised complexes were collected, washed five times with 1 ml of RIPA buffer, eluted from protein G with 30 μl of 2.5× Laemmli sample buffer. Samples (20 μl) were separated by SDS-PAGE and subjected to immunoblot analysis.

Endocytosis assays by indirect immunofluorescence microscopy analysis

HT1080 cells seeded onto 13 mm sterile round glass coverslips and grown to 60-70% confluence were washed twice with PBS. After 30 minutes incubation at 37°C in a serum-free CO_2 independent medium (CIM) containing 1% Insulin-Transferrin-Selenium (ITS) supplement, cells were washed with ice-cold CIM/ITS and incubated for a further 15 minutes at 4°C. Intact cells were then incubated for 1 hour at 4°C with 10 μg/ml affinity-purified anti-MT1-MMP sheep pAb (N175/6) or 10 μg/ml anti-TIMP-2 mouse mAb (67.4H11) in CIM/ITS. Cells were extensively washed with ice cold serum-free CIM/ITS to remove unbound antibody and surface-bound material was internalised by incubating the cells at 37°C in CIM/ITS for indicated time points. Texas red-labelled transferrin (25 ug/ml, Molecular Probes, Eugene, OR) was internalised in serum-free CIM/ITS for 20 minutes at 37°C. Cells were then fixed and permeabilised (see below) and internalised primary antibody was detected using the appropriate fluorescently labelled secondary antibody and samples were then processed as described under Indirect Immunofluorescence Microscopy.

Indirect immunofluorescence microscopy

Cells were seeded on 13 mm round glass coverslips and grown at 37°C until reaching 70-80% confluence. Cells were washed in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4), fixed either with methanol at –20°C for 5 minutes or with 4% paraformaldehyde in PBS for 25 minutes at room temperature. In the latter case, cells were permeabilised for 4 minutes in 0.1% Triton X-100 (Surfact-Amps X-100 grade, Pierce, Rockford, IL) in PBS. Staining was carried out as described by Roghi and Allan (Roghi and Allan, 1999). After extensive washing in PBS, coverslips were briefly rinsed in H_2O and then mounted on glass slides in Mowiol containing 25 mg/ml 1,4-diazobicyclo-[2.2.2]-octane to reduce photo-bleaching. Pictures of fluorescently labelled cells were collected using a cooled, slow scan CCD camera (Micromax 1401E, Roper Scientific, Harlow, UK) attached to a Nikon Eclipse E800 microscope (Nikon UK, Kingston upon Thames, UK) using a 60× Plan-Apo (NA 1.4) oil objective. Images were also acquired using a Photometrics Coolsnap HQ CCD camera (Roper Scientific, Harlow, UK) attached to a Zeiss Axioplan 2 imaging microscope equipped with a motorised stage and fitted with 63× and 100× plan apochromat objectives (Carl Zeiss, Welwyn Garden City, UK). Image acquisition was performed using Metamorph software (Universal Imaging Corporation, Downingtown, PA). Images were transferred to Photoshop (Adobe Systems, San Jose, CA) imaging software for digital processing.

Drug treatments

Cells were incubated for 1 hour at 37°C in CIM/ITS containing 30 μg/ml nystatin or 50 μg/ml concanavalin A (ConA) or for 30 minutes at 37°C in CIM/ITS containing 4 mM methyl-β-cyclodextrin before cell-surface biotinylation or antibody uptake assay. bbHT1080 cells were incubated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 16 hours. Microtubule-disrupting drug nocodazole (10 μg/ml in DMSO) was added to the cells for 1 hour. As controls, carriers were added to the cells at a final concentration of 0.1% and did not have any effect on the internalisation (data not shown).

Detergent-free isolation of caveolae-enriched membrane fraction

Caveolae-enriched membranes were prepared from HT1080 cells using carbonate extraction according to the protocol described by Song et al. (Song et al., 1996). Fractions (1 ml) were collected from the top of the gradient and transferred into new ultracentrifuge tubes containing 8 ml of MBS [25 mM MES (2[N-morpholino] ethanesulfonic acid), pH 6.5, 150 mM NaCl]. After 90 minutes at 208,429 g_{max} (Ti70.1 rotor, Beckman Coulter, High Wycombe, UK) at 4°C, pellets were resuspended with 150 μl of Laemmli sample buffer and 15 μl aliquots were separated by SDS-PAGE and subjected to immunoblot analysis as described below.

SDS-PAGE and immunoblot analysis

All reagents for electrophoresis were purchased from Bio-Rad (High Wycombe, UK). Proteins were electrophoretically transfered for 1 hour at 15 Volts onto Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech) using a Mini Trans-Blot Electrophoretic transfer cell (Bio-Rad). After blocking with 5% (wt/vol) nonfat dry milk in PBS (2× 20 minutes at room temperature), blots were probed with 1.5 μg/ml affinity purified anti-MT1-MMP IgGs or 50 ng/ml anti-caveolin-1 pAb in 2.5% milk in PBS for 16 hours at 4°C. After several washings (PBS containing 0.05% (vol/vol) Tween 20), membranes were incubated for 1 hour at room temperature with appropriate HRP-conjugated secondary antibody diluted in 2.5% milk in PBS. After extensive washes, specific immunocomplexes were detected using an ECL western blotting detection kit (Pierce).

Results

Active MT1-MMP is internalised from the cell surface of HT1080

In an attempt to study the internalisation of MT1-MMP and its proteolytically inactivated 45 kDa degradation product in unstimulated cells, we developed a cell-surface biotinylation assay. HT1080 cells were rapidly cooled to 0°C and cell-surface proteins were biotinylated using a cleavable biotin derivative (Fig. 1A, biotinylation). Afterwards, cells were warmed to 37°C for 15 minutes to allow internalisation (Fig. 1A, uptake), and then cooled on ice to arrest further transport. Biotin present at the cell surface was removed by incubation with the membrane-impermeable reducing agent MESNA (Fig. 1A, cleavage 1). Internalised biotinylated proteins were selectively immunoprecipitated from the cell extract using an anti-biotin pAb and analysed by immunoblotting using the affinity-purified anti-MT1-MMP antibody (N175/6). Using this protocol, both active 60 kDa and the 45 kDa form of MT1-MMP were detected at the surface of HT1080 cells incubated at 0°C (Fig. 1B, lane 2). Removal of biotin was quantitative as indicated by the complete loss of biotinylated MT1-MMP forms in cells that were MESNA-stripped at 0° C (Fig. 1B, lane 3). When the cells were warmed to 37°C for 15 minutes before de-biotinylation, active MT1-MMP was now detected in the MESNA protected fraction (Fig. 1B, lane 5) demonstrating that 60 kDa MT1-MMP was internalised from HT1080 cell surface. Interestingly, the 45 kDa form of MT1-MMP was not detected in the MESNA protected fraction (Fig. 1B, lane 5) probably because of the low amount of this form at the cell surface of nonstimulated HT1080 cells (Fig. 1B, lane 2). To control whether or not 45 kDa MT1-MMP could eventually be internalised from the cell surface, we used HT1080 cells stably transfected with full-length wild-type human MT1-MMP cDNA (bbHT1080). These cells express higher amounts of 45 kDa and 60 kDa MT1-MMP at their surface. bbHT1080 cell-surface proteins were biotinylated at 0°C. Cells were warmed

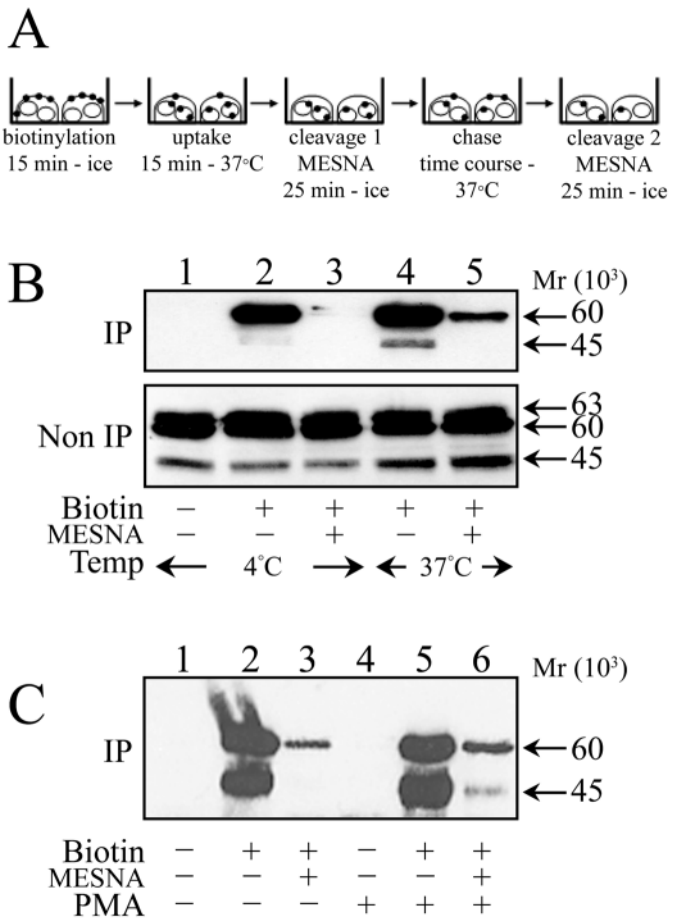

Fig. 1. Active MT1-MMP is internalised in nonstimulated HT1080 cells. (A) The biotinylation experiment. Plasma membrane proteins were biotinylated on ice (black spots; biotinylation) and then internalised for 15 minutes at 37°C (uptake). Cells were cooled on ice and surface biotin was removed by treating the cells with MESNA for 25 minutes at 4°C as described in Materials and Methods (cleavage 1). Recycling of internalised biotinylated proteins was performed by placing the cells at 37°C for various times (chase), followed by a second MESNA treatment to cleave the biotin present on internalised proteins re-exposed at the cell surface (cleavage 2). (B) Active MT1-MMP is internalised in HT1080 cells. HT1080 cells were incubated on ice in the presence (lanes 2-5) or absence (lane 1) of biotin to label cell-surface proteins. Biotinylated HT1080 cells were kept for 15 minutes on ice (lanes 2 and 3) or warmed to 37°C (lanes 4 and 5) to allow internalisation and then treated with MESNA to remove cell-surface biotin (lanes 3 and 5). Biotinylated proteins were immunoprecipitated (IP) using an anti-biotin antibody, separated on an SDS-PAGE and analysed by immunoblotting using anti-MT1-MMP affinity-purified IgGs (IP). Unbound materials (non-IP) were also analysed. (C) bbHT1080 cells treated with (lanes 4, 5 and 6) or without (lanes 1, 2 and 3) PMA for 16 hours at 37°C were incubated with (lanes 2, 3, 5 and 6) or without biotin (lanes 1 and 4) on ice to label cell-surface proteins. Cells were warmed to 37°C to allow internalisation and treated with (lanes 3 and 6) or without (lanes 2 and 5) MESNA. Biotinylated proteins were immunoprecipitated, separated on an SDS-PAGE and analysed by immunoblotting.

to 37°C and treated without (Fig. 1C, lane 2) or with MESNA (Fig. 1C, lane 3). Despite the presence of a large amount of the 45 kDa MT1-MMP form at the cell surface of bbHT1080 (Fig. 1C, lane 2), we did not detect any internalisation of this form when cells were warmed up for only 15 minutes at 37°C (Fig. 1C, lane 3), suggesting that the 45 kDa MT1-MMP form was either not internalised at this time or was internalised but immediately degraded. Internalisation of the 45 kDa MT1-MMP proteolytically inactivated form could, however, be detected after 15 minutes internalisation when bbHT1080 cells were treated with PMA. In addition to a slight increase in the amount of the 45 kDa MT1-MMP form detected at the cell surface of PMA-treated bbHT1080 cells (Fig. 1C, lane 5) compared with untreated cells (Fig. 1C, lane 3), a small amount of the 45 kDa MT1-MMP form could now be detected in the MESNA protected fraction suggesting that the proteolytically inactivated 45 kDa MT1-MMP form could, under these conditions, be internalised from the cell surface (Fig. 1C, lane 6). PMA stimulation of endocytosis has previously been reported – for example, in human neutrophils (Keller, 1990). PMA has also been suggested to induce rapid internalisation and thus downmodulation of the chemokine receptor CXCR4 (Signoret et al., 1997); it has also been found to stimulate the transcytosis and recycling of the polymeric immunoglobulin receptor (pIgR) in Madin-Darby canine kidney (MDCK) cells (Cardone et al., 1994). Endocytosis of 45 kDa MT1-MMP has also been observed in untreated bbHT1080 cells when the internalisation time was raised to 40 minutes (S. Atkinson and G. Murphy, personal communication). This result agrees with the observation made by Munshi et al. (Munshi et al., 2002) using squamous cell carcinoma line SCC25 cells.

Internalised MT1-MMP accumulates in a perinuclear located vesicular compartment in bbHT1080 cells

On the basis of our previous observation, we decided to assess MT1-MMP internalisation in bbHT1080 using an antibody uptake assay to visualize MT1-MMP localisation after its internalisation. bbHT1080 cells were incubated with affinity-purified anti-MT1-MMP antibody for 1 hour at 4°C. Unbound antibody was washed off and cells were warmed to 37°C for varying times. Cells were fixed and IgG-bound MT1-MMP complexes were detected after permeabilisation using a fluorescently labelled secondary antibody. Using this protocol, we observed that IgG-bound MT1-MMP complexes originally present at the cell surface (Fig. 2A) were quickly internalised after switching the cells from 4°C to 37°C (Fig. 2B-E). Vesicles containing IgG-bound MT1-MMP complexes accumulated in the cell cytoplasm and concentrated with time in the perinuclear region of the cell (Fig. 2C-E). Addition of the microtubule-depolymerising drug nocodazole during the uptake assay did not inhibit internalisation of IgG-bound MT1-MMP complexes but induced the redistribution of perinuclear IgG-bound MT1-MMP complexes to the cell periphery (Fig. 2F), suggesting the accumulation of internalised MT1-MMP in the endocytic pathway. MT1-MMP internalisation was also observed using a Fab fraction prepared from the affinity-purified anti-MT1-MMP IgGs. This shows that MT1-MMP internalisation did not result from an antibody-mediated clustering effect (data not shown). In addition, no intracellular vesicular staining was observed when internalisation experiments were carried out in MCF7 and T47D cells, which do not express MT1-MMP (data not shown).

MT1-MMP is found in the endocytic compartment

To characterise the vesicular structures containing IgG-bound MT1-MMP complexes, we performed double-immunofluorescence microscopy experiments. Nonstimulated bbHT1080 cells, which were allowed to internalise IgG-bound MT1-MMP complexes for 30, 45 and 60 minutes, were fixed, permeabilised and incubated with primary antibodies directed against membrane markers for various endocytic compartments. The early endosomal antigen EEA1 interacts with Rab5 and phosphatidylinositol-3-phosphate, facilitating early endosome fusion (Christoforidis et al., 1999). After 30 minutes internalisation, IgG-bound MT1-MMP complexes were found to extensively colocalise with EEA1- (Fig. 3A-C, arrowheads) and, to a certain extent, with Eps15-containing early endosomes (Fig. 3D-F, arrowheads). We did not observe

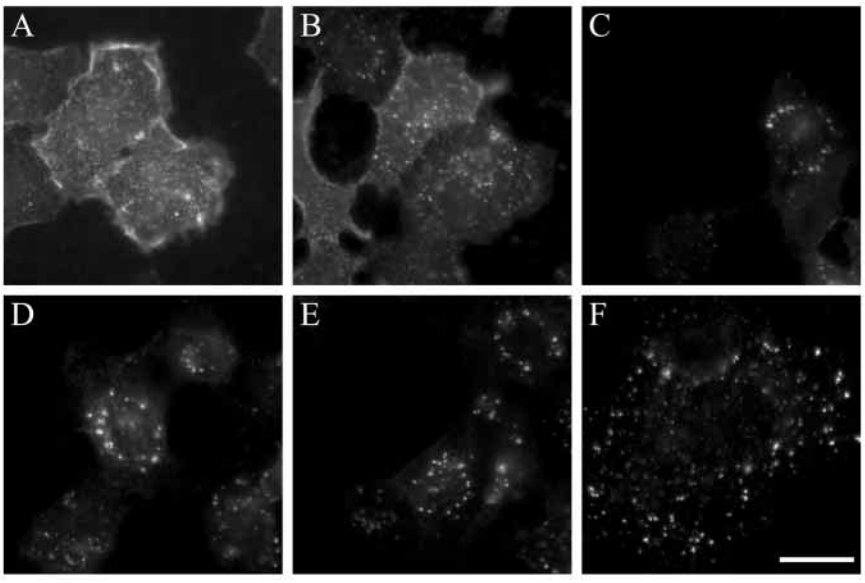

Fig. 2. Internalisation of IgG-bound MT1-MMP complexes in bbHT1080 cells visualised by immunofluorescence microscopy. Intact and nonstimulated bbHT1080 cells were incubated with affinity-purified anti-MT1-MMP IgGs for 1 hour at 4°C. Cells were then washed and fixed immediately with paraformaldehyde (A) or warmed to 37°C for 10 (B), 20 (C), 30 (D) or 60 minutes (E) before fixation. In F, bbHT1080 cells were warmed to 37°C for 60 minutes in the presence of nocodazole to depolymerise microtubules. IgG-bound MT1-MMP complexes were detected using fluorescently labelled secondary antibody in TX-100-permeabilised cells. Bar, 20 μm.

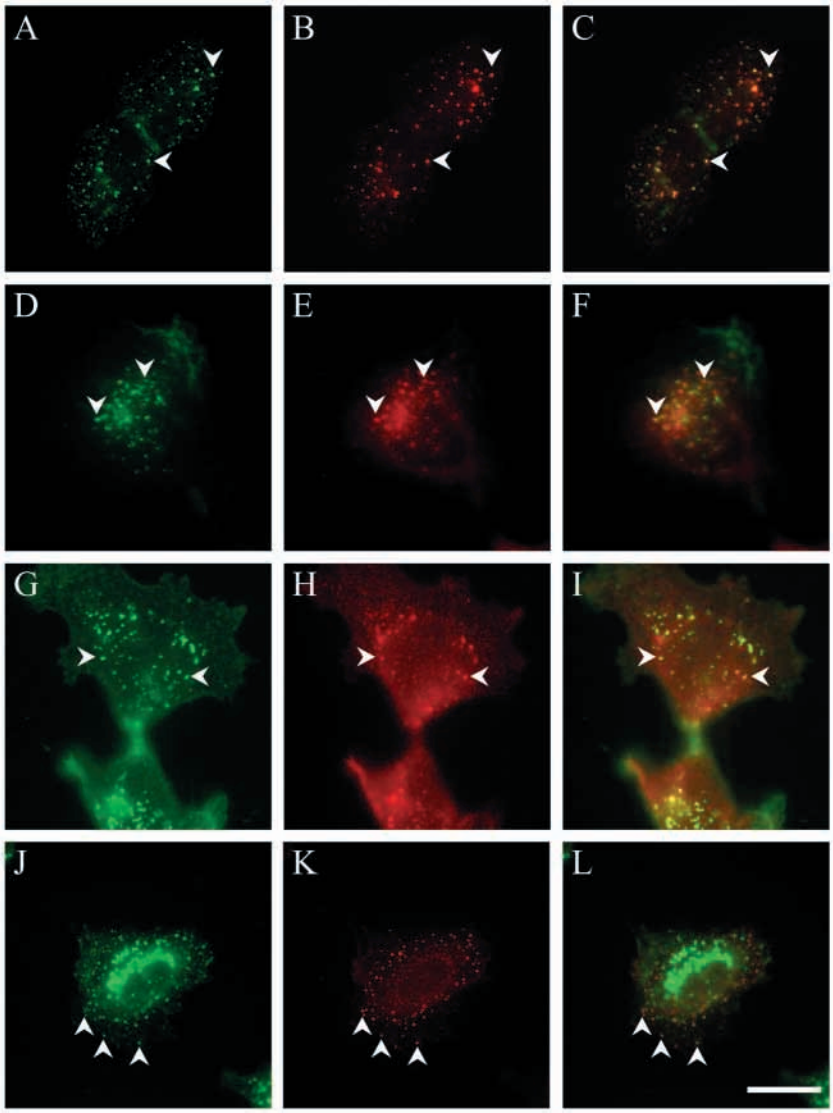

Fig. 3. Internalised MT1-MMP colocalises with different endocytic markers. bbHT1080 cells that had internalised IgG-bound MT1-MMP complexes for 30 (A-F), 45 (G-I) or 60 minutes (J-L) were fixed, permeabilised and incubated with antibodies directed against EEA1 (B), Eps15 (E), Rab4 (H) and LAMP-1 (K). Arrowheads are examples of colocalising structures. The merged panels (C, F, I and L) depict colocalisation between MT1-MMP and the appropriate endocytic marker. Bar, 20 μm.

colocalisation with Rab4, a marker for recycling endosomes (Sönnichsen et al., 2000) or with LAMP-1, a marker for late endosomes/lysosomes (Hunziker and Geuze, 1996) at this particular time point (data not shown). When IgG-bound MT1-MMP complexes were internalised for 45 minutes, IgG-bound MT1-MMP complexes were still largely present in EEA1-positive endosomes (data not shown) but in addition, they were also found in rab4-positive recycling endosomes (Fig. 3G-I, arrowheads). Colocalisation with LAMP-1 could not be detected at this time point but could, however, be observed when IgG-bound MT1-MMP complexes were internalised for 60 minutes (Fig. 3J-L, arrowheads). We did not observe colocalisation between MT1-MMP and TGN43, a specific marker for the TGN, suggesting that internalized MT1-MMP is probably not recycled to the TGN.

Taken together, this set of experiments clearly shows that, in bbHT1080, cell-surface IgG-bound MT1-MMP complexes are internalised from the cell surface and distributed with time in various compartments of the endocytic pathway. Our results correlate well with observations reported previously by other groups (Jiang et al., 2001; Uekita et al., 2001), enabling us to pursue our studies with bbHT1080 cells.

Exocytosis of internalised active MT1-MMP

The presence of IgG-bound MT1-MMP in Rab4-positives structures (Fig. 3G-I) suggests that internalised MT1-MMP may recycle back to the cell surface. To test this hypothesis, wild-type nontransfected HT1080 cells were biotinylated at 0°C (Fig. 1A, biotinylation, and Fig. 4, lane 2). After 15 minutes internalisation at 37°C (Fig. 1A, uptake), cells were cooled again to 0°C and MT1-MMP remaining at the cell surface was de-biotinylated with MESNA (Fig. 1A, cleavage 1, and Fig. 4, lane 3). In parallel, cells with internalised biotinylated MT1-MMP were warmed again to 37°C for 15, 30 and 45 minutes (Fig. 1A, chase) to allow recycling of internalised biotinylated proteins to the cell surface, cooled to 0°C and treated (Fig. 1A, cleavage 2, and Fig. 4, lanes 5, 7 and 9) or not (Fig. 4, lanes 4, 6 and 8) with MESNA. In this case, the difference between MESNA-treated and MESNA-nontreated samples corresponds to the fraction of biotinylated MT1-MMP that has been re-exported to the cell surface during the second warming to 37°C. This experiment clearly shows for the first time that internalised MT1-MMP is recycled to the surface of the cell.

Selective inhibition of the clathrin-dependent pathway does not completely inhibit active MT1-MMP internalisation

Previous reports have shown that active MT1-MMP internalisation is completely blocked in cells expressing the dyn^{K44A} dominant-negative dynamin mutant (Jiang et al., 2001; Uekita et al., 2001). Because dyn^{K44A} overexpression was shown to block both clathrin-dependent (Pearse and Robinson, 1990; De Camilli et al., 1995) and clathrin-independent endocytosis (Oh et al., 1998; Schnitzer, 2001), the previously reported experiments were unable to precisely identify the pathway(s) involved in active MT1-MMP internalisation. In bbHT1080, expression of EGFP-dyn^{K44A} completely inhibited MT1-MMP (Fig. 5A,B) and transferrin receptor (Fig. 5C,D) internalisation. Interestingly, when the cells were transiently transfected with the EΔ95/295 dominant-negative Eps15 mutant tagged with EGFP (EGFP-$Eps^{EΔ95/295}$), which has been previously described to specifically block clathrin-dependent endocytosis without disrupting clathrin-independent endocytosis

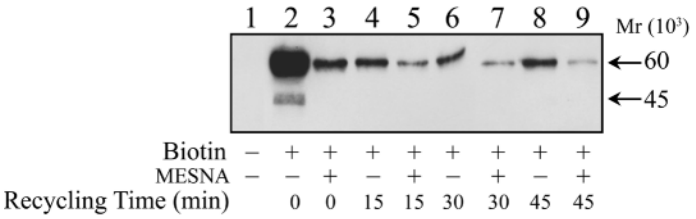

Fig. 4. Active MT1-MMP is recycled to the cell surface in HT1080 cells. HT1080 cells were incubated on ice in the presence (lanes 2-9) or absence (lane 1) of biotin to label cell-surface proteins. Biotinylated cells were warmed to 37°C for 15 minutes to allow internalisation then cooled to 0°C and treated with (lane 3) or without (lane 2) MESNA. Cells were warmed to 37°C for 15 (lanes 4 and 5), 30 (lanes 6 and 7) and 45 minutes (lanes 8 and 9) to allow recycling and then treated with (lanes 5, 7 and 9) or without (lanes 4, 6 and 8) MESNA. Biotinylated proteins were immunoprecipitated, separated on an SDS-PAGE and analysed by immunoblotting using anti-MT1-MMP affinity-purified IgGs.

(Benmerah et al., 1999), we observed a significant uptake of active MT1-MMP from the cell surface (Fig. 5E,F), whereas Texas red-labelled transferrin internalisation was completely inhibited (Fig. 5G,H). No effects were observed when EGFP-Eps15^{wt} was expressed before MT1-MMP internalisation (data not shown). In addition, IgG-bound MT1-MMP complexes were observed at the cell surface of EGFP-Eps$^{E\Delta 95/295}$-transfected cells (Fig. 5F) compared with untransfected cells, suggesting that internalisation of MT1-MMP also required a fully functional clathrin-dependent pathway. Interestingly, the expected plasma membrane staining observed in EGFP-Eps$^{E\Delta 95/295}$-expressing cells was not found in EGFP-dyn^{K44A}-expressing cells (Fig. 5B), indicating that expression of dyn^{K44A} has actually a more profound effect on MT1-MMP expression at the cell surface than previously described (Jiang et al., 2001).

Taken together, our observations showed that active MT1-MMP was still internalised when the clathrin-dependent pathway was selectively disrupted, suggesting that both clathrin-dependent and -independent pathways are probably involved in the endocytosis of active MT1-MMP.

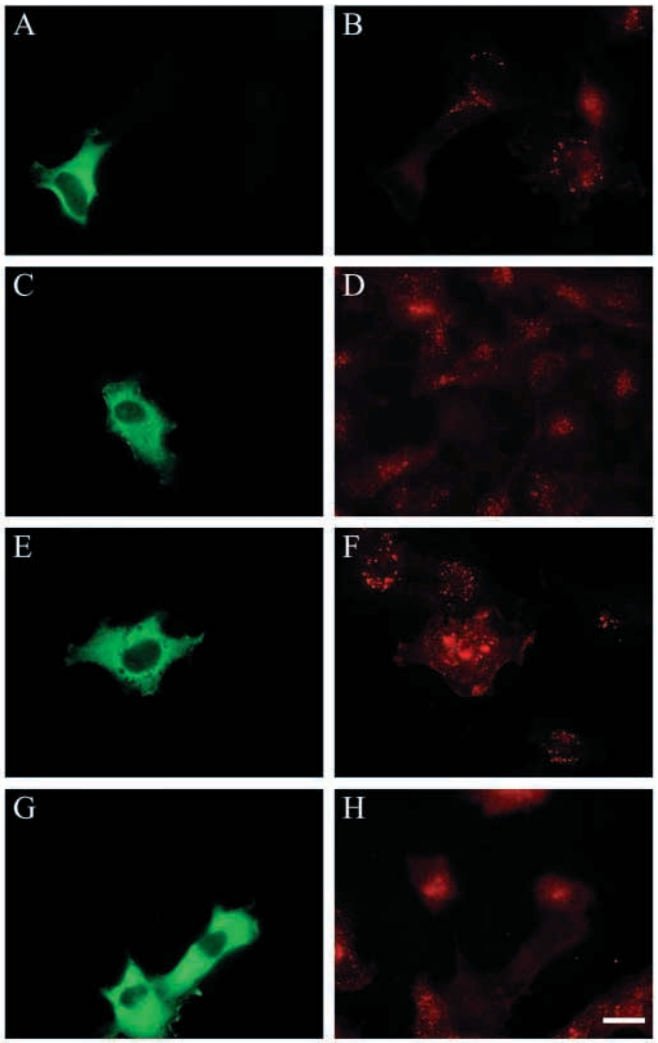

Fig. 5. Active MT1-MMP is internalised in EGFP-Eps$^{E\Delta 95/295}$-expressing cells. bbHT1080 cells transiently transfected with a dyn^{K44A} dominant-negative dynamin mutant (A-D) or with an EGFP-Eps$^{E\Delta 95/295}$ dominant-negative Eps15 mutant (E-H) were allowed to internalise anti-MT1-MMP pAb (A,B,E,F) or Texas red-conjugated transferrin (C,D,G,H) at 37°C. After fixation and TX-100 permeabilisation, IgG-bound MT1-MMP complexes (B,F) were revealed using a Texas-red conjugated secondary antibody. Bar, 20 μm.

Active MT1-MMP form is internalised through a cholesterol-dependent pathway

We next focused our interest on the analysis of the previously identified clathrin-independent pathway involved in active MT1-MMP internalisation. Previous reports have shown the presence of MT1-MMP in caveolae (Puyraimond et al., 2001; Annabi et al., 2001), and to assess whether or not active MT1-MMP could be endocytosed through a caveolae-mediated pathway, we examined IgG-bound MT1-MMP internalisation in cells with perturbed caveolae. bbHT1080 cells were treated with nystatin, a cholesterol-binding drug that inhibits endocytosis from caveolae and caveolae-like domains by impairing invagination, and thereby internalisation (Schnitzer et al., 1994; Deckert et al., 1996), without interfering with clathrin-coated pit formation. In cells treated with nystatin (Fig. 6B) or with methyl-β-cyclodextrin (Fig. 6D), we observed a more profound cell-surface staining compared with untreated cells (Fig. 6A,C), suggesting that cholesterol-sensitive structures are probably involved in the internalisation of MT1-MMP from the cell surface. Internalised IgG-bound MT1-MMP complexes can be seen in nystatin-treated cells (Fig. 6B, arrowheads), as well as in methyl-β-cyclodextrin-treated cells (Fig. 6D, arrowheads), suggesting that some internalisation still occurred in these cells most probably via the clathrin-mediated pathway. Texas-red-labelled transferrin internalisation was not affected in nystatin-treated cells (data not shown) as reported previously by others (Torgersen et al., 2001).

The involvement of both pathways was also assessed using our biotinylation approach using concanavalin A- or nystatin-treated bbHT1080 cells. In nontreated cells (Fig. 6E, lanes 2 and 3), we observed, as previously described in this paper, only the internalisation of 60 kDa active MT1-MMP (Fig. 6E, lane

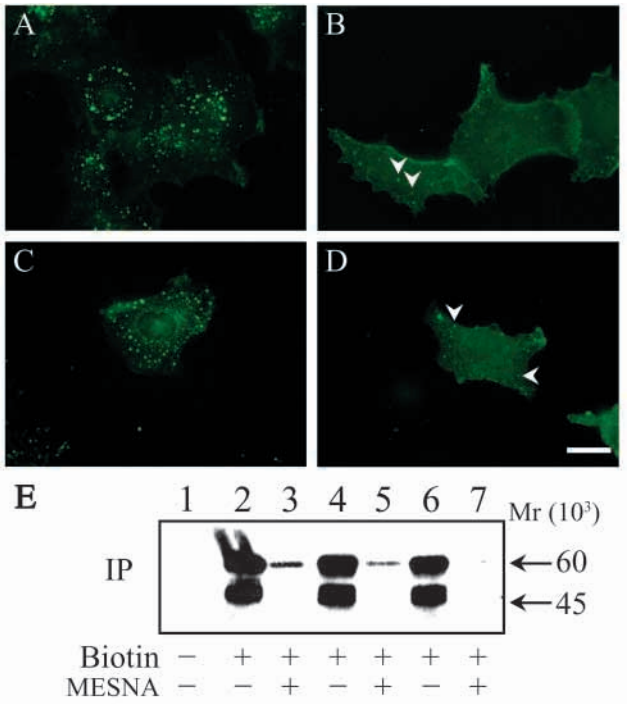

Fig. 6. MT1-MMP internalisation is greatly reduced in cells with perturbed caveolae function. bbHT1080 cells treated in the absence (A,C) or in the presence of 30 μg/ml nystatin (B) or 4 mM methyl-β-cyclodextrin (D) were subjected to an antibody uptake assay using anti-MT1-MMP affinity-purified IgGs. After 15 minutes, cells were fixed and TX-100 permeabilised, and IgG-bound MT1-MMP complexes were revealed using a FITC-conjugated secondary antibody. Arrowheads are examples of internalised MT1-MMP-positive structures. Bar, 20 μm. (E) bbHT1080 cells were not treated (lanes 2 and 3) or treated for 1 hour with 30 μg/ml nystatin (lanes 4 and 5) or for 16 hours with 50 μg/ml concanavalin A (lanes 6 and 7) before cell-surface biotinylation as described in Materials and Methods. After 15 minutes internalisation, cells were treated with (lanes 3, 5 and 7) or without (lanes 2, 4 and 6) MESNA to remove cell-surface biotin. Biotinylated proteins were immunoprecipitated, separated on a SDS-PAGE and analysed by immunoblotting using anti-MT1-MMP affinity-purified IgGs.

3). ConA has previously been reported to block both caveolae- and clathrin-coated pits-mediated pathways (for a review, see Lefkowitz, 1998) and, as expected, completely inhibited internalisation of active MT1-MMP from the cell surface (Fig. 6E, lane 7). Interestingly, when bbHT1080 cells were treated with nystatin (Fig. 6E, lane 5) we observed that 60 kDa active MT1-MMP was still internalised but the amount of internalised enzyme was clearly reduced compared with untreated cells (Fig. 6E, lane 3). This approach provides further evidence that MT1-MMP internalisation requires a combination of clathrin-dependent and -independent pathways.

To support this result, unstimulated bbHT1080 cells with internalised IgG-bound active MT1-MMP complexes were permeabilised and stained with antibodies directed against caveolin-1 and clathrin heavy chain. We observed that MT1-MMP was found in caveolin-1-positive vesicular structures (Fig. 7C, arrowheads), as well as in clathrin-positive structures (Fig. 7F, arrowheads), again suggesting the involvement of the caveolae system and clathrin-coated pits in MT1-MMP internalisation.

Finally, to have a more biochemical confirmation that caveolae might be involved in MT1-MMP internalisation, we isolated caveolae-enriched membrane domains using a detergent-free procedure (Song et al., 1996) from nonstimulated HT1080 cells. Isolated crude membranes were subjected to a carbonate extraction, followed by a subcellular fractionation

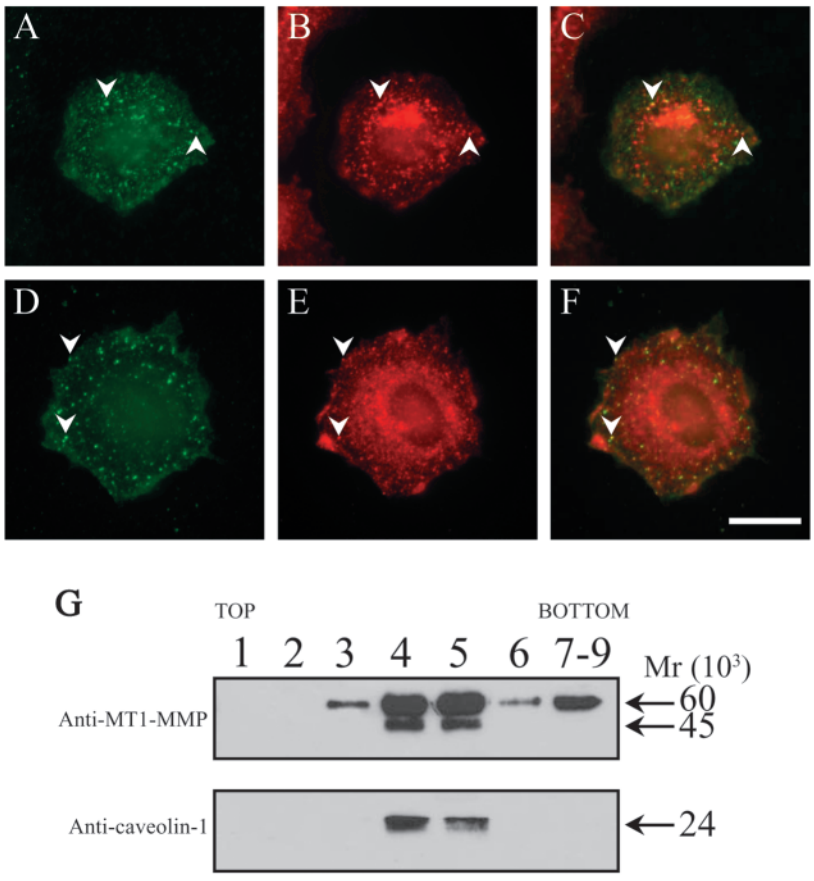

Fig. 7. Internalised MT1-MMP colocalised with caveolin-1 and clathrin heavy chain and is present in caveolin-1 containing fractions. bbHT1080 cells, which had internalised IgG-bound MT1-MMP complexes for 10 minutes (A,D), were fixed, permeabilised and incubated with antibodies directed against caveolin-1 (B) and clathrin heavy chain (E) followed by a fluorescently labelled secondary antibody. Merged panels (C,F) are shown in the right-hand column. Arrowheads are examples of colocalising structures. Bar, 20 μm. (G) After a detergent-free carbonate-based extraction, HT1080 cells were homogenised and subjected to subcellular fractionation. Fractions (1 ml) were collected from the top of the gradient, separated on a 10% SDS-PAGE gel and immunoblotted using anti-MT1-MMP affinity-purified IgG (upper panel) or with an anti-caveolin-1 pAb (lower panel).

using a discontinuous sucrose-density gradient. Analysis of the gradient fractions by immunoblotting (Fig. 7G) revealed that active MT1-MMP was present in the caveolin-1-positive fractions (Fig. 7G, lanes 4 and 5), as well as in other fractions (Fig. 7G, lanes 3, 6 and 7-9), suggesting its distribution within multiple membrane compartments. Interestingly, the proteolytically inactivated 45 kDa MT1-MMP form was only found in the caveolin-1-enriched membrane fraction (Fig. 7G, lanes 4 and 5).

Taken altogether, our results suggest that in HT1080, MT1-MMP is internalised by a clathrin-independent pathway, most probably caveolae, alongside the clathrin-mediated endocytosis.

TIMP-2 is co-internalised with MT1-MMP

Numerous reports have described the binding of TIMP-2 to the MT1-MMP catalytic domain and the role of the complex as the physiological plasma membrane receptor for proMMP-2 activation (Strongin et al., 1995; Butler et al., 1998). Recently, Maquoi et al. (Maquoi et al., 2000) have reported that, in PMA-treated cells, TIMP-2 was first internalised before being partially degraded. We therefore examined whether or not TIMP-2 internalisation could be mediated by MT1-MMP. HeLa cells, which express low levels of MT1-MMP and a substantial amount of TIMP-2, were transiently transfected with full-length MT1-MMP or cytotail truncated MT1ΔC cDNAs. Transfected cells were then subjected to an antibody uptake assay using affinity-purified anti-MT1-MMP IgGs together with anti-TIMP-2 antibody. After 30 minutes at 37°C, cells were fixed and permeabilised and IgG-bound active MT1-MMP and IgG-bound TIMP-2 complexes were revealed using fluorescently labelled secondary antibodies. Using this strategy, we observed a very strong colocalisation between internalised TIMP-2 and internalised active MT1-MMP (Fig. 8B) in MT1-MMP-transfected HeLa cells. Interestingly, most, if not all, intracellular TIMP-2-positive vesicles were MT1-MMP positive, suggesting that TIMP-2 internalisation is a MT1-MMP-mediated event in which TIMP-2 and MT1-MMP could be internalised as a bimolecular complex. In addition, a few MT1-MMP-positive vesicles were TIMP-2-negative, indicating the presence of TIMP-2-free MT1-MMP in the cell cytoplasm (Fig. 8B, arrowhead, for an example). It is still not known whether this TIMP-2 free MT1-MMP resulted from the endocytosis of uncomplexed MT1-MMP or the endocytosis of the bimolecular complex, followed by the dissociation of the complex due to the low pH of the endocytic compartment. To confirm that TIMP-2 endocytosis is indeed MT1-MMP mediated, HeLa cells were transiently transfected with MT1-MMP deleted from its cytoplasmic tail (MT1ΔC). MT1ΔC can traffic to the cell surface but was then poorly endocytosed and mainly retained at the cell surface (Jiang et al., 2001; Uekita et al., 2001). In MT1ΔC-transfected HeLa cells, IgG-bound MT1-MMP and IgG-bound TIMP2 complexes were mainly detected at the cell surface (Fig. 8D). Taken together, our data suggest that TIMP-2 internalisation is exclusively and specifically mediated by MT1-MMP.

Discussion

In the present study, we have investigated the internalisation of endogenous MT1-MMP from the cell surface of fibrosarcoma cells HT1080. As previously described by Jiang et al. (Jiang et al., 2001) and Uekita et al. (Uekita et al., 2001) in transfected MDCK and CHO-K1 cells, respectively, we observed that endogenous MT1-MMP is endocytosed from the cell surface of HT1080 fibrosarcoma cells. Interestingly, in our conditions we did not observe the internalisation of 45 kDa catalytically inactive MT1-MMP, even when present in a large amount at the cell surface of unstimulated bbHT1080 cells. The presence of this molecule at the cell surface may have important biological properties because it retains both hemopexin and

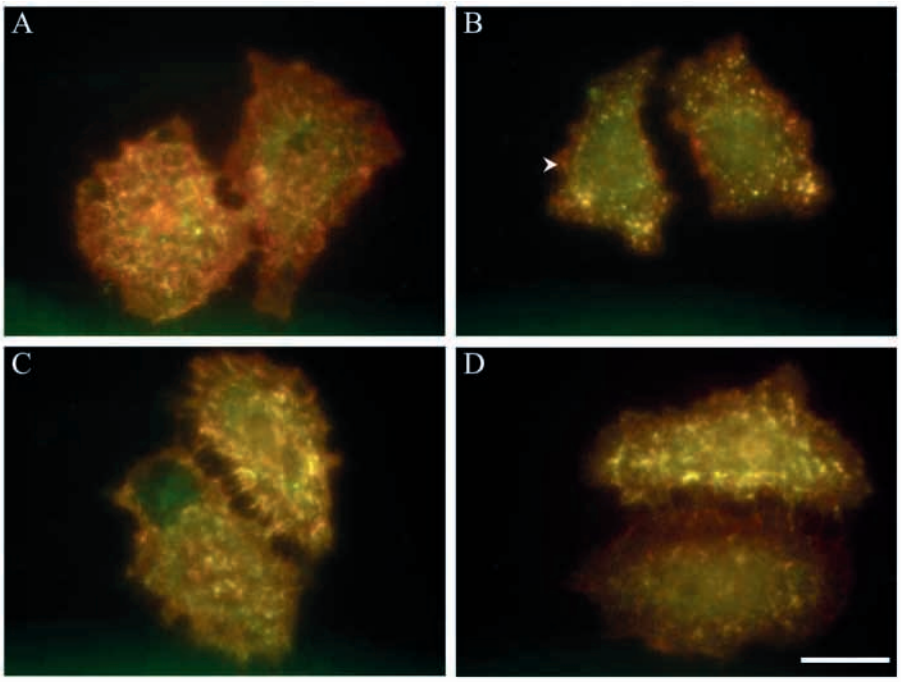

Fig. 8. TIMP-2-mediated MT1-MMP internalisation. HeLa cells transiently transfected with full-length (A,B) or with cytoplasmic tail-deleted MT1-MMP construct (MT1ΔC; C,D) were subjected to an antibody uptake assay using anti-MT1-MMP affinity-purified IgGs, together with anti-TIMP2 67.4H11 antibodies. Internalised MT1-MMP-bound and TIMP2-bound IgG complexes were revealed at the cell surface of non-permeabilised cells (A,C) and in TX-100 permeabilised cells (B,D). All panels are *z*-projections of a stack or 10 images. Bar, 10 μm.

probably its cytoplasmic domain. The proteolytically inactive 45 kDa MT1-MMP form was internalised only in PMA-treated cells. PMA has been reported to prevent the formation of caveolar invaginations but does not inhibit clathrin-mediated endocytosis (Smart et al., 1994), suggesting that, in PMA-treated cells, this form is internalised in a clathrin-dependent pathway.

Active MT1-MMP is internalised by two different pathways

Previous work has shown the clathrin-mediated and dynamin-dependent internalisation of MT1-MMP internalisation from the cell surface (Uekita et al., 2001; Jiang et al., 2001). Our results confirm and extend these recent findings by showing clearly that MT1-MMP is internalised by a clathrin-independent mechanism, which we suspect to be caveaole, in addition to a the clathrin-mediated endocytic pathway. The most direct evidence is the continued internalisation of MT1-MMP in bbHT1080 cells expressing EGFP-Eps$^{E\Delta 95/295}$ in which clathrin-coated pit endocytosis is selectively blocked. Moreover, the nondetection, using immunofluorescence techniques, of IgG-bound complexes in EGFP-dyn^{K44A} transfected bbHT1080 cells suggests that most, if not all, MT1-MMP internalisation is dynamin-dependent and a third internalisation pathway is not likely be involved in MT1-MMP internalisation.

Clathrin-dependent and -independent pathways seem to co-exist in nonstimulated HT1080, and they are probably nonredundant internalisation pathways for MT1-MMP. It will be interesting to assess whether or not these two pathways co-exist in a migrating fibroblast. Obviously, the definitive role of each pathway is as yet unknown and remains to be identified, thus their potential function can only be hypothesised. It may be that both pathways internalise different subpopulations of MT1-MMP present at the cell surface. Another difference between caveolae and clathrin-mediated internalisations is speed and localisation of internalisation. In a resting cell, the speed of internalisation, as well as the localisation, may not be that important for the cell. However, in a migratory situation, speed may be an issue. It has been shown previously that internalisation by caveolae is two to four times slower that clathrin-mediated endocytosis (Fishman, 1982; Tran et al., 1987). Clathrin-mediated and caveolae-dependent pathways may be involved in the differential localisation of internalisation at the cell surface. In migrating endothelial cells, caveolae have been found to relocalise at the trailing edge of the migrating cell (Isshiki et al., 2002). MT1-MMP is internalised at the adherent edge of cells plated on gelatin (Uekita et al., 2001). Recent studies have shown a functional interaction between MT1-MMP and integrins. However, the mechanisms by which ECM and integrins might regulate MT1-MMP functionality remain unexplored. Recently, Gálvez et al. (Gálvez et al., 2002) have shown that β1 integrin interacts with MT1-MMP and appears to be involved in the modulation of MT1-MMP internalisation together with ECM components. Integrin β1 has been found to immunoprecipitate with caveolin-1, a defining protein component of caveolae, and uPAR in uPAR-transfected 293 cells (Wei et al., 1999) and in detergent-lysed A431cells (Wary et al., 1996). In addition in WI-38 human lung fibroblasts, a fraction of β1 coprecipitates with caveolin-1 and promotes Fyn-dependent Shc phosphorylation in response to integrin ligation, leading to mitogen-activated protein kinase (MAPK) activation and cellular growth (Wary et al., 1998). Caveolin-1 is also important to β1 integrin-dependent fibronectin adhesion and focal adhesion kinase activation (Wei et al., 1999). It is possible that MT1-MMP internalisation from the adherent edge could be caveolae and/or clathrin mediated. The absence of internalization of cytotail depleted MT1-MMP from the adherent edge implies the absence of determinant signal sequence(s) which direct MT1-MMP to caveolar structures in these cells.

Caveolae have been described as chemical switchboards enriched in molecules that play pivotal roles in intracellular signal transduction. MT1-MMP is present in caveolae located at the cell surface, as well as in internalised caveolae that have been pinched off from the cell surface. Signal transduction can certainly happen in both situations. Caveolae-located MT1-MMP could be involved in extracellular signal-related kinase (ERK)-dependent activation of transcription via its cytoplasmic tail (Gingras et al., 2001). Overexpression of caveolin-1 has been reported to inhibit caveolae formation and, in MT1-MMP-expressing COS-7 cells, suppresses the increased migration on gelatin promoted by MT1-MMP expression (Annabi et al., 2001). The absence of caveolae in these cells could reflect the absence of signalling between cell-surface MT1-MMP and the inside of the cell, and thus explain why these cells are unable to achieve MT1-MMP-induced migration.

Postendocytic sorting of MT1-MMP

Internalised MT1-MMP is also recycled to the cell surface. The presence of MT1-MMP in a rab4-positive endocytic compartment suggests that the recycling is probably effected from this intracellular compartment. However, it is possible that recycling may also occur from internalized caveolae. Studies on the internalisation of the folate receptor (Smart et al., 1994), cholera toxin and alkaline phosphatase (Parton et al., 1994) have indicated that membrane recycling occurs during caveolae-mediated endocytosis.

In our resting HT1080 cells, we suspect that internalised MT1-MMP is randomly re-inserted with internalised membranes at the cell surface. However, reinsertion may be redirected to the sites of protusion when migration is induced by motogenic stimuli (Bretscher and Aguado-Velasco, 1998). Cell migration is driven by the protrusive activity at the leading edge of the cell, where continuous remodelling of actin and adhesive contacts is required. It has been hypothesised that membrane internalised from the cell surface is recycled to the front of migrating cells to contribute to the extension of the cell border (Bretscher and Aguado-Velasco, 1998). Given the rapid rate of membrane internalization (Hao and Maxfield, 2000), large amounts of recycling membrane would be made available for polarized delivery by such a mechanism.

MT1-MMP recycling could also be involved in the dissociation of the MT1-MMP/TIMP-2 complex. The co-internalisation experiment of MT1-MMP and TIMP-2 has revealed the presence of both molecules in the same vesicular structure, probably early endosomes. It is not known whether or not both molecules are still interacting together, but the

low pH of this endosomal compartment may provoke the dissociation of the complex and both proteins may recycle independently and to different regions of the cell surface. In addition, it would be interesting to investigate whether or not pro-MMP-2 is found in the same compartment as TIMP-2 and MT1-MMP after internalisation and thus could be activated in an intracellular endocytic membrane compartment and released as an active MMP-2 in the extracellular milieu.

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