The cytoplasmic tail peptide sequence of membrane type-1 matrix metalloproteinase (MT1-MMP) directly binds to gC1qR, a compartment-specific chaperone-like regulatory protein

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Abstract Membrane type-1 matrix metalloproteinase (MT1-MMP), a key enzyme in cell locomotion, is known to be primarily recruited to the leading edge of migrating cells. This raises a possibility that the C-terminal cytoplasmic tail of MT1-MMP interacts with intracellular regulatory proteins, which modulate translocations of the protease across the cell. Here, we demonstrated that MT1-MMP via its cytoplasmic tail directly associates with a chaperone-like compartment-specific regulator gC1qR. Although a direct functional link between these two proteins remains uncertain, our observations suggest that the transient associations of gC1qR with the cytoplasmic tail of MT1-MMP are likely to be involved in the mechanisms regulating presentation of the protease at the tumor cell surface.

Key words: Extracellular matrix; Trafficking; Membrane protein; Membrane type-1 matrix metalloproteinase; gC1qR; Internalization

1. Introduction

Matrix metalloproteinases (MMPs) are a comprehensive family of zinc enzymes that degrade the extracellular matrix and cell surface molecules [1,2]. Understanding the function of these enzymes in carcinogenesis is critical for the design of anti-cancer pharmaceuticals. A transmembrane domain and a cytoplasmic tail (CT) of membrane type-1 (MT1)-MMP associate this abundant membrane-tethered protease with discrete regions of the plasma membrane and the intracellular milieu, respectively [3]. MT1-MMP has been shown to be directly involved in cell locomotion and matrix degradation [4–6] and in the activation pathway of soluble secretory MMPs, proMMP-2 and proMMP-13 [7–9]. MT1-MMP was primarily localized to the leading edge of migrating cells where the protease directly cleaves certain matrix substrates [10,11]. These observations raise a possibility that the CT of MT1-MMP interacts with certain proteins of the intracellular milieu and plays a role in the mechanisms regulating MT1-MMP function [12,13].

In this report we present evidence that both in vitro and in breast carcinoma MCF7 cells the CT of MT1-MMP specifically interacts with a multifunctional, ubiquitously expressed mature gC1qR protein (named also as p33 or p32) [14]. Earlier, we have demonstrated that gC1qR directly associates with the extracellular domain of MT1-MMP and that gC1qR is susceptible to MT1-MMP proteolysis [15]. Our current results indicate that there are two distinct gC1qR-binding sites in the MT1-MMP molecule, the first in the extracellular part and the second in the intracellular CT sequence of the protease. These independent lines of evidence linking MT1-MMP to gC1qR suggest a functional significance of interactions existing between these proteins in tumor cells.

2. Materials and methods

2.1. Antibodies and reagents

The recombinant gC1qR protein and anti-gC1qR murine mAb 60.11 were described earlier [16,17]. Goat anti-rabbit IgG and donkey anti-mouse IgG conjugated with horseradish peroxidase (HRP), rabbit antibody AB815 against the hinge region of MT1-MMP, and TMB/M were from Chemicon International (Temecula, CA, USA). Rabbit antibody against the recombinant catalytic domain of MT1-MMP (catMT) [18] was generated in our laboratory. Control rabbit and murine IgG, and ExtrAvidin-HRP were from Sigma (St. Louis, MO, USA).

2.2. Purification of gC1qR

The (C)RRHGTPRRLLYSERSLLDKV peptide derived from the sequence of the MT1-MMP's CT and the (C)YRSRDHRVKTRLPLRQSLGL scrambled peptide (1 mg each) were dissolved in 50 μl dimethyl sulfoxide (DMSO), mixed with 1 ml 50 mM Tris–HCl, 5 mM ethylenediamine tetraacetic acid (EDTA), pH 8.5, and covalently linked through the cysteine residue (shown in parentheses in the peptide sequence) to 1 ml of Sulfo-link Coupling gel (Pierce, Rockford, IL, USA). Human breast carcinoma MCF7 cells (1×10^9) grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS) were lysed for 1 h in 20 ml of phosphate-buffered saline (PBS), 1% Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, pH 7.4. Insoluble material was removed by centrifugation at $25\,000\times g$ for 15 min. The supernatant (about 350 mg of total protein) was split in two equal parts. One part was chromatographed on a 1 ml CT column, and the other was chromatographed on a 1 ml scrambled peptide column. After loading of the superna-

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Abbreviations: CT, cytoplasmic tail; catMT, the catalytic domain of MT1-MMP; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP; TFA, trifluoroacetic acid

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tant, the columns were consecutively washed with 20 volumes of the lysis buffer, 20 volumes of the lysis buffer containing 0.5 M NaCl and 10 volumes of 10 mM Tris–HCl, pH 8.5. The bound material was eluted with 0.05% trifluoroacetic acid (TFA) and collected in 10 fractions (1 ml each). The pH in each fraction was adjusted by adding 5 μl of 10% NH_4OH. Fractions were 10-fold concentrated on a Speed-Vac. One tenth of each fraction was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis.

2.3. Sequence determination

Following SDS–PAGE, the protein band was transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) and subjected to N-terminal microsequencing. In separate experiments, the protein band was cleaved directly in gel with trypsin. Tryptic peptides were extracted from the gel and their mass spectra were determined by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry using Perseptive Biosystems Voyager DE-RP mass spectrometer. The ProFound software at http://129.85.19.192/profound_bin/WebProFound.exe was used to identify the sequence of the tryptic peptides.

2.4. MT1-MMP constructs and cell transfection

The wild-type MT1-MMP (WT; GeneBank Accession Number U41078) and the catalytically inactive mutant (E240A) where the Ala replaced the Glu-240 residue of the enzyme's active site were described earlier [13]. The mutagenic primers, 5′-CCTTGCAGTC-TTCTTCTTC**TA**ACGCCATGGGACCCCC-3′ (direct primer) and 5′-GGGGGTCCCATGGCGT**TA**GAAGAAGAAGACTGCAAGG-3′ (reverse primer), were used to construct MT1-MMP mutant lacking the CT (ΔCT) where the AGA triplet encoding the Arg-563 had been replaced with the TAA stop codon (nucleotides shown in bold indicate the altered codons). Mutagenesis was performed using the QuickChange system (Stratagene, San Diego, CA, USA). The cDNA coding for ΔCT mutant was further recloned into the pcDNA3-zeo plasmid (Invitrogen, San Diego, CA, USA) and its structure was confirmed by sequencing.

MCF7 cell lines stably expressing the WT, E240A and ΔCT constructs were isolated as described previously [19,20]. The MT1-MMP-transfected cell pools of three to six respective positive clones were generated to avoid any clone-specific effects. Mock cells transfected with the original pcDNA3-zeo plasmid were generated as a pool of zeocin-resistant cells. Transfected cells were routinely grown in DMEM/10% FCS and 0.2 mg/ml zeocin.

2.5. Immunoprecipitation

Cells were surface biotinylated for 1 h with 0.1 mg/ml Sulfo-NHS-LC-biotin. Labeled cells were solubilized at 5×10^6–1×10^7 cells/ml in triethanolamine-buffered saline (TBS), pH 7.4, containing 50 mM *n*-octyl-β-D-glucopyranoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin for 1 h at 4°C. Insoluble material was removed by centrifugation. Supernatants were pre-cleared for 2 h at 4°C with protein A-Agarose beads (Calbiochem, San Diego, CA, USA). Aliquots (1 mg of total protein) of pre-cleared supernatants were incubated overnight at 4°C with 1–3 μg of anti-MT1-MMP AB815 or anti-gC1qR mAb 60.11 and 30 μl of a 50% protein A-Agarose slurry. Following washing, the beads were boiled with 2×SDS sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 0.005% bromophenol blue and 20% glycerol) with 50 mM DTT for 5 min. Eluted proteins were separated by SDS–PAGE and transferred onto an Immobilon-P membrane. Bands containing biotin-labeled proteins were visualized by using ExtrAvidin-HRP and TMB/M as a substrate.

2.6. Immunostaining and confocal microscopy

Cells were grown for 48 h in DMEM/10% FCS at 3×10^4 cells/well on 8-well Lab-Tek™ II chamber glass slides (Nalge Nunc International, Naperville, IL, USA) pre-coated overnight with 10 μg/ml type I collagen. Next, cells were fixed in 4% formaldehyde in PBS for 15 min at room temperature and washed with 0.02% glycine PBS to quench free aldehyde residues. Cells were permeabilized with 0.1% saponin (Sigma) in PBS, consecutively blocked with 2.5% normal donkey serum, 1.0% bovine serum albumin (BSA) and 0.1% saponin in PBS for 20 min at room temperature and further incubated with gC1qR-specific mAb 60.11 and MT1-MMP-specific antibodies (both at 10 μg/ml) for 1 h at room temperature. Slides were washed and successively incubated for 45 min each with a 1:150 dilution of Alexa-488-conjugated donkey anti-mouse IgG and a 1:150 dilution of Alexa-594-conjugated donkey or goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). Following washing with PBS, the slides were mounted in VectaShield antifading embedding medium (Vector Lab, Burlingame, CA, USA). The samples were studied in the laser scanning confocal microscope MRC 1024 MP (Bio-Rad) equipped with Kr/Ar laser. The levels of co-localization of MT1-MMP with gC1qR were quantified by the LaserPix program (Bio-Rad) and expressed as the Pearson's correlation coefficient.

3. Results

3.1. The CT peptide sequence of MT1-MMP binds gC1qR

Earlier, we have hypothesized that the C-terminal CT of MT1-MMP directly interacts with certain intracellular proteins [13]. To isolate a putative intracellular partner, we synthesized the (C)RRHGTPRRLLY**S**ERSLLDKV bait peptide derived from the sequence of the MT1-MMP's CT and the (C)YR**S**RDHRVKTRLPLRQSLGL scrambled control peptide which has the distinct charge distribution. To avoid dimerization of the peptide and to ensure its correct orientation on the matrix, the unique Cys-574 of the CT was replaced with the Ser in both the bait and control peptides (shown in bold in the peptide sequence). Earlier, the Cys-574 has been demonstrated to be essential for a disulfide bridge that links monomers of MT1-MMP on cell surfaces [13]. We hypothesized that homodimerization of MT1-MMP could eliminate a complex formation of the protease monomer with its putative, heterologous intracellular partner. Accordingly, we specifically selected the CT sequence incapable of stable dimerization and replaced the Cys-574 in the peptide sequence with the Ser, the most chemically similar amino acid residue. This approach was successfully used in the past [21] and, consequently, was not expected to sharply modify the recognition profile of the CT peptide. Since Lehti et al. [12] reported mislocalization at the cell surface of the C-terminally truncated MT1-MMP mutants bearing deletions in the CT, we did not use shortened peptides as bait in our studies.

Peptides were each immobilized on a solid matrix support via the thiol group of the corresponding Cys (shown in parentheses). The resulting sorbents were used for isolation of the CT-binding proteins from MCF7 cells. Equal aliquots of the lysate were chromatographed on each column. After washing of the columns, the bound material was eluted with 0.05% TFA and analyzed by SDS–PAGE (Fig. 1A). There was no specific protein binding to the scrambled peptide. On the contrary, a prevalent 33 kDa protein was eluted from the CT column. The 33 kDa band was subjected to the N-terminal microsequencing (Fig. 1B). Additionally, the 33 kDa band was hydrolyzed with trypsin and the resulting proteolytic fragments were analyzed by MALDI-TOF mass spectrometry. Both the N-terminal sequencing and MALDI-TOF data demonstrated that the 33 kDa band represented the mature gC1qR protein.

To further substantiate the ability of the CT bait to directly interact with gC1qR, the recombinant gC1qR protein was chromatographed on each the CT and scrambled peptide columns. Following washing, the bound material was eluted and analyzed by SDS–PAGE (Fig. 1C). gC1qR was found in the sample eluted from the CT column but not in that obtained from the scrambled peptide column. These results confirmed that gC1qR was capable of binding to the CT of MT1-MMP.

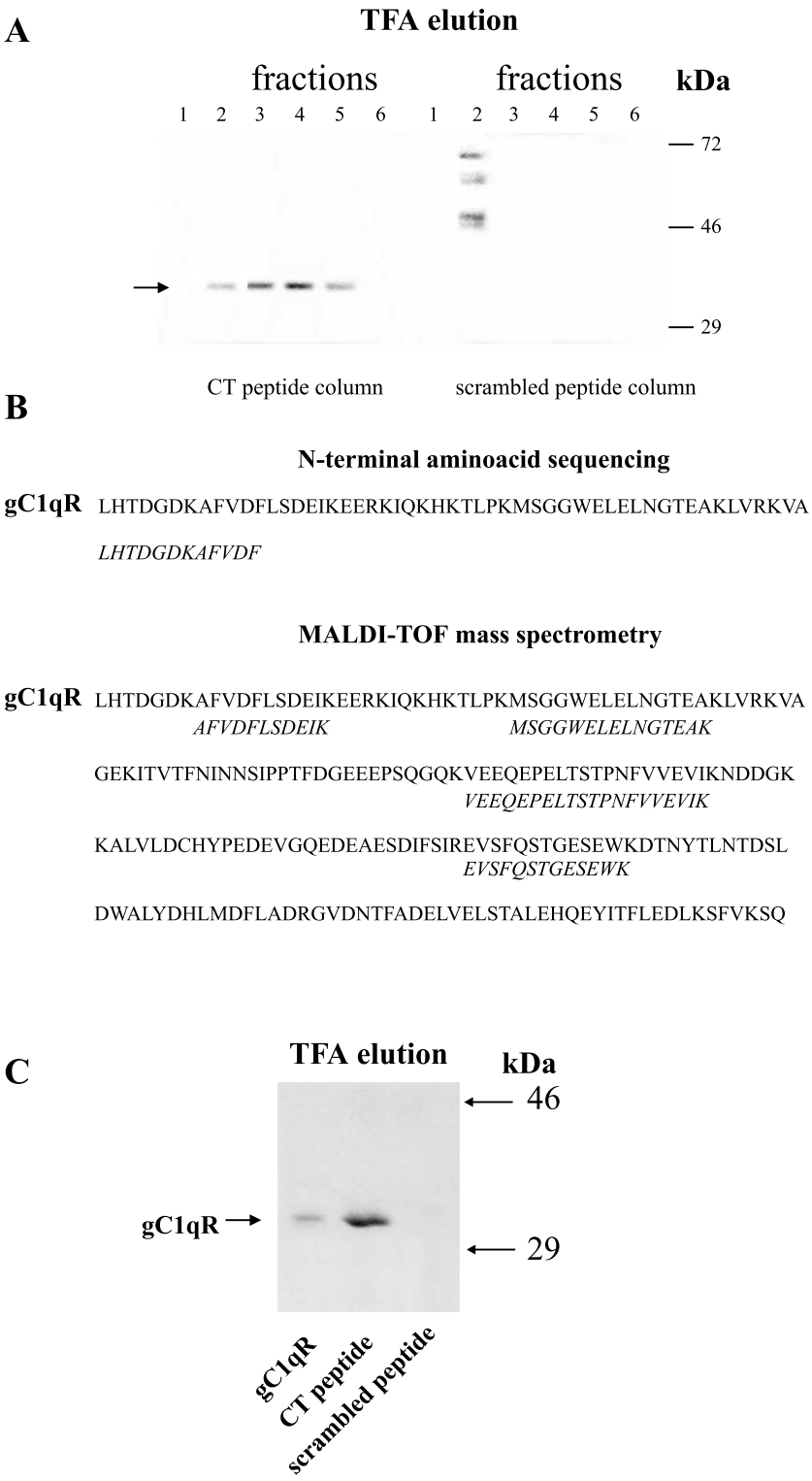

Fig. 1. gC1qR specifically binds the CT peptide bait. A: Purification of gC1qR from MCF7 cells. The equal aliquots of the cell lysate were loaded onto the CT and the scrambled peptide column. After washing, the bound proteins were eluted with 0.05% TFA. Fractions were collected and analyzed by reducing SDS–PAGE. Arrow indicates the 33 kDa band subjected to sequence determination. The positions of the molecular weight markers are on the right in kDa. B: The sequence analysis of the 33 kDa protein. The upper panel: The upper and lower lanes represent the known N-terminal sequence of gC1qR (GenBank Accession Number CAA53512) and the N-terminal sequence of the 33 kDa band (italicized), respectively. The bottom panel: MALDI-TOF mass spectrometry analysis of the 33 kDa band. The upper sequence represents gC1qR. The peptide sequence of the 33 kDa tryptic peptides (italicized) is shown below. C: Chromatography of gC1qR. Equal aliquots of recombinant gC1qR (2 μg each) in 1 ml PBS, pH 7.4, containing 0.005% Brij 35, 1 mM $CaCl_2$, 1 mM $MgCl_2$ were loaded onto each the CT and the scrambled peptide columns. Columns were consecutively washed with 20 volumes of PBS, pH 7.4, containing 0.005% Brij 35, 1 mM $CaCl_2$, 1 mM $MgCl_2$, with 10 volumes of the same buffer with 0.5 M NaCl and with 10 volumes of 10 mM Tris–HCl buffer, pH 8.5. The protein was eluted with 0.05% TFA. Fractions were collected, concentrated on a Speed-Vac and analyzed by SDS–PAGE. Left, middle and right lanes show purified gC1qR, and the samples eluted from the CT and the scrambled peptide columns, respectively. The positions of the molecular weight markers are on the right in kDa.

3.2. The CT is essential for co-precipitation of gC1qR with MT1-MMP

To analyze whether MT1-MMP interacts with cellular gC1qR, cells stably expressing the WT, E240A or ΔCT constructs were surface labeled with membrane-impermeable biotin and lysed. The cell lysates were immunoprecipitated with either MT1-MMP- or gC1qR-specific antibodies. Immunoprecipitation detected no MT1-MMP in mock cells (Fig. 2A). In turn, MT1-MMP was readily identified in cells transfected with the wild-type and mutant constructs. Consistent with the earlier observations [12,13,22], the WT protease was represented by the prominent 39 and 42 kDa stable, catalytically inactive ectodomain forms. The 72 kDa band was non-specific since it was present also in mock cells expressing no MT1-MMP (Fig. 2A, WT and mock). Cells expressing the E240A mutant exhibited the 60 kDa MT1-MMP since self-proteolysis of the enzyme was significantly suppressed. Truncated MT1-MMP was observed in cells transfected with the ΔCT construct.

Intriguingly, in cells expressing the proteolytically inert E240A construct, anti-gC1qR antibody recovered a biotin-labeled protein highly similar to MT1-MMP. The relatively low efficiency of co-precipitation may be explained by the dimerization of MT1-MMP and by the preferential association of E240A monomers with gC1qR. The same anti-gC1qR antibody relatively inefficiently recovered the degraded, catalytically inactive ectodomain forms of MT1-MMP in WT cells and failed to precipitate the ΔCT mutant in ΔCT cells (Fig. 2A). These findings suggest that intracellular gC1qR was capable of efficiently associating the CT of the inactive E240A construct. In agreement, the ΔCT mutant lacking the CT sequence was unable to bind intracellular gC1qR.

Anti-gC1qR antibody mAb 60.11 did not precipitate biotin-labeled gC1qR from any tested cell lines (Fig. 2A). Concomitantly, flow cytometry analysis has not revealed any surface expression of gC1qR in MCF7 cells (data not shown). However, this antibody efficiently immunoprecipitated unlabeled gC1qR from cell lysates (Fig. 2B). To verify the presence of gC1qR in the intracellular compartment, the plasma membrane and cytosol fractions of E240A cells were separated by the ultracentrifugation at 100 000 × *g* for 1 h. Immunoblotting employing anti-gC1qR mAb 60.11 demonstrated the presence of soluble and membrane-integrated gC1qR (Fig. 2B). According to these results, the total amount of soluble gC1qR exceeded that of the membrane-associated gC1qR about 40–50-fold. Taken together, these findings suggested that there was no gC1qR associated with the surface of MCF7 cells and that the gC1qR protein observed in our studies originated from the intracellular compartment.

3.3. Subcellular localization and internalization of MT1-MMP

Given the recent reports that the CT regulates activity of MT1-MMP by controlling it trafficking to and from the cell surface [23–25] and our findings that the CT sequence directly interacts with gC1qR, we investigated the subcellular localization of MT1-MMP and gC1qR by high-resolution confocal microscopy. Here, we employed affinity-purified antibody against the catMT which recognizes the full-length enzyme but not the inactive 39 and 42 kDa ectodomain forms lacking the catalytic domain sequence [22].

Immunostaining experiments demonstrated a predominantly membrane localization of the proteinase in permeabilized WT, E240A and ΔCT cells (Fig. 3A–C). Furthermore, there was an intense staining of the intracellular pool of MT1-MMP in WT and E240A cells (*XZ* plans at the bottom part of Fig. 3A and C). MT1-MMP immunolabeling in E240A cells resembled the pattern observed in WT cells.

Remarkably, no intracellular pool of MT1-MMP was observed in cells expressing the ΔCT construct (Fig. 3B; see *XZ* plan at the bottom). Further, no MT1-MMP staining was found at the basal side of the membrane at the sites involved

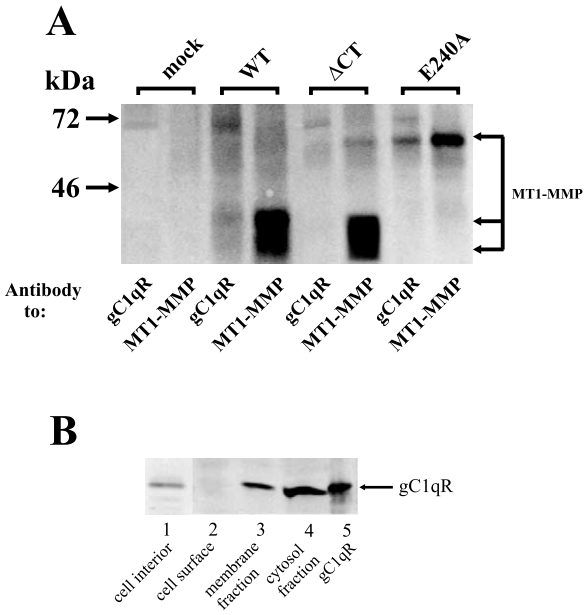

Fig. 2. gC1qR recovers biotin-labeled MT1-MMP. A: gC1qR co-precipitates with the catalytically inert full-length E240A mutant. Mock, WT, E240A and ΔCT cells were surface labeled with membrane-impermeable biotin and lysed. Cell lysates were each immunoprecipitated with MT1-MMP- or gC1qR-specific antibodies. The precipitated material was separated by SDS–PAGE and transferred to a membrane support. Biotin-labeled proteins were detected by incubating the membrane with ExtrAvidin-HRP followed by incubation with TMB/M. Note that gC1qR recovers the WT and especially the E240A but not the CT construct. No specific bands were detected with control rabbit and murine IgG (data not shown). The positions of the molecular weight markers are on the left in kDa. B: gC1qR is present in the membrane and cytosol fractions but not on the surface of E240A cells. Cells were left untreated (lanes 1, 3, 4) or surface labeled with biotin (lane 2). The gC1qR protein was immunoprecipitated from the lysates of unlabeled cells with anti-gC1qR mAb 60.11 and detected after SDS–PAGE and transfer to a membrane with biotin-labeled mAb 60.11, followed by incubation with ExtrAvidin-HRP (lane 1). The lysate of biotin-labeled cells (1 mg total protein) was immunoprecipitated with anti-gC1qR mAb 60.11, the precipitate was electrophoretically separated, and the proteins were identified by using ExtrAvidin-HRP (lane 2). Unlabeled 2.5×10^8 cells (lanes 3 and 4) were re-suspended in 20 ml TBS and the cell suspension was transferred to a Parr cell disruption bomb (PARP Instrument Co., Moline, IL, USA). The bomb was pressurized with nitrogen to 500 psi for 10 min. Cells were disrupted as the suspension was released to atmospheric pressure through the discharge valve. The lysate was centrifuged for 15 min at 10 000 × *g* to pellet cell debris. Further, the supernatant was spun at 100 000 × *g* for 1 h in a Beckman XL-80 ultracentrifuge to separate the membrane fraction from the supernatant (20 ml) largely containing the soluble cytosolic proteins. The membrane fraction was re-suspended in 1 ml TBS. The equal aliquots of the membrane fraction (lane 3, membranes) and the supernatant (lane 4, cytosol) were analyzed by Western blotting employing anti-gC1qR mAb 60.11 followed by donkey anti-mouse IgG-HRP. Lane 5, the purified recombinant gC1qR (100 ng) stained with mAb 60.11 as described above.

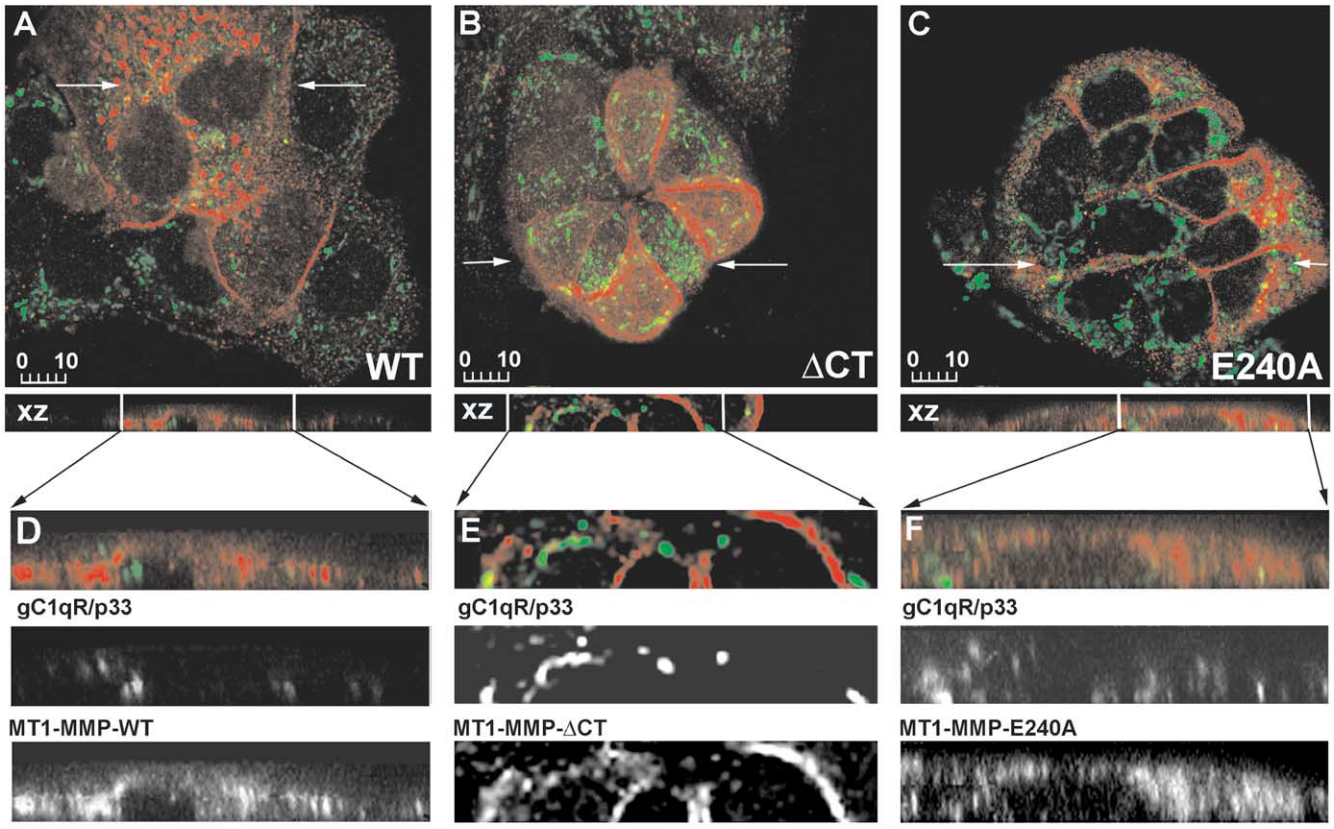

Fig. 3. Immunofluorescence of cells. WT, E240A and ΔCT cells were each stained with anti-gC1qR mAb 60.11 and anti-catMT antibody (green and red, respectively). A, B and C: Images of *XY* projections of cellular clusters. Arrows locate the *Y* coordinate of the vertical *XZ* single section made through cells. D, E and F: Enlarged images of *XZ* sections presented at the bottom of A, B and C. Note the presence of the intracellular MT1-MMP pool in WT and E240A cells. Staining with control rabbit and murine IgG was clearly negative (data not shown). The bar, 10 μm.

in attachment of ΔCT cells to the matrix. The morphology of ΔCT cells was clearly distinct from that of other cell types. Consistent with our earlier observations [13], ΔCT cells were rounded suggesting that these mutant cells were incapable of properly maintaining the cytoskeletal scaffold (Fig. 3B). Similar observations were reported recently by other groups [12,23,24,26].

The immunocytochemical examination of cells has shown a conservative distribution of naturally expressed gC1qR at the regions corresponding to mitochondria, the lumens of endoplasmic reticulum and Golgi in mock, WT, E240A and ΔCT cells. Intracellular localization of gC1qR was demonstrated by additional co-staining experiments with mitochondrial, Golgi and endoplasmic reticulum markers (data not shown). Both WT and E240A cells demonstrated the similar staining pattern of gC1qR, especially near the cell membrane where gC1qR partially co-localized with MT1-MMP (*XZ* plans at the bottom part of Fig. 3A and C). The gC1qR staining of ΔCT cells was distinct from that in other cell types. In ΔCT cells gC1qR largely resided in the vicinity of the plasma membrane beneath the layer of MT1-MMP rather than in the cytoplasm (*XZ* plan at the bottom part of Fig. 3B).

To enhance the visual effect of MT1-MMP co-localization with gC1qR, color inserts of vertical sections (*XZ* planes) on Fig. 3A, B and C were enlarged, split in single monochromatic channels and shown in Fig. 3D, E and F. In WT and E240A cells the strong proteinase staining obscured that of gC1qR, thereby complicating the visual assessment of the images. To quantitatively evaluate the levels of co-localization of MT1-MMP with gC1qR, the Pearson's correlation coefficient was calculated on stacks of optical sections scanned within single cells. The values were 0.43, 0.31 and 0.26 for E240A, WT and ΔCT cells, respectively, indicating the least antigen overlap between the ΔCT mutant and gC1qR.

Since our findings suggest that the CT is essential both to the associations with gC1qR and to the presentation of MT1-MMP at the cell surface, apparently, through regulating the mechanisms involved in internalization of the protease, we further assessed internalization of MT1-MMP in WT, E240A and ΔCT cells. For these purposes, cells were pre-incubated for 48 h with a potent hydroxamate inhibitor GM6001 to suppress autolytic processing and shedding of the protease [22,25] and then surface biotinylated. Further, labeled cells were each incubated alone or with the inhibitors of the lysosomal degradation (chloroquine) and of the protein synthesis (cycloheximide). Under our experimental conditions, MT1-MMP was predominantly internalized in WT and E240A cells but not in ΔCT cells (Fig. 4A). The blocking of protein synthesis prevented efficient internalization of WT and E240A constructs suggesting the de novo protein synthesis is essential for internalization of MT1-MMP. The inhibition of lysosomal degradation by chloroquine also significantly elevated the residual levels of membrane-tethered biotin-labeled MT1-MMP in WT and E240A cells. The effect of chloroquine indicated that the lysosomal degradation pathway is at least partially involved in the regulation of MT1-MMP presenta-

tion on cell surfaces. In contrast, internalization of ΔCT was less prominent relative to that of two other constructs. Both cycloheximide and chloroquine failed to strongly affect the presentation of the ΔCT construct on the cell surface suggesting less efficient internalization of ΔCT.

However, the absence of the CT weakens associations of MT1-MMP with the plasma membrane, promotes shedding and accumulation of the soluble forms of the protease in the cultured medium. Thus, only ΔCT cells exhibited the soluble MT1-MMP species in medium while WT and E240 cells failed to extensive shed the protease (Fig. 4B). Similarly to the recent findings of other authors [25], inhibition of MT1-MMP activity by a hydroxamate stabilized the protease, and repressed its autoproteolytic shedding (data not shown). Apparently, by affecting both internalization and shedding, the CT sequence plays a pleiotropic role in cell surface presentation of MT1-MMP.

4. Discussion

MT1-MMP plays a major role in matrix degradation and tumor progression [3,4,10,27]. Following stimulation with concanavalin A or phorbol esters, MT1-MMP is thought to be recruited to the plasma membrane by yet unidentified mechanisms [26,28–30]. In view of these data it was interesting to speculate whether the CT of MT1-MMP is capable of specifically associating with the individual components(s) of the intracellular milieu. This prompted us to use the CT sequence as bait in our attempts to identify the putative intracellular partner(s) of MT1-MMP. We have succeeded in identifying a novel cellular protein, gC1qR, that interacts with MT1-MMP in vitro and in cultured cells.

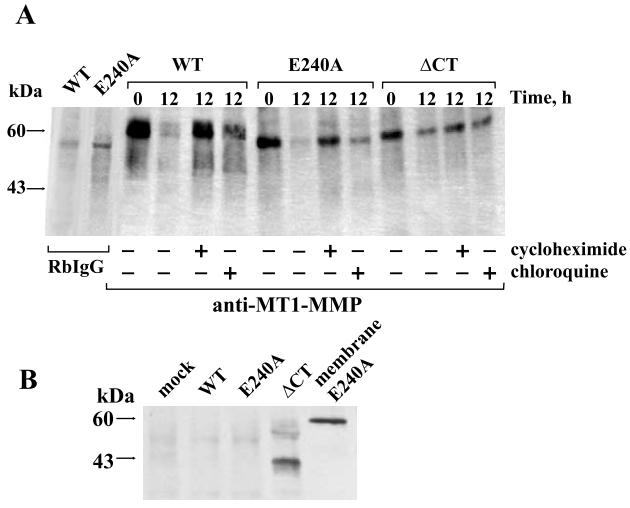

Fig. 4. The CT affects internalization and shedding of MT1-MMP. A: The CT-deleted MT1-MMP is less efficiently internalized. Following incubation of WT, E240A and ΔCT cells (2×10^6 each) for 48 h in DMEM/10% FCS with hydroxamate inhibitor GM6001 (50 μM), cells were surface labeled with membrane-impermeable biotin. Labeled cells were further incubated alone or with chloroquine (50 μM) in DMEM/10% FCS for 12 h. Separately, labeled cells were pre-incubated for 2 h in serum-free DMEM in the presence of cycloheximide (80 μg/ml) and further incubated for 10 h in DMEM/10% FCS supplemented with 20 μg/ml cycloheximide. Next, cell lysates were each immunoprecipitated with control rabbit IgG or MT1-MMP-specific antibody AB815. The precipitates were separated by SDS–PAGE and transferred to a membrane support. Biotin-labeled proteins were detected by incubating the membrane with ExtrAvidin-HRP and the TMB/M substrate. Note less efficient internalization and the failure of chloroquine to affect ΔCT. B: The CT-deleted MT1-MMP is extensively shed. WT, E240A, ΔCT and mock cells (5×10^6) were each incubated for 24 h in 5 ml DMEM. The aliquots (0.5 ml) of medium were immunoprecipitated with the antibody specific to catMT. The precipitates were separated by SDS–PAGE and transferred to a membrane support. Soluble MT1-MMP form was detected by incubating the membrane with biotin-labeled antibody to catMT followed by incubation with ExtrAvidin-HRP and the TMB/M substrate. The E240A construct (right lane) was used as a marker representing the full-length MT1-MMP. The positions of the molecular weight markers are on the right.

MT1-MMP 559–582	VFFF**RR**HGTP**RR**LLYCQ**R**-SLLDKV
REV 34–56	TRQA**RR**NRR-**RR**WRERQ**R**QIHSIS
HBcAg 148–172	VVRR**R**GRSPR**RR**TPSP**RR**RRSQSPR
GABA (type A) receptor β-subunit 414–439	RALD**RH**GVPS**K**GR**I**-R**RR**ASQLKVK
$Alpha_{1B}$-adrenergic receptor 364–386	GCQC**R**GRRRR**RRR**--R**RR**LGGCAYTT

Fig. 5. Alignment of the 559–582 sequence of MT1-MMP with the Rev, HBcAg, GABA type A receptor β-subunit and α_{1B}-adrenergic receptor sequence. The peptide sequence of the CT of MT1-MMP is underlined. Positively charged similar residue positions are in bold. HBcAg, hepatitis B core antigen; GABA, γ-aminobutyric acid.

The gC1qR is known to exist in two molecular forms, the 1–282 precursor and the 74–282 mature proteins. The mature gC1qR is generated by post-transcriptional proteolytic processing of the precursor. The gC1qR protein may be found in mitochondria as well as at the cell membrane, in the cytosol, in the nucleus and in cell microenvironment [14,31]. gC1qR associates with and functions as a compartment-specific regulator of numerous cellular and viral proteins [14,32–37]. The alignment of the protein sequences which have been demonstrated to be involved in the binding with gC1qR such as the Rev of HIV1, HBcAg, the cytoplasmic portion of adrenergic receptor and the neurotransmitter-gated ion channel segment of GABA (type A) receptor β-subunit with the CT sequence of MT1-MMP demonstrates their similarity, especially in relative positions of the Arg residues (Fig. 5). The sequence alignment suggests that the Arg cluster (nos. 563, 564, 569, 570, and 576) of the MT1-MMP's CT is specifically involved in the binding to gC1qR. Since it has been documented that gC1qR is a regulator of protein kinase C location and function in rat hepatocytes [33], it is possible that gC1qR plays a similar function in regulating MT1-MMP.

Our chromatography and co-precipitation studies suggest that MT1-MMP via its CT peptide sequence is capable of binding to mature gC1qR, thereby suggesting a role of the CT in MT1-MMP·gC1qR interactions. These findings as well as our data regarding cell surface expression, internalization and shedding of MT1-MMP extend the recent studies by Jiang et al. [23] who demonstrated the function of the CT of MT1-MMP in cell surface presentation of the protease.

Although a direct functional link between gC1qR and MT1-MMP is still uncertain, our data allow us to speculate that this membrane-tethered protease and cellular gC1qR may work in concert in vivo. There are a few other potentially important structural features such as di-leucines which are likely clathrin-dependent internalization motifs [26] and the C-terminal Val [30], those might affect trafficking and presentation of MT1-MMP in cells. The significance of these motifs is currently under investigation in our laboratory.

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