Calcium Regulation of Matrix Metalloproteinase-mediated Migration in Oral Squamous Cell Carcinoma Cells*

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Activation of matrix metalloproteinase 2 (MMP-2) has been shown to play a significant role in the behavior of cancer cells, affecting both migration and invasion. The activation process requires multimolecular complex formation involving pro-MMP-2, membrane type 1-MMP (MT1-MMP), and tissue inhibitor of metalloproteinases-2 (TIMP-2). Because calcium is an important regulator of keratinocyte function, we evaluated the effect of calcium on MMP regulation in an oral squamous cell carcinoma line (SCC25). Increasing extracellular calcium (0.09–1.2 mM) resulted in a dose-dependent increase in MT1-MMP-dependent pro-MMP-2 activation. Despite the requirement for MT1-MMP in the activation process, no change in MT1-MMP expression, cell surface localization, or endocytosis were apparent. However, increased generation of the catalytically inactive 43-kDa MT1-MMP autolysis product and decline in TIMP-2 levels in conditioned media were observed. The decrease in TIMP-2 levels in the conditioned media was prevented by a broad spectrum MMP inhibitor, suggesting that calcium promotes recruitment of TIMP-2 to MT1-MMP on the cell surface. Despite the decline in soluble TIMP-2, no accumulation of TIMP-2 in cell lysates was seen. Blocking TIMP-2 degradation with bafilomycin A1 significantly increased cell-associated TIMP-2 levels in the presence of high calcium. These data suggest that the decline in TIMP-2 is because of increased calcium-mediated MT1-MMP-dependent degradation of TIMP-2. In functional studies, increasing calcium enhanced MMP-dependent cellular migration on laminin-5-rich matrix using an in vitro colony dispersion assay. Taken together, these results suggest that changes in extracellular calcium can regulate post-translational MMP dynamics and thus affect the cellular behavior of oral squamous cell carcinoma.

Oral squamous cell carcinoma (OSCC) is characterized by local, regional, and distant spread of the disease; however, the

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§ The abbreviations used are: OSCC, oral squamous cell carcinoma; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; cellular and molecular events that control the invasive behavior are poorly understood (1, 2). Immunohistochesismal studies have implicated enzymes belonging to the matrix metalloproteinase (MMP) family in basement membrane proteolysis and tissue invasion in OSCC (3). MMPs are a large family of metallo-endopeptidases with activity directed against a variety of extracellular matrix substrates (4–7). Expression of MMP-2 (gelatinerase A, a 72-kDa type IV collagenase) is observed in invasive and metastatic cases of OSCC (3, 8). Furthermore, increased expression of MMP-2 is associated with decreased staining of extracellular matrix in OSCC, suggesting that MMP-2 promotes matrix breakdown (3, 8). MMP-2 is secreted from cells as azymogen (pro-MMP-2) and is activated posttranslationally by a trans-membrane MMP designated as membrane type 1-MMP (MT1-MMP) (9–11). MT1-MMP is also up-regulated in OSCC, and increased expression is observed in highly invasive and metastatic cases (3, 8). Pro-MT1-MMP is synthesized as a 63–66-kDa zymogen and is activated intracellularly to a 55-kDa species by the serine proteinase furin, a member of the proprotein convertase family (12–14).

The activation of pro-MMP-2 is regulated by a complex mechanism involving formation of a trimolecular complex with MT1-MMP and tissue inhibitor of metalloproteinase-2 (TIMP-2) (10, 15–17). In this model, TIMP-2 plays a dual role in the regulation of MMP-2 activation, functioning both to promote and to inhibit the activation process in a concentration-dependent manner (16, 18). TIMP-2 bridges the interaction between the MMP-2 zymogen and MT1-MMP via N-terminal binding to the active site of MT1-MMP with the concomitant C-terminal binding to the pro-MMP-2 hemopexin domain (10, 15–20). Thus, at low TIMP-2 concentration, an adjacent TIMP-2-free MT1-MMP can effectively process the cell surface-bound pro-MMP-2 to a 68-kDa intermediate species, which undergoes autolytic processing to the mature 62-kDa active species. However at high TIMP-2 concentration, all of the cell surface MT1-MMPs undergo complex formation with TIMP-2, thereby inhibiting pro-MMP-2 activation (10, 15–20).

As stringent control of MMP activity plays an important role in keratinocyte behavior (21–23) and dysregulation of MMP activity has been correlated with metastatic progression, factors that control acquisition of net MMP activity in OSCC were evaluated. Of the many agents that are known to affect keratinocyte behavior, calcium is one of the key factors (24–27). There is a steep calcium gradient within the epidermis, with

MTIP, tissue of inhibitor of metalloproteinases; RVK, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone; DMEM, Dulbecco’s modified Eagle’s media; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; MESNA, mercaptoethanesulfonic acid; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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higher calcium present in the uppermost layers (28–30). Moreover, altering extracellular calcium has been used to effectively model in vitro physiologic changes in keratinocytes that occur within the epidermis as cells migrate from the basal to the uppermost layers. Interestingly, recent studies (31–33) have demonstrated a relationship between extracellular calcium and enhanced matrix metalloproteinase gene expression in primary human keratinocytes.

Because calcium is an important regulator of keratinocyte function, we evaluated the effect of calcium on MMP regulation in an oral squamous cell carcinoma line (SCC25). Increasing extracellular calcium resulted in a dose-dependent increase in pro-MMP-2 activation, accompanied by enhanced MT1-MMP autolytic processing and a decline in the levels of soluble TIMP-2. The decrease in TIMP-2 levels in the conditioned media was prevented by a broad spectrum MMP inhibitor, suggesting that calcium promotes recruitment of TIMP-2 to MT1-MMP on the cell surface. Despite the decline in soluble TIMP-2, no accumulation of TIMP-2 in cell lysates was seen. However, blocking TIMP-2 degradation with bafilomycin A1 significantly increased cell-associated TIMP-2 levels in the presence of high calcium. These data suggest that the decline in TIMP-2 is due to increased calcium-mediated MT1-MMP-dependent degradation of TIMP-2. Moreover, calcium enhanced MMP-dependent cellular migration on laminin-5-rich matrix. These results suggest that changes in extracellular calcium can regulate post-translational MMP dynamics and thus affect the cellular behavior of OSCC.

EXPERIMENTAL PROCEDURES

Materials—Gelatin, type I collagen, cell culture reagents, Chelex 100, MESNA, peroxidase-conjugated secondary antibodies, and the MT1-MMP antibody directed against the hinge region were purchased from Sigma. Phorbol 12-myristate 13-acetate (PMA), epidermal growth factor (EGF), and bafilomycin A1 were from Calbiochem. Dulbecco’s modified Eagle’s media (DMEM), DMEM without calcium, Ham’s F-12, G418, Trizol, and One-step RT-PCR kits were purchased from Invitrogen. Purified TIMP-1 and TIMP-2 proteins, rabbit polyclonal TIMP-2 antibody, and the broad spectrum MMP inhibitor GM6001 were purchased from Chemicon (Temecula, CA). TIMP-2 ELISA kit was from Oncogene Research Products (Boston, MA). SuperSignal enhanced chemiluminescence (ECL) reagent, EZ-Link Sulfo-NHS-LC-Biotin, EZ-Link Sulfo-NHS-SS-Biotin, and UltraLink immobilized streptavidin gel were obtained from Pierce. The furin inhibitor decapeptyl-Arg-Val-Lys-Arg-chloromethyl ketone (BVR) was from Alexis Biochemicals (San Diego, CA). Microcon 10 microconcentrators and polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA). FuGENE 6 was obtained from Roche Molecular Biochemicals. RQI DNase was from Promega (Madison, WI).

Cell Cultures—SCC25 cells were obtained from American Type Culture Collection (ATCC). SCC25 cells were routinely maintained in DMEM/Ham’s F-12 = 1:1 media containing 10% fetal calf serum and supplemented with 100 units/ml penicillin. SCC25 and SCC25-MT (defined below) cells were plated in DMEM containing 0.9 mM calcium and supplemented with Chelex-treated 10% fetal calf serum. After overnight serum starvation, the cells were switched to serum-free DMEM containing an indicated calcium concentration. In additional experiments, inhibitors or other chemical reagents were added 30 min prior to the medium change.

In some experiments, cells were cultured on thin layer or three-dimensional collagen surfaces (34). Briefly, acid-solubilized rat tail type I collagen was diluted to 50 μg/ml in 0.02 M acetic acid and added to the tissue culture dish. The indicated calcium concentration. In additional experiments, inhibitors or other chemical reagents were added 30 min prior to the medium change.

Cell Surface Biotinylation—To label cell surface proteins, SCC25-MT cells were grown to confluence in a 6-well plate, washed with ice-cold PBS, and incubated at 4°C for 30 min with 0.5 mg/ml cell-impermeable Sulfo-NHS-LC-Biotin in ice-cold PBS, followed by incubation at room temperature to quench free biotin. Cells were detached by scraping, lysed in modified RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 0.1% SDS) with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin), and clarified by centrifugation. To isolate biotinylated cell surface proteins, equal amounts of protein from each sample were incubated with streptavidin beads at 4°C for 14 h, followed by centrifugation. After boiling in Laemmli sample dilution buffer (35) to dissociate streptavidin-bead-biotin complexes, the biotin-labeled samples were analyzed by SDS-PAGE (9%) gels and immunoblotted for MT1-MMP.

MT1-MMP Endocytosis—To determine whether calcium affects MT1-MMP endocytosis, SCC25-MT cells grown to confluence in a 6-well plate were washed with ice-cold PBS and then incubated at 4°C for 30 min with 0.5 mg/ml cell-impermeable Sulfo-NHS-LC-Biotin in ice-cold PBS, followed by incubation at room temperature to quench free biotin. Cells were then incubated with DMEM containing either 0.89 mM or 1.2 mM calcium at 37°C for 60 min to initiate endocytosis. Endocytosis was assessed by the amount of biotinylated cell surface proteins that were then stopped by placing the cells on ice and washing them with ice-cold PBS. Biotin was then cleaved off the exposed cell surface by incubating the cells with membrane-impermeable reducing agent MESNA (100 mM) for 30 min at 37°C (37). The cells were lysed in RIPA buffer with proteinase inhibitors (1 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin) and clarified by centrifugation. To isolate biotinylated proteins (representing endocytosed surface-labeled species), equal amounts of protein from each of the samples were incubated with streptavidin beads at 4°C for 14 h, followed by centrifugation. After boiling in Laemmli sample dilution buffer (35) to dissociate streptavidin-bead-biotin complexes, the biotin-labeled samples were analyzed by SDS-PAGE (9%) gels and immunoblotted for MT1-MMP.
citate streptavidin–biotin complexes, the samples were analyzed by SDS-PAGE (9% gels) and immunoblotted for MT1-MMP. In control experiments to determine the efficiency of surface stripping with MESNA, cells were maintained on ice for the duration of the experiment and were not induced to undergo endocytosis via a temperature shift. In additional control experiments, the MESNA stripping step was omitted such that total labeled protein (endocytosed and the cell surface pool) was assayed.

Generation of Laminin-5-Enriched Matrix and Cell Dispersion Assays—The extracellular matrix deposited by SCC25 cells was generated as described previously (34, 38). Briefly, SCC25 cells were grown in 12-well plates to 48–72 h post-confluence prior to treatment for 7 min with 20 mM ammonium hydroxide to remove cells. After 3 rapid washes each in sterile distilled water and PBS, the laminin-5-enriched matrix was then used for in vitro migration assays. The effect of calcium on laminin-5-induced migration was assessed using a cell dispersion assay as described previously (39). Briefly, SCC25 and SCC25-MT cells (3 × 10⁵) were plated in DMEM (0.09 mM calcium) inside a cloning cylinder placed in the middle of a 12-well plate coated with laminin-5-enriched matrix. After the cells have attached and spread, the cloning cylinder was removed, and the cells were washed twice with DMEM containing 0.09 mM calcium and serum-starved for an additional 3 h. The media were then switched to DMEM containing either 0.09 or 1.2 mM calcium supplemented with 20 ng/ml EGF. In selected experiments, the proteinase dependence of migration was determined by adding the MMP inhibitor GM6001 (10 μM). To quantify the relative motility, the migratory front was photographed every 12 h for 48 h, and the percentage of cells crossing a line designated “migratory max” was enumerated.

RESULTS

Extracellular Calcium Regulates Pro-MMP-2 Activation—MMP activity is subject to complex post-translational regulation by a number of processes including zymogen activation, enzyme–inhibitor binding, endocytosis, and shedding (4–7, 40, 41); however, the biologic factors that control and coordinate these processes are poorly understood. As keratinocytes are subjected to fluctuations in extracellular calcium in the epidermal milieu, the effect of calcium on MMP activation was evaluated in SCC25 cells. The predominant soluble MMP expressed by SCC25 cells is MMP-2, with low level expression of MMP-9. SCC25 cells were plated in low calcium media (0.09 mM), serum-starved, and incubated with fresh serum-free media containing increasing calcium. Conditioned media were collected at 24 h and analyzed for MMP activity by gelatin zymography. Whereas cells cultured in 0.09 mM calcium concentration expressed pro-MMP-2 (Fig. 1A, 1st lane), increasing calcium concentration resulted in a dose-dependent MMP-2 activation (Fig. 1A, 2nd to 4th lanes). There was no change in MMP-9 expression with increasing calcium concentration in these cells (data not shown). Because collagen has been shown to affect MMP-2 expression and/or processing (42–48), the effect of calcium on collagen-induced MMP-2 activation was examined. Similar to the results obtained with SCC25 cells on plastic (Fig. 1A), cells plated on thin layer collagen demonstrated MMP-2 activation with increasing calcium concentration (Fig. 1B, 1st to 3rd lanes). Although cells cultured on three-dimensional collagen gels had a more pronounced base-line MMP-2 activation (Fig. 1B, 4th lane), a calcium-dependent increase in MMP-2 activation was observed (Fig. 1B, 5th and 6th lanes). These data indicate that extracellular calcium-mediated regulation of MMP-2 activation in SCC25 cells may act in synergy with collagen-induced pro-MMP-2 processing.

To investigate the proteolytic process leading to MMP-2 activation, SCC25 cells were treated with a broad spectrum MMP inhibitor, GM6001, or vehicle (Me₂SO) control. GM6001 inhibited calcium-induced pro-MMP-2 activation, demonstrating the involvement of an MMP in the activation process (Fig. 2A, lanes 3 and 4). To investigate MMP dependence further, SCC25 cells were treated with TIMP-1 and TIMP-2. TIMP-2 blocks both MMP-2 and MT1-MMP activities, whereas MT1-MMP activity is not inhibited by TIMP-1 (49, 50). TIMP-1 had no effect on calcium-induced MMP-2 activation (Fig. 2A, lanes 5 and 6), whereas TIMP-2 completely abrogated the response (Fig. 2A, lanes 7 and 8), implicating MT1-MMP in the calcium-induced pro-MMP-2 activation reaction.

To investigate further the involvement of MT1-MMP in calcium-dependent pro-MMP-2 activation, SCC25 cells were treated with a furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (RVKR), which has been shown to block activation of pro-MT1-MMP (51). Treatment of SCC25 cells with RVKR inhibited calcium-mediated MMP-2 activation, further implicating MT1-MMP in the calcium-induced pro-MMP-2 activation (compare Fig. 2B, lanes 1 and 3, with Fig. 2B, lanes 5 and 7). To determine whether calcium can act in synergy with PMA, another agent that has been shown to induce MMP-2 activation via MT1-MMP (11, 52), cells were cultured in low versus high calcium concentration in the presence of PMA and various proteinase inhibitors. As reported previously (52, 53), PMA induced pro-MMP-9 expression, irrespective of calcium concentration (Fig. 2C, compare lanes 1 and 2 with lanes 5 and 6). In contrast, expression of pro-MMP-2 was not affected; however, activation was stimulated (Fig. 2C, lanes 1 and 2 and lanes 5 and 6). Addition of calcium further increased PMA-induced pro-MMP-2 activation (compare Fig. 2C, lanes 2 and 6), indicative of synergistic stimulation of MMP processing. In control experiments, activation was blocked by both RVKR and GM6001 (Fig. 2C, lanes 3 and 7 and 4 and 8, respectively).

Overexpression of MT1-MMP in SCC25 Cells—To investigate further the involvement of MT1-MMP in calcium-dependent pro-MMP-2 activation, SCC25 cells overexpressing MT1-MMP (designated SCC25-MT) were generated (Fig. 3A). Overexpression of MT1-MMP in SCC25-MT cells was verified by Western blotting of whole cell lysates, indicating the presence of the 55-kDa active species and the 43-kDa catalytically inactive autolysis product (Fig. 3A). As reported previously (54, 55), GM6001 prevents autolysis of MT1-MMP and thus increases the accumulation of the 55-kDa species (Fig. 3A, 2nd lane). Correlating with the enhanced MT1-MMP expression in SCC25-MT cells (Fig. 3A, 4th lane), a significantly increased MMP-2 activation is observed (Fig. 3A, lower panel, 4th lane). Similar to wild-type SCC25 cells, calcium increased pro-MMP-2 activation by SCC25-MT cells in a dose-dependent manner (Fig.
were plated on plastic in medium containing 0.09 mM calcium, subjected to overnight serum starvation, and transferred to medium containing the indicated calcium concentration. At the time of calcium switch, the cells were treated with MeSO<sub>4</sub> (DMSO, vehicle control), MMP inhibitor GM6001 (10 μM), TIMP-1 (20 ng/ml), or TIMP-2 (20 ng/ml). The conditioned media were collected at 24 h and analyzed for MMP activity by gelatin zymography. Rapid MMP-2 activation was detected at 24 h in all treatment groups except those treated with GM6001, where further support for increased pro-MMP-2 activity was provided by zymogram data showing increased pro-MMP-2 activation (Fig. 4B, lower panel, 3rd lane).

To determine whether MT1-MMP activity is enhanced via increased cell surface association, surface biotinylation was used to probe calcium-induced changes in the cell surface MT1-MMP species. Serum-starved SCC25-MT cells were maintained in low or high calcium for 24 h as indicated and then incubated with cell-impermeable NHS-biotin to label cell surface proteins and lysed in modified RIPA buffer. Following precipitation of surface-labeled proteins with streptavidin beads, samples were electrophoresed and probed for MT1-MMP by immunoblotting. Similar to the results obtained with whole cell lysates, calcium had no effect on the surface expression of MT1-MMP (Fig. 4C).

Rapid Kinetics of Calcium-induced Pro-MMP-2 Activation—To evaluate the kinetics of pro-MMP-2 activation, serum-starved SCC25 and SCC25-MT cells were first incubated in low calcium medium (0.09 mM) to accumulate pro-MMP-2 and TIMP-2 in the conditioned media. After 24 h, activation was initiated by the addition of calcium from a concentrated stock solution to a final concentration of 1.2 mM. At various time points, conditioned media and cell lysates were collected, and the relative kinetics of pro-MMP-2 activation were analyzed by gelatin zymography. Rapid MMP-2 activation was detected at the cell surface within 30 min following calcium restoration in both wild-type and MT1-MMP-overexpressing SCC25 cells (Fig. 5, A and B). Surface activation was followed by a more gradual release of MMP-2 as evidenced by accumulation of activated MMP-2 in the conditioned media (Fig. 5, C and D). The rapid calcium-induced activation of pro-MMP-2 was blocked by GM6001 (data not shown).
Because MMP-2 activation occurs rapidly following calcium addition, two distinct approaches were utilized to address the potential for rapid calcium-induced changes in the surface localization of MT1-MMP. In initial experiments, cells were cultured in low calcium medium (0.09 mM), switched to high calcium (1.2 mM), and at the indicated times cell surface proteins were labeled with NHS-biotin followed by lysis in modified RIPA buffer. The surface-labeled proteins were immunoprecipitated with streptavidin, and electrophoresed on a 9% acrylamide gel. The membranes were immunoblotted with anti-MT1-MMP antibody, followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The results are representative of at least two independent experiments.

Recent data demonstrate that MT1-MMP can be regulated post-translationally via internalization from the cell surface (56, 57). To determine whether calcium induces dynamic turnover of MT1-MMP and thereby regulates MMP-2 activation, we evaluated MT1-MMP endocytosis in SCC25-MT cells. SCC25-MT cells were surface-biotinylated with cleavable cell-impermeable NHS-SS-biotin at 4°C to block endocytosis and then transferred to 37°C in 0.09 or 1.2 mM calcium-containing medium to allow for internalization (Fig. 7A, [1]). Control cells were maintained at 4°C to prevent internalization (Fig. 7A, [2]). After a 40-min incubation at 37°C, the cells were returned to 4°C to stabilize surface protein profiles and block further internalization (Fig. 7A, [3]). Biotin on the remaining cell surface proteins was then removed using the reducing agent MESNA (Fig. 7A, [4]). In control experiments, MESNA was omitted to enable evaluation of total labeled proteins (i.e., surface and internalized) (Fig. 7A, [5]). Cells were washed with ice-cold PBS and lysed, and labeled proteins were precipitated with streptavidin beads (Fig. 7A, [6–8]), electrophoresed, and probed for MT1-MMP by immunoblotting. No significant changes in the surface levels of the 55-kDa MT1-MMP species were induced by calcium supplementation (Fig. 6A), although GM6001 stabilized cell surface MT1-MMP against autolysis (Fig. 6B).

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Calcium Regulates TIMP-2 Levels—TIMP-2 plays an important role in pro-MMP-2 activation (10, 15–20); at low concentrations it facilitates activation by bridging trimolecular-activation complex formation, whereas at higher concentrations the activation is inhibited via interaction of TIMP-2 with the catalytically competent MT1-MMP active site. Because the calcium-induced pro-MMP-2 activation could not be attributed to changes in either MT1-MMP expression or to surface localization, the remaining component of the trimolecular complex, TIMP-2, was evaluated. Following serum starvation in low calcium, SCC25-MT cells were maintained in medium containing the indicated calcium concentration in the presence or absence of GM6001. Conditioned media were collected at 24 h, concentrated 15–20-fold, and TIMP-2 analyzed by Western blotting. A dose-dependent decrease in soluble TIMP-2 was observed (Fig. 8A, 1st to 4th lanes). To determine whether the calcium-induced decrease in soluble TIMP-2 results from decreased expression, the TIMP-2 message levels from SCC25 and SCC25-MT cells were analyzed by RT-PCR. No calcium-induced changes in TIMP-2 message levels were observed either in SCC25 or SCC25-MT cells (Fig. 8B). However, analysis of soluble TIMP-2 protein levels in cells cultured with GM6001 indicated that GM6001 blocked the calcium-mediated decline in soluble TIMP-2 (Fig. 8A, 5th to 8th lanes). Together, these data suggest that blocking the MT1-MMP active site with GM6001 may prevent the loss of soluble TIMP-2 by affecting recruitment of TIMP-2 to the cell surface-activation complex, providing evidence that calcium regulates TIMP-2 at the post-translational level.

To determine whether the rapid calcium-mediated induction of pro-MMP-2 activation reflects changes in surface-associated TIMP-2 levels, the effect of calcium on the kinetics of TIMP-2 loss from the conditioned media was examined. SCC25-MT cells were incubated in low calcium (0.09 mM) medium to acu-
bafilomycin A1 (75 nM), and conditioned media were collected, 41486

Baf 0.09, a final concentration of 1.2 mM. At the indicated times, the 24 h, calcium from a concentrated stock solution was added to

cells were serum-starved overnight and incubated for an additional 24 h at the indicated calcium concentration in the presence or absence of bafilomycin A1 (75 nM). A, conditioned media were concentrated 15–20-fold using Micron 10 microconcentrators and electrophoresed on a 15% SDS-polyacrylamide gel. The membranes were immunoblotted with anti-TIMP-2 antibody followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. B, TIMP-2 levels in the cell lysates were quantified using ELISA according to the manufacturer's specifications. The results are representative of at least two independent experiments. *p significantly different from control (Ca²⁺ 0.09, Baf⁻) with p < 0.05.

mulate pro-MMP-2 and TIMP-2 in the conditioned media. After 24 h, calcium from a concentrated stock solution was added to a final concentration of 1.2 mM. At the indicated times, the conditioned media were collected, concentrated, and TIMP-2 analyzed by Western blotting. TIMP-2 levels in the conditioned media changed with time, with the decrease apparent at 2 h following calcium restoration (Fig. 5C, upper panel). The presence of GM6001 blocked the decline (Fig. 5C, lower panel), further supporting the hypothesis that calcium promotes MT1-MMP dependent recruitment of TIMP-2 to the cell surface trimolecular activation complex.

To differentiate whether the calcium-induced decrease in soluble TIMP-2 levels reflected enhanced surface accumulation versus increased degradation of TIMP-2, the vacuolar ATPase inhibitor bafilomycin A1 was utilized. The rationale for this experiment was based on previous studies (58) showing that PMA-induced stimulation of pro-MMP-2 activation and corresponding loss of soluble TIMP-2 resulted from MT1-MMP-mediated TIMP-2 internalization and subsequent intracellular degradation in endosomal and/or lysosomal compartments. Increasing the pH of these compartments with bafilomycin A1 blocked TIMP-2 degradation, leading to a build-up of cellular TIMP-2 levels (58). Thus, SCC25-MT cells were cultured in 0.09 or 1.2 mM calcium for 24 h in the presence or absence of bafilomycin A1 (75 nM), and conditioned media were collected, concentrated 15–20-fold, and evaluated for TIMP-2 by Western blotting. In addition, the cell lysates at 24 h were collected and analyzed for TIMP-2 by ELISA. As shown above (Fig. 8A), calcium decreased the TIMP-2 levels in the conditioned media (Fig. 9A, 1st and 3rd lanes) but did not affect the levels of TIMP-2 in the cell lysates (Fig. 9B). Together, these data indicate that the calcium-mediated decline in soluble TIMP-2 is not due to cell surface accumulation of the inhibitor and suggest that TIMP-2 degradation is increased in high calcium. This is supported by experiments using bafilomycin A1, which par-
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generation of 43-kDa catalytically inactive MT1-MMP species and a decline in the levels of soluble TIMP-2. Calcium did not affect the steady state levels of TIMP-2 in the cell lysates, suggesting that calcium induces TIMP-2 degradation. As a functional consequence, calcium promoted cellular migration, suggesting that calcium may control keratinocyte migration via regulation of MMP-2 activation.

Calcium-mediated MMP-2 activation was MT1-MMP-dependent; however, calcium did not affect MT1-MMP message or cell surface protein levels, consistent with the observation that calcium-mediated activation of MMP-2 occurs rapidly at the cell surface. Increased generation of the catalytically inactive 43-kDa MT1-MMP species autolysis product was also observed. These data are in agreement with the recent reports (58, 69, 70) showing that MMP-2 activation induced by fibronectin and PMA increased accumulation of the 43-kDa MT1-MMP species without affecting the levels of the 55-kDa MT1-MMP species. Despite the rapid activation of MMP-2 at the cell surface, calcium did not affect MT1-MMP endocytosis.

Calcium-induced MMP-2 activation was associated with an MT1-MMP-dependent decline in soluble TIMP-2. A similar phenomenon has been reported recently (58, 69, 71) in other model systems. For example, PMA and type IV collagen-induced MMP-2 activation in HT1080 cells is coupled with TIMP-2 degradation (58, 69). In SCC25 cells, the calcium-induced decline in TIMP-2 also likely results from degradation as treatment with bafilomycin A1, a highly specific inhibitor of vacuolar ATPase that was previously shown to block MT1-MMP-mediated degradation of TIMP-2 (58), restored soluble TIMP-2 levels. This is in contrast to the loss of soluble TIMP-2 that accompanies concanavalin A-induced pro-MMP-2 activation, which results from enhanced cell surface binding rather than degradation (58, 69, 72). The mechanism by which changes in extracellular calcium promote TIMP-2 internalization and degradation is currently under investigation. Nevertheless, it is interesting to note that an inverse relationship between MMP-2 activation and soluble TIMP-2 has been observed in many human cancer cell lines (22, 51, 72).

The calcium-induced changes in post-translational MMP regulation correlated with increased migration over laminin-5-enriched matrix. Several reports (63, 64, 74, 75) have demonstrated involvement of active MMP-2 in cellular migration, including laminin-5-driven motility (73, 76). MT1-MMP has also been implicated in epithelial cell migration over laminin-5 matrix (39, 73). Our data demonstrate that migration on laminin-5 is enhanced both in MT1-MMP-overexpressing cells and under conditions that promote MMP-2 activation. Because calcium is a key regulator of keratinocyte function, these data suggest that localized changes in calcium in the extracellular milieu may function as a fine regulatory mechanism for post-translational control of MMP activity and MMP-influenced cellular behaviors such as migration and invasion.

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