Membrane type 1 matrix metalloproteinase (MT1-MMP) is a collagenolytic enzyme that has been implicated in normal development and in pathological processes such as cancer metastasis. The activity of MT1-MMP is regulated extensively at the post-translational level, and the current data support the hypothesis that MT1-MMP activity is modulated by glycosylation. Enzymatic deglycosylation, site-directed mutagenesis, and lectin precipitation assays were used to demonstrate that MT1-MMP contains O-linked complex carbohydrates on the Thr291, Thr299, Thr300, and/or Ser301 residues in the proline-rich linker region. MT1-MMP glycoforms were detected in human cancer cell lines, suggesting that MT1-MMP activity may be regulated by differential glycosylation in vivo. Although the autolytic processing and interstitial collagenase activity of MT1-MMP were not impaired in glycosylation-deficient mutants, cell surface MT1-MMP-catalyzed activation of pro-matrix metalloproteinase-2 (proMMP-2) required proper glycosylation of MT1-MMP. The inability of carbohydrate-free MT1-MMP to activate proMMP-2 was not a result of defective MT1-MMP zymogen activation, aberrant protein stability, or inability of the mature enzyme to oligomerize. Rather, our data support a mechanism whereby glycosylation affects the recruitment of tissue inhibitor of metalloproteinases-2 (TIMP-2) to the cell surface, resulting in defective formation of the MT1-MMP/TIMP-2/proMMP-2 trimeric activation complex. These data provide evidence for an additional mechanism for post-translational control of MT1-MMP activity and suggest that glycosylation of MT1-MMP may regulate its substrate targeting.
secretory pathway by furin or furin-like proprotein convertases (PCs) (41–43). These proprotein convertases cleave proMT1-MMP after the R₁₀₀KR₁₁₃ sequence to generate the active species (55 kDa). This is supported by studies using an engineered mutant of α1-proteinase inhibitor (α1-PI) with furin/PC inhibitory activity, designated α1-PI₉₆₄ (44), to block proMT1-MMP zymogen activation (43). Active MT1-MMP is inhibited by TIMP-2, but is unaffected by TIMP-1 (34). MT1-MMP also undergoes autolytic degradation at Gly²⁸⁴ and Gly²⁸⁵ in the linker region, generating a peptidomimetic inactive species (calculated molecular mass 34 kDa) (39). Finally, as a trans-membrane protein, MT1-MMP is also regulated by membrane trafficking and cell surface localization (37, 45–47).

The current study tested the hypothesis that glycosylation of MT1-MMP functions as an additional mechanism for post-translational regulation of enzymatic activity and demonstrates that MT1-MMP is an O-glycoprotein. Although glycosylation was not required for collagenase activity, formation of a stable MT1-MMP/TIMP-2/proMMP-2 ternary complex and subsequent cell surface activation of MMP-2 was blocked in glycosylation-defective mutants. Distinct glycoforms of MT1-MMP were detected in human cancer cell lines, suggesting that MT1-MMP activity may be regulated by differential glycosylation in vivo. These data support a model in which glycosylation regulates substrate targeting and suggest a cellular mechanism for controlling the initiation of MMP-2 dependent proteolysis.

EXPERIMENTAL PROCEDURES

Materials—Anti-FLAG monoclonal M1 and M2 antibody, anti-Myc monoclonal antibody (9E10), rabbit polyclonal antibody that recognizes -glycoprotein. Although glycosylation was not required for collagenase activity, formation of a stable MT1-MMP/TIMP-2/proMMP-2 ternary complex and subsequent cell surface activation of MMP-2 was blocked in glycosylation-defective mutants. Distinct glycoforms of MT1-MMP were detected in human cancer cell lines, suggesting that MT1-MMP activity may be regulated by differential glycosylation in vivo. These data support a model in which glycosylation regulates substrate targeting and suggest a cellular mechanism for controlling the initiation of MMP-2 dependent proteolysis.

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Results.

Lectin Precipitation Assay—Biotinylated lectins, including concanavalin A, soybean agglutinin, Ulex europaeus agglutinin I, wheat germ agglutinin, sialic acid-containing lectins (Maackia amurensis lectin II, and Sambucus nigra lectin) were purchased from Vector Laboratories (Burlingame, CA). These biotinylated lectins were immobilized on streptavidin-coated magnetic beads and used to capture MT1-MMP from cell lysates. The precipitated MT1-MMP was eluted by boiling in SDS sample dilution buffer and analyzed by Western blot.

Colagen Invasion Assay—Collagen invasion assays were performed as described above (24, 52). The purified protein was heat-denatured in SDS buffer provided, neutralized with Triton X-100, and digested with buffer control or different glycosidases for 24 h. Several glycosidases, including sialidase A, endo-O-glycosidase, β(1–4)-galactosidase, glucosaminidase, and peptide-N-glycosidase F were used either individually or in combinations as described under “Results.” The digestion was stopped by boiling in SDS sample dilution buffer, and the samples were analyzed by Western blot.

Enzymatic Deglycosylation—Enzymatic deglycosylation was performed according to instructions from the manufacturer (Prozyme, San Leandro, CA). Briefly, FLAG-tagged MT1-MMP or glycosylation mutants were purified using M2 affinity gel as described above. The purified protein was heat-denatured in SDS buffer provided, neutralized with Triton X-100, and digested with buffer control or different glycosidases for 24 h. Several glycosidases, including sialidase A, endo-O-glycosidase, β(1–4)- galactosidase, glucosaminidase, and peptide-N-glycosidase F were used either individually or in combinations as described under “Results.” The digestion was stopped by boiling in SDS sample dilution buffer, and the samples were analyzed by Western blot.

Colagen Invasion Assay—Collagen invasion assays were performed as described above (24, 52). Briefly, cell culture inserts (24 wells, 8.0-μm pore, Becton Dickinson, Bedford, MA) were
coated with rat tail type I collagen (10 μg/well) in 100 mM Na2CO3 and allowed to air-dry overnight. Collagen-coated inserts were then washed with Dulbecco’s modified Eagle’s medium three times to remove salts and used immediately. Transfected COS-7 cells were trypsinized, washed with cultured medium, and 1 x 10^5 cells were added to the inner invasion chamber in 250 μl of culture medium. The outer wells contained 400 μl of culture medium. To evaluate the MMP dependence of invasion, 25 μM GM6001 was added to the inner and outer chambers as indicated. Cells were allowed to invade for 24 h, non-invading cells were removed from inner wells using a cotton swab, and invading cells adherent to the bottom of membrane were fixed and stained using a Diff-Quick staining kit (Dade AG, Miami, FL). Invading cells were enumerated as described previously (24, 40).

**Transverse Urea Gradient (TUG) PAGE**—TUG gel electrophoresis

**FIG. 1.** MT1-MMP is O-glycosylated with complex carbohydrates. A, the amino acid sequences from the linker domains of MT-MMPs are aligned in a clustal format, with the conserved residues denoted with asterisks. The predicted O- and N-glycosylation sites within these sequences are highlighted with ▼ and ▽, respectively. B, schematic diagram of MT1-MMP (Ser^24_–Val^582) consisting of propeptide (Pro), catalytic, linker, hemopexin-like, and transmembrane/cytoplasmic (TM/CT) domains. The predicted O-glycosylation sites, Thrs^291, Thrs^299, Thr^301, and Ser^301, were indicated with ▽ on the linker domain. Different FLAG- and Myc-tagged MT1-MMP plasmid constructs were generated in the following studies. The tagging sites are, as indicated in each experiment, either after furin/PC cleavage site (RRKR)^291_Y^292A, f^292) or in the stalk region between hemopexin-like and TM/CT domains. The site of autolysis is also indicated (YG^284_G^285ES). C, FLAG-tagged (stalk) MT1-MMP purified from transiently transfected COS-7 cells digested with sialidase A, endo-0-glycosidase, β1–4-galactosidase, glucosaminidase, and peptide-N-glycosidase F (PNGase F) as indicated. The reaction mixtures were fractionated on a 10% SDS-PAGE followed by Western blot (WB) with anti-FLAG M2 antibody. D, FLAG-tagged (stalk) glycosylation mutants CHO-1 (T291A), CHO-3 (T299A/T300A/S301A), and CHO-4 (T291A/T299A/T300A/S301A), as well as wild type MT1-MMP were purified from COS-7 cells, digested with sialidase A, and analyzed by Western blot with anti-FLAG M2 antibody. E, lysates from COS-7 cells expressing FLAG-tagged (stalk) wild type or CHO-4 mutant MT1-MMP were incubated with BSA, biotinylated lectins, including concanavalin A (ConA), soybean agglutinin (SBA), U. europaeus agglutinin I (UEA I), wheat germ agglutinin (WGA), succinylated wheat germ agglutinin (sWGA), M. amurensis lectin II (MAL II), or S. nigra lectin (SNA), followed by neutravidin precipitation as described under “Experimental Procedures.” The carbohydrate group(s) that interacts with each lectin was indicated. Glc, glucose; Man, mannose; Gal, galactose; GalNAc, N-acetylgalactosamine; Fuc, fucose; GlcNAc, N-acetylglucosamine; and Sia, sialic acid. The precipitates along with 10% of corresponding lysate input were analyzed by Western blot with anti-FLAG M2 antibody. The pro- and active forms of MT1-MMP are indicated as ◼ and ◼, respectively.
was performed as previously described (53). Briefly, 7% polyacrylamide gels containing a continuous 0–8 m urea gradient were cast in batch using a multiple gradient caster (Owl Scientific, Woburn, MA). The gels were rotated 90°, and a single sample of purified protein, FLAG-tagged (f112) sMT1-MMP or sCHO-4 in a 200–μl total volume was loaded evenly across the top of the gel. Electrophoresis was performed in the presence of SDS. The fractionated proteins were transferred to polyvinylidene difluoride membranes and analyzed by Western blot analysis using anti-FLAG M1 antibody (1:1000 dilution) and peroxidase-conjugated secondary antibody (1:10,000 dilution) followed by ECL detection (Pierce).

Labeling and Purification of Cell Surface Proteins—Labeling of cell surface proteins was performed as described previously (40). Briefly, cells were washed with phosphate-buffered saline and incubated with Sulfo-NHS-LC-LC-Biotin (Pierce) for 10 min on ice and washed with 100 mM glycine to quench remaining NHS groups. The cells were then lysed in lysis buffer, incubated with neutravidin-conjugated beads (Pierce) for 1 h, and eluted by boiling in SDS sample dilution buffer for 15 min followed by Western blot analysis.

Immunostaining of Endocytosed MT1-MMP—COS-7 cells were transfected with FLAG-tagged (stalk) MT1-MMP, CHO-4, and E240A mutant in the presence of 25 μM GM6001. Cell surface FLAG-tagged proteins were labeled with FLAG/M2 antibody (1:1000 dilution) on ice for 1 h. The unbound antibody was washed off with medium, and 25 μM GM6001 alone or 25 μM GM6001 and 25 μM TIMP-2 were added back to the chamber slides. Cells were shifted to 37°C to allow endocytosis to take place. After 15 min, the cells were plated on ice, washed with wash buffer (500 mM acetic acid and 150 mM NaCl) (47) and phosphate-buffered saline, fixed with 3.7% formaldehyde, and permeabilized with 0.2% Triton X-100. The cells were further stained with rabbit polyclonal antibody (Sigma) that recognizes the linker domain of MT1-MMP (1:2000 dilution). The bound FLAG/M2 antibody and linker antibody were detected with corresponding secondary antibody (1:5000 dilution) conjugated with Alexa Fluor 488 and 546 (Molecular Probes, Eugene, OR), respectively. The nucleus was counterstained with TO-PRO-3 iodide (Molecular Probes). The images were taken using a Zeiss LSM510 laser scanning confocal microscope at the Northwestern University Cell Imaging Facility, and edited using Adobe PhotoShop 7.0 software.

RESULTS

MT1-MMP Is Post-translationally Modified by O-Glycosylation—To determine whether MT1-MMP is post-translationally modified by glycosylation, the glycosylation potentials of MT1-MMP were examined on the Center for Biological Sequence Analysis server (NetNGlyc and NetOGlyc programs for N-linked and O-linked glycosylation, respectively). The results of analysis indicated that MT1-MMP is not likely to be N-glycosylated; however, four potential O-glycosylation sites (Thr291, Thr299, Thr300, and Ser301) were identified (Fig. 1, A and B). These sites were all located in the proline-rich linker (also known as hinge) region, proposed to be critical for the proteinase activity of MT1-MMP (54). Similar to MT1-MMP, MT2- to MT6-MMP were all predicted to be glycosylated in the linker region (Fig. 1A), suggesting a conserved post-translational modification indicative of a potential regulatory function. Enzymatic deglycosylation was subsequently used to test the prediction that MT1-MMP is a glycoprotein. Affinity-purified MT1-MMP from COS-7 cells was digested with specific glycosidases and evaluated by SDS-PAGE. Because glycosylation can cause a change in the mass and charge of a protein, both positive and negative changes in relative electrophoretic mobility were interpreted in support of carbohydrate removal. Without digestion, the purified protein migrated as a 64-kDa proMT1-MMP species and a 50-kDa active MT1-MMP species (Fig. 1C, lane 1). Treatment of MT1-MMP with peptide-N-glycosidase F, which efficiently removes N-linked sugars, did not result in any detectable mobility shift in either the pro-or active MT1-MMP (Fig. 1C, lane 7), suggesting the prediction that MT1-MMP is not N-glycosylated. Similarly, treatment with several O-glycosidases (endo-O-glycosidase, β1–4)-galactosidase, and glucosaminidase) did not alter the electrophoretic

FIG. 2. Differential glycosylation of MT1-MMP in cancer cells. A, COS-7 cells were transfected with FLAG-tagged (stalk) wild type (left panel) or CHO-4 mutant (right panel) MT1-MMP in the absence or presence of 2 mM O-glycosylation inhibitor BGN. The cell lysates were fractionated on a 10% SDS-PAGE followed by Western blot with anti-FLAG M2 antibody. B, breast cancer MDA-MB-231 cells, and ovarian cancer OVCA-R3, DOV13, and OVCA433 cells were cultured in the absence or presence of O-glycosylation inhibitor BGN for 48 h. GM6001 was added 24 h before lysing the cells. The cell surface proteins from these cells were purified as described under “Experimental Procedures” and analyzed by Western blot (WB) with anti-MT1-MMP antibody. The pro- and active forms of MT1-MMP are indicated as △ and ⌧, respectively.

MT1-MMP activity was measured on the Center for Biological Sequence Analysis server (NetNGlyc and NetOGlyc programs for N-linked and O-linked glycosylation, respectively). The results of analysis indicated that MT1-MMP is not likely to be N-glycosylated; however, four potential O-glycosylation sites (Thr291, Thr299, Thr300, and Ser301) were identified (Fig. 1, A and B). These sites were all located in the proline-rich linker (also known as hinge) region, proposed to be critical for the proteinase activity of MT1-MMP (54). Similar to MT1-MMP, MT2- to MT6-MMP were all predicted to be glycosylated in the linker region (Fig. 1A), suggesting a conserved post-translational modification indicative of a potential regulatory function.
migration of MT1-MMPs (Fig. 1C, lanes 3–5), potentially because of the poor efficiency of these O-glycosidases against complex O-linked carbohydrates. However, treatment of MT1-MMP with sialidase A consistently resulted in an altered relative mobility of the active MT1-MMP species (Fig. 1C, lanes 2, 6, and 8). A similar decrease in relative mobility after desialylation has been shown in several other glycoproteins including MUC1, endolyn, and CD44 (55–57) and presumably reflects alterations in SDS binding properties of the modified proteins. Of note, there is no mobility shift detected in the proMT1-MMP species following treatment with sialidase A, suggesting that the sialylation likely follows activation of proMT1-MMP in the trans-Golgi network (58).

To confirm the prediction that MT1-MMP contains O-linked carbohydrate, three alanine mutants were generated, in which Thr291 (designated CHO-1), Thr299-Thr-Ser301 (CHO-3), or all four predicted O-glycosylation sites (Thr291 and Thr299-Thr-Ser301, designated CHO-4) were mutated to alanine(s) (schematic of linker region shown in Fig. 1D, top panel). The CHO-1 mutant, which preserves three predicted glycosylation sites, exhibited a relative electrophoretic mobility shift following desialylation similar to the wild type protein (Fig. 1D, lanes 2 and 4). Removal of all four potential O-glycosylation sites (CHO-4, Fig. 1D, lane 7) resulted in a mutant with electrophoretic mobility similar to desialylated wild type MT1-MMP (Fig. 1D, lane 2). Further, this mutant was insensitive to sialidase A treatment (Fig. 1D, lane 8), suggesting that sialic acids are added to MT1-MMP via O-linked carbohydrates and all potential O-glycosylation sites were identified. To further character-
Glycosylation Regulates MT1-MMP Activity

Fig. 5. Glycosylation of MT1-MMP does not affect collagen invasion. COS-7 cells were transfected with vector, MT1-MMP, E240A, and different glycosylation mutants as indicated. After 12 h, these cells were trypsinized, seeded (1 × 10⁵/well) onto cell culture inserts (24 well, 8.0 µm pore) coated with a type I collagen (10 µg/well), and allowed to invade for 24 h as described under “Experimental Procedures.” Non-invading cells were removed from the upper chamber with a cotton swab. Filters were then stained, and cells, adherent to the underside of the filter, were enumerated using an ocular micrometer. The average values of triplicate experiments were normalized to cells transfected with vector alone (designated J) and were presented with S.D. error bar. GM6001 (25 µM) was added in the well indicated.

ize the glycosylation of MT1-MMP, cells were transfected with either wild type or CHO-4 mutant MT1-MMP and the lysates were precipitated with various immobilized lectins. Negligible CHO-4 MT1-MMP was precipitated by any lectin (Fig. 1E, lower panel), suggesting that MT1-MMP has very low carbohydrate-independent interaction with lectins. In contrast wild type MT1-MMP was precipitated with both concanavalin A, which binds to glucose and mannose (Fig. 1E, lane 2), and soybean agglutinin, which binds to galactose and N-acetylglucosamine (Fig. 1E, lane 3). Interestingly, a preferential interaction with proMT1-MMP was observed, suggesting that partial deglycosylation of the proenzyme may accompany zymogen activation. Wheat germ agglutinin, which binds to N-acetylglucosamine and sialic acid, also precipitated MT1-MMP, with preferential binding to the active MT1-MMP species (Fig. 1E, lane 5). Succinylated wheat germ agglutinin, which no longer binds to sialic acid but preserves its interaction with N-acetylglucosamine, differentially recognizes proMT1-MMP (Fig. 1E, lane 6), providing additional evidence that active MT1-MMP is sialylated. To determine the subtype of sialic acid on MT1-MMP, interaction with the (α-2,3) linkage-specific lectin M. amurensis lectin II and the (α-2,6) linkage-specific lectin S. nigra lectin was evaluated. Only M. amurensis lectin II was found to interact with MT1-MMP (Fig. 1E, lanes 7 and 8), indicating the sialic acid was added via (α-2,3) linkage. These data support the conclusion that MT1-MMP is a glycoprotein with O-linked complex carbohydrates.

In addition to mutational analysis and lectin precipitation, the O-glycosylation inhibitor benzyl-2-acetamido-2-deoxy-α-β-galactopyranoside (GalNAc-α-O-benzyl, or BGN) (59, 60) and the N-glycosylation inhibitor tunicamycin were used to evaluate glycosylation of MT1-MMP. Culture of cells overexpressing wild type MT1-MMP with BGN resulted in expression of an MT1-MMP species (Fig. 2A, lane 2) with relative electrophoretic migration similar to the carbohydrate-free CHO-4 mutant (Fig. 2A, lane 3), providing additional evidence that MT1-MMP is O-glycosylated. No change in mobility was observed upon treatment of CHO-4-expressing cells with BGN (Fig. 2A, lane 4). In control experiments, treatment with the N-glycosylation inhibitor tunicamycin did not alter the electrophoretic mobility of either wild type or CHO-4 MT1-MMP (data not shown). To determine whether endogenously expressed MT1-MMP is also glycosylated in cancer cells, breast and ovarian cancer cell lines were cultured in the presence and absence of BGN. Because of low expression levels, the endogenous MT1-MMP was enriched by purification of cell surface proteins. The active species of endogenous MT1-MMP was detected when probed with an antibody that recognizes the linker region in Western blot analysis (Fig. 2B). The treatment with BGN resulted in a similar mobility shift of the active MT1-MMP species in MDA-MB-231, DOV13, and OVCA433 cells, suggesting that endogenous MT1-MMP is also glycosylated. Interestingly, no mobility shift was detected upon BGN treatment of OVCA433 cells, with the active species of MT1-MMP detected at the higher apparent molecular weight corresponding to underglycosylated MT1-MMP. These data demonstrate that endogenous MT1-MMP is O-glycosylated and indicate the presence of differential MT1-MMP glycoforms in human cancer cells.

Glycosylation Does Not Affect Zymogen Activation and Folding of MT1-MMP—The distinct lectin binding properties of pro- and active MT1-MMP (Fig. 1E) suggest a temporal relationship between MT1-MMP activation and glycosylation. ProMT1-MMP is activated by a specific cleavage following the R108RKR111 sequence by furin or other PCs and zymogen activation can be inhibited by an engineered mutant of αl-PI designated αl-PIHUX (43). To evaluate the relationship between proMT1-MMP activation and glycosylation, wild type or glycosylation-defective MT1-MMP was co-expressed with wild type αl-PI or αl-PIHUX. In the presence of wild type αl-PI (inactive against furin/PC), all proMT1-MMP glycoforms were converted to the active species in the same relative ratio as the wild type protein, suggesting glycosylation is not required for MT1-MMP activation (Fig. 3A, lanes 1, 3, 5, and 7). Co-expression of αl-PIHUX inhibited proMT1-MMP activation in all glycoforms (Fig. 3A, lanes 2, 4, 6, and 8), indicating the conversion was furin/PC-dependent. The accumulated proMT1-MMP appeared to be glycosylated at the same sites in the linker region, because the pro-form of the CHO-4 mutant did not exhibit a similar mobility shift (Fig. 3A, lane 8). These data indicate that glycosylation of MT1-MMP is not required for efficient zymogen activation.

Because differential NH2-terminal proteolytic processing of active MT1-MMP has been reported (61, 62), control experiments were performed to examine whether the altered mobility of CHO-4 was the result of unusual NH2-terminal processing. This was achieved by taking advantage of the anti-FLAG M1 antibody, which recognizes the FLAG epitope only when it is at the NH2 terminus. Additional tagged wild type MT1-MMP and CHO-4 constructs were generated in which the FLAG sequence was inserted immediately after furin/PC cleavage site (designated f112), instead of the stalk region (Fig. 1B). The f112-tagged constructs were expressed in COS-7 cells and initially probed with M2 antibody, which detects the FLAG epitope irrespective of location in the protein primary structure. Both the pro- and active species of wild type and CHO-4 MT1-MMP were recognized (Fig. 3B, lower panel). When probed with M1 antibody, only the active species were detected, because of exposure of the NH2-terminal FLAG epitope following furin/PC processing (Fig. 3B, upper panel). The CHO-4 mutant as well as wild type MT1-MMP was recognized, indicating that the amino terminus of the CHO-4 mutant is identical to that of wild type MT1-MMP. These data confirm that the altered relative electrophoretic mobility observed in the CHO-4 mutant reflects lack of glycosylation rather than differential proteolysis. Because glycosylation has been shown to be important for protein folding in many proteins, the stability of the carbohydrate-free
Fig. 6. Glycosylation of MT1-MMP is required for MMP-2 activation. The transfected COS-7 cells in Fig. 5 were also plated on 6-well plates coated with thin layer of type I collagen at the same time. A, after attachment, cells were incubated with 25 μM GM6001 for 12 h. Cell surface proteins were labeled with Sulfo-NHS-LC-LC-Biotin, purified with neutravidin as described under “Experimental Procedures,” and analyzed by Western blot (WB) using anti-FLAG M2 antibody and anti-transferrin receptor (7R) antibody. The active MT1-MMP are indicated as ●, A, cells were also incubated with 1 μM of purified proMMP-2 in serum-free media for 24 h. The conditioned media were collected and analyzed by zymography as described under “Experimental Procedures.” The pro-, intermediate, and active form of MMP-2 are indicated as ▲, —, and ●, respectively.

CHO-4 mutant relative to wild-type MT1-MMP was evaluated. Soluble MT1-MMP was generated on the background of either wild type (sMT1-MMP) or CHO-4 mutant (sCHO-4). Comparison of sMT1-MMP and sCHO-4 by TUG-gel electrophoresis showed an identical unfolding transition, indicating similarity in stability and folding of the wild type and mutant soluble proteins (Fig. 3C).

Glycosylation Regulates Substrate Targeting of MT1-MMP—To evaluate the potential functional consequences of post-translational glycosylation of MT1-MMP, the proteolytic activity of the enzyme was evaluated against several key substrates. MT1-MMP activity can be down-regulated by autolytic cleavage at the start of the linker region (Gly284↓Gly285, Fig. 1B), generating a catalytically inactive species (39). Because the predicted glycosylation sites are close to the reported cleavage site, the effect of glycosylation on MT1-MMP autolysis was investigated. In initial control experiments, FLAG-tagged MT1-MMP was co-expressed with untagged wild type MT1-MMP, inactive MT1-MMP (E240A) or control vector. The cell lysates were probed with anti-FLAG antibody to monitor processing of only the FLAG-tagged proteins. The conversion of active wild-type MT1-MMP (50 kDa) to a 37-kDa species was blocked by GM6001 (Fig. 4A, lanes 1 and 2). Co-expression of an untagged MT1-MMP increased the cleavage of the FLAG-tagged protein (Fig. 4A, lane 3), whereas co-transfection of the catalytically inactive E240A mutant did not affect conversion (Fig. 4A, lane 4), supporting a model of autolysis in trans. To further examine the autolysis model, similar experiments were performed with FLAG-tagged E240A MT1-MMP mutant. Consistent with the loss of proteinase activity in the E240A mutant, no autolysis was detected in the presence or absence of GM6001 (Fig. 4A, lanes 5 and 6). Co-expression of the untagged wild type MT1-MMP, but not the untagged E240A mutant, however, substantially converted the active species to the deg-
incubated with purified proMMP-2, followed by analysis of zymogen activation by gelatin zymography. Cells transfected with wild type MT1-MMP processed proMMP-2 (72 kDa) to a 68-kDa intermediate and a 62-kDa active species (Fig. 6B, lane 5). The catalytic activity of MT1-MMP is required, because the activation of proMMP-2 was blocked by GM6001 (Fig. 6B, lane 3) and transfection of the MT1-MMP E240A mutant failed to activate proMMP-2 (Fig. 6B, lane 4). Cells transfected with glycosylation-deficient mutants demonstrated a distinct activation profile. Whereas the CHO-1 mutant activated proMMP-2...
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Fig. 8. Glycosylation of MT1-MMP is required for MT1-MMP/TIMP-2/MMP-2 trimeric complex formation. A, COS-7 cells were transfected with FLAG-tagged MT1-MMP or CHO-4 mutant in the presence of 25 μM GM6001. After 24 h, cells were incubated with 10 nM TIMP-2 and 10 nM proMMP-2 in the presence of 25 μM GM6001 for 1 h. Unbound TIMP-2 and proMMP-2, as well as GM6001, were then removed to allow activation of cell surface-bound MMP-2. Cell lysates were obtained at 0, 15, 30, and 60 min and analyzed by gelatin zymography as described under “Experimental Procedures.” The pro-, intermediate, and active form of MMP-2 are indicated as <, –, and >, respectively. B, COS-7 cells were transfected with vector, Δ Cat, E240A, and different glycosylation variants of MT1-MMP in the presence of 25 μM GM6001. After 24 h, cells were incubated with 10 nM TIMP-2 and 10 nM proMMP-2 for 1 h followed by co-immunoprecipitation with anti-FLAG M2 antibody. The immunoprecipitates were analyzed by Western blot (WB) using anti-FLAG M2 and anti-TIMP-2 antibodies, and analyzed by gelatin zymography. The pro-, active, and autolytic products of MT1-MMP are indicated as <, >, and >, respectively.

Oligomerization of MT1-MMP Is Not Regulated by Glycosylation—It was recently reported that hemopexin domain-dependent oligomerization of MT1-MMP is required for efficient proMMP-2 activation (35). To determine whether the defect in proMMP-2 activation described above results from the inability of the CHO-deficient mutants to oligomerize, differentially epitope-tagged MT1-MMP constructs were generated. Wild-type or CHO-4 MT1-MMP expressing the Myc epitope tag was generated and co-expressed with FLAG-tagged MT1-MMP or the individual CHO-1, CHO-3, or CHO-4 mutants. Following expression, cellular extracts were immunoprecipitated with immobilized FLAG antibody (M2), electrophoresed, and blots were probed with either the FLAG or Myc epitope tag antibodies. Potential dimer pairings between wild type and CHO-deficient MT1-MMP species are shown schematically in Fig. 7A (only one CHO chain is included for simplicity). Consistent with the observation of Itoh and co-workers (35), immunoprecipitation through the FLAG epitope tag also precipitated Myc-tagged MT1-MMP (Fig. 7B, lane 4), supporting the hypothesis that protein-protein interactions occur between adjacent MT1-MMP species. This interaction was not affected by the glycosylation status of MT1-MMP, as similar amounts of Myc-tagged MT1-MMP were co-immunoprecipitated with the FLAG-tagged CHO-1, -3, and -4 glycosylation-defective mutants (Fig. 7B, lanes 5–7). Similar results were obtained using Myc-tagged CHO-4 MT1-MMP (Fig. 7C), demonstrating that oligomerization is independent of glycosylation. Further, these data indicate that the inability of the CHO-3 and CHO-4 mutants to effectively catalyze proMMP-2 activation is not a result of inefficient oligomerization.

Glycosylation Affects the Presentation of a Stable MT1-MMP/TIMP-2/MMP-2 Trimeric Complex and Modulates TIMP-2/MT1-MMP Interaction—MT1-MMP mediates MMP-2 activation through the formation of a trimeric complex consisting MT1-MMP, TIMP-2 and proMMP-2. To examine the effect of glycosylation on the formation of the trimeric complex, FLAG-tagged MT1-MMP species were expressed in COS-7 cells in the presence of GM6001 to prevent autolytic degradation. After 24 h, the cells were incubated with TIMP-2 and proMMP-2 in the presence of GM6001 for 1 h. Cells were washed to remove unbound protein and inhibitors, and cellular activation of MMP-2 was monitored with time. As indicated in Fig. 8A, proMMP-2 initially associates with MT1-MMP transfected cells, and is processed to the intermediate and active species within 1 h (Fig. 8C, lanes 1–4). In contrast, interaction of proMMP-2 with CHO-4 mutant MT1-MMP transfected cells was substantially decreased (Fig. 8A, lanes 5–8), suggesting altered association and/or dissociation kinetics. This was con-
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Fig. 9. Glycosylation of MT1-MMP affects TIMP-2 inhibition of autolysis and TIMP-2-dependent endocytosis of MT1-MMP. COS-7 cells were transfected with FLAG-tagged (stalk) MT1-MMP or CHO-4 mutant in the presence of increasing concentrations of GM6001 (A, 0, 1, 10, and 100 nM), or in B, TIMP-1 (100 nM) or increasing concentrations of TIMP-2 (1, 10, and 100 nM). After 24 h, cell lysates were fractionated on 10% SDS-PAGE and analyzed by Western blot using anti-FLAG M2 antibody. The pro-, active, and autolytic products of MT1-MMP are indicated as <, , and <, respectively. C–H, COS-7 cells were transfected with FLAG-tagged (stalk) MT1-MMP (C and F), CHO-4 (D and G), and E240A (E and H) mutant in the presence of 25 nM GM6001. Cell surface FLAG-tagged proteins were labeled with anti-FLAG M2 antibody on ice for 1 h. After washing off unbound antibody, cells were put back to 37 °C to allow endocytosis for 15 min. Cells were then fixed with formaldehyde and permeabilized with saponin, followed by Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (in green). Total expression of MT1-MMP or its mutants were also stained with anti-linker antibody followed by Alexa Fluor 546-conjugated goat anti-rabbit secondary antibody (inset, in red). The nuclei were counterstained using TO-PRO iodide (642/661) (in blue). The images were taken using a Zeiss LSM510 laser scanning confocal microscope.

firmed by co-immunoprecipitation experiments in which wild type or mutant MT1-MMP species were immunoprecipitated with anti-FLAG antibody, and analyzed for the presence of TIMP-2 by Western blot and MMP-2 by gelatin zymography. Both TIMP-2 and MMP-2 were co-precipitated with wild type MT1-MMP (Fig. 8B, lanes 4 and 5). In control experiments, no complex was generated with the ΔCat mutant lacking the active site (Fig. 8B, lane 2) and negligible MMP-2 was associated with the inactive E240A mutant, presumably through direct interaction with the catalytic domain of MT1-MMP (63) (Fig. 8B, lane 3). Although similar levels of expression of CHO-deficient MT1-MMP mutants were obtained, no TIMP-2 was precipitated with the CHO-3 and CHO-4 mutants, and associated MMP-2 was substantially decreased to the level of E240A mutant (Fig. 8B, lanes 7 and 8). In control experiments, purified soluble MT1-MMP or the CHO-4 mutant (lacking the transmembrane and cytoplasmic domains) bound to TIMP-2 with similar efficiency (data not shown). These data suggest an inability to efficiently recruit TIMP-2 and proMMP-2 to cell surface localized carbohydrate-deficient MT1-MMP, indicating that glycosylation directly affects the presentation of a functional MT1-MMP/TIMP-2/proMMP-2 ternary complex on the cell surface and thereby inhibits cell surface proMMP-2 activation.

To further characterize the effect of glycosylation on the MT1-MMP/TIMP-2 interaction, the interaction of MT1-MMP with synthetic metalloproteinase inhibitor GM6001 and the endogenous inhibitor TIMP-2 was examined. GM6001 inhibited the autolysis of both wild type and CHO-4 MT1-MMP in a dose-dependent manner and with a similar inhibitory profile,
with partial inhibition at 1 and 10 μM, and complete blockade at 100 μM (Fig. 9A). In contrast, the inhibitory profiles of TIMP-2 against the two glycoforms were quite distinct. Although TIMP-2 stabilized the 50-kDa active species of wild type MT1-MMP in a dose-dependent manner (Fig. 9B, lanes 2–4), very little of the active species of CHO-4 mutant MT1-MMP was preserved even at 100 nM concentration of TIMP-2 (Fig. 9B, lanes 6–8). These data suggest that under in vitro conditions wherein TIMP-2 is the primary inhibitor of MT1-MMP, glycosylation of MT1-MMP may protect against autolysis and thus stabilize active MT1-MMP.

As a recent report demonstrated that TIMP-2 undergoes endocytosis with MT1-MMP (47, 64, 65), the effect of TIMP-2 on wild type and CHO-4 mutant MT1-MMP endocytosis was evaluated. Cells expressing FLAG epitope-tagged wild type, CHO-4, or E240A-MT1-MMP were incubated at 4 °C to prevent endocytosis and labeled with anti-FLAG antibody. Cells were then incubated in the presence or absence of TIMP-2 and shifted to 37 °C for 15 min to promote endocytosis. To prevent autolysis and shedding of MT1-MMP, GM6001 was kept throughout the experiment (38, 39). After endocytosis, antibody remaining on the cell surface was removed by low pH washing, and endocytosed antibody (indicative of endocytosed MT1-MMP) was detected with a secondary antibody probe. Control experiments using an antibody directed against the MT1-MMP linker domain demonstrated similar levels of expression of the various MT1-MMP species (Fig. 9, C–H, index box). Similar patterns of endocytosis were observed among wild type, carbohydrate-free, and catalytically inactive MT1-MMP (Fig. 9, C–E). Addition of TIMP-2 to wild-type MT1-MMP significantly increased endocytosis (Fig. 9F), whereas TIMP-2 had no effect on endocytosis of either inactive E240A MT1-MMP or carbohydrate-free CHO-4 MT1-MMP (Fig. 9, G and H). These data suggest that the inability of cell surface CHO-4 MT1-MMP to bind TIMP-2 may modulate the cell surface retention of the protein.

Discussion

Results of the current study demonstrate that MT1-MMP is post-translationally modified by O-glycosylation at Thr^{301}, Thr^{299}, and/or Ser^{303} residues in the proline-rich linker region. Although the detailed carbohydrate composition was not analyzed, lectin precipitation experiments suggest that the carbohydrate moiety contains complex sugar structures. Using glycosylation inhibitors, evidence for glycosylation of endogenously expressed MT1-MMP in human cancer cells was provided. Distinct glycoforms of MT1-MMP were detected in human cancer cell lines, suggesting that MT1-MMP activity may be regulated by differential glycosylation in vivo. Moreover, putative glycosylation sites are predicted to exist in the linker regions of all known MT-MMPs, suggesting a conserved post-translational modification indicative of a potential regulatory function. Although the role of glycosylation among MMP family members has not been extensively evaluated, the secreted proteinase MMP-9 is known to be both O- and N-glycosylated and terminally sialylated. Interestingly, desialylation of MMP-9 has shown to increase the hydrolysis of peptide substrate in the presence of TIMP-1 (66), suggesting TIMP-dependent inhibition of MMP activity could be modulated by glycosylation.

To evaluate the potential role of glycosylation in modifying MT1-MMP function, initial studies focused on analysis of collagenase activity, as recent studies have proposed that the linker domains of MMPs may play a crucial role in collagenolysis. For example, introduction of a single amino acid mutation in the linker region of MMP-1 (collagenase-1) dramatically decreased collagenase activity without affecting gelatinolysis, suggesting a direct role of this domain in collagen cleavage (67). Regulation of collagenase activity by the MT1-MMP linker region has also been demonstrated. MT1-MMP-mediated collagen cleavage was blocked by a recombinant protein containing the linker and hemopexin-like domains of MT1-MMP, whereas the hemopexin-like domain fragment in the absence of the linker was ineffective (54). Results of the current study show effective collagen gel invasion, regardless of the glycosylation status of the MT1-MMP linker region, suggesting that the relative efficiency of pericellular collagenolysis is not altered by carbohydrate.

In addition to interstitial collagen, proMMP-2 is a predominant MT1-MMP substrate, and results of the current study demonstrate that cell surface activation of proMMP-2 is blocked in glycosylation-defective mutants. The mechanism by which glycosylation of MT1-MMP affects MMP-2 activation was explored in detail. Experiments using α1-PI_{box} to block furin/PC activity indicated that the zymogen of MT1-MMP was activated with equal efficiency in the wild type enzyme or carbohydrate deficient mutants. To determine whether the inability to activate MMP-2 resulted from failure of the carbohydrate-free mutant to oligomerize, differentially epitope-tagged MT1-MMP species were employed as recently described (35). Our results confirm published reports that protein-protein interactions between neighboring MT1-MMP species accompany proMMP-2 activation; however, MT1-MMP oligomerization was independent of glycosylation status. The current data support the hypothesis that carbohydrate-free MT1-MMP does not form an effective ternary activation complex owing to an inability to recruit TIMP-2 to the cell surface proteinase. Although soluble recombinant MT1-MMP lacking the transmembrane and cytoplasmic domains can bind to TIMP-2 in solution, co-precipitation analyses demonstrate a lack of ternary complex formation with the cell-associated carbohydrate-free mutant. This result is supported by data showing an inability of TIMP-2 to block autolysis of cell surface carbohydrate-free MT1-MMP. Although the biochemical basis for the lack of TIMP-2 binding by the cell surface carbohydrate-free MT1-MMP species is unclear, an unidentified carbohydrate-binding protein may be necessary to induce or stabilize the active conformation of MT1-MMP prior to TIMP-2 binding. Alternatively, the carbohydrate moiety may participate in the trafficking of MT1-MMP (55, 68). Indeed, our data demonstrate that, although TIMP-2 promotes endocytosis of wild-type MT1-MMP, internalization of the carbohydrate-defective MT1-MMP mutant is unaltered by TIMP-2, suggesting that glycosylation may regulate TIMP-2-driven endocytosis of MT1-MMP. Together these data support a model wherein glycosylation regulates substrate targeting and suggest a cellular mechanism by which post-translational processing may control MT1-MMP surface presentation and proMMP-2 activation.

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