

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 changes 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 the 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)¹ is characterized by local, regional, and distant spread of the disease; however, the

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¹ 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). Immunohistochemical 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 (gelatinase 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 a zymogen (pro-MMP-2) and is activated post-translationally 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

TIMP, tissue inhibitor of metalloproteinases; RVKR, 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.

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 decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (RVKR) 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. RQ1 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 0.09 mM calcium and supplemented with Chelex-treated 10% fetal calf serum. After overnight serum starvation, the cells were switched to serum-free DMEM containing the 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 μ g/ml in 0.02 N acetic acid and added to the tissue culture plate for 1 h at 18 °C. The solution was aspirated, and the plate was rinsed three times with PBS. Three-dimensional collagen gels were prepared by diluting acid-solubilized type I collagen to a concentration of 1 mg/ml in cold DMEM (calcium-free), neutralizing with sodium hydroxide, and then allowing to gel (700 μ l in 12-well plates) for 30 min at 37 °C prior to plating cells.

Generation of SCC25-MT Cells—Human MT1-MMP cDNA (a kind gift from Duanqing Pei, University of Minnesota) was cloned into pCR3.1-Uni (Invitrogen) mammalian expression vector. SCC25 cells were stably transfected using FuGENE 6 according to the manufacturer's instructions. Cell clones resistant to 0.8 mg/ml G418 were chosen

and screened for MMP-2 activation by zymography as described below and for MT1-MMP expression by Western blotting using antibody directed against the hinge region. Six different clones with high levels of MT1-MMP expression were selected for subsequent experiments. The cells were then maintained in DMEM/Ham's F12 media = 1:1 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.65 mg/ml G418.

Analysis of MMP-2 and TIMP-2 Expression—Gelatinase activities in 24-h serum-conditioned media were determined using SDS-PAGE gelatin zymography as described previously (34). Briefly, SDS-PAGE gels (9% acrylamide) were co-polymerized with 0.1% gelatin, and samples were electrophoresed without reduction or boiling using 5 \times Laemmli sample buffer (35). SDS was removed through a 30-min incubation in 2.5% Triton X-100, and gels were incubated in 20 mM glycine, pH 8.3, 10 mM CaCl₂, 1 μ M ZnCl₂ at 37 °C for 24–36 h. The gels were stained with Coomassie Blue to visualize zones of gelatinolytic activity. The conditioned media were concentrated 15–20-fold using Microcon 10 microconcentrators, boiled in Laemmli sample dilution buffer (35), analyzed for TIMP-2 by SDS-PAGE (15% gels), and immunoblotted with rabbit polyclonal antibody (Chemicon). Levels of TIMP-2 protein in the cell lysates were quantified by ELISA (Oncogene Research Products) according to the manufacturer's specifications.

MT1-MMP and TIMP-2 RNA Levels—Total RNA was isolated from SCC25 and SCC25-MT cells using Trizol reagent according to the manufacturer's instructions. Following digestion with RQ1 DNase for 30 min at 37 °C, the total RNA concentration was determined by spectrophotometric measurement. Primer pairs for human MT1-MMP, human TIMP-2, and human GAPDH were as follows: forward primer 5'-GCC-CATTGGCCAGTTCTGGCGGG-3' and reverse primer 5'-CCTCGTCC-ACCTCAATGATGATC-3' for MT1-MMP; forward primer 5'-GGCGTT-TTGCAATGCAGATGTAG-3' and reverse primer 5'-CACAGGAGCCG-TCACITCTCTTG-3' for TIMP-2; and forward primer 5'-CGGAGTCA-ACGGATTTGGTTCGTAT-3' and reverse primer 5'-AGCCTTCTCCATG-TGGTGGAAGAC-3' for GAPDH (36). The length of the MT1-MMP, TIMP-2, and GAPDH amplicons were 530, 497, and 307 bp, respectively. RT-PCR was performed using the One-step RT-PCR kit where reverse transcription and DNA amplification occur in the same reaction. Briefly, 1 μ g of total RNA was used as template in a reaction that included the appropriate primers in the presence of both reverse transcriptase and *Taq* polymerase. The mixture was incubated at 45 °C for 30 min and cycled 30 times at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. Appropriate negative controls of amplification included reactions without reverse transcriptase. PCR products were visualized by UV transillumination of 1.5% agarose gels stained with ethidium bromide.

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 with gentle shaking for 30 min with 0.5 mg/ml cell-impermeable Sulfo-NHS-LC-Biotin in ice-cold PBS, followed by washing with 100 mM glycine 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 proteinase inhibitors (1 μ g/ml aprotinin, 1 μ M pepstatin, and 10 μ M 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-cm dish were washed with ice-cold PBS and then incubated with cleavable cell-impermeable sulfo-NHS-SS-biotin (1 mg/ml) for 20 min in an ice bath. Biotinylation was stopped by washing with ice-cold PBS followed by 100 mM glycine in PBS to quench free biotin. Cells were then incubated with DMEM containing either 0.09 mM or 1.2 mM calcium at 37 °C for 40 min to initiate endocytosis. Endocytosis of cell surface proteins was 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 4 °C (37). The cells were lysed in modified RIPA buffer with proteinase inhibitors (1 μ g/ml aprotinin, 1 μ M pepstatin, and 10 μ M 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 disso-

ciate streptavidin bead-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 analyzed.

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 (3×10^4) 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 μ M). 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

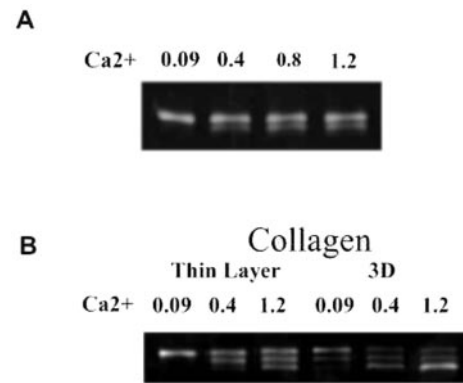


FIG. 1. Extracellular calcium regulates pro-MMP-2 activation. A, SCC25 cells were plated on plastic in medium containing 0.09 mM calcium. Following overnight serum starvation, cells were transferred to medium containing the indicated calcium concentration (0.09–1.2 mM). The conditioned media were collected at 24 h and analyzed for MMP activity by gelatin zymography. B, SCC25 cells were plated in medium containing 0.09 mM calcium on thin layer or three-dimensional (3-D) collagen as described under “Experimental Procedures.” Following serum starvation, media were replaced with serum-free medium at the indicated calcium concentration. The conditioned media were collected at 24 h and analyzed for MMP activity by gelatin zymography. The results are representative of at least four independent experiments.

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.

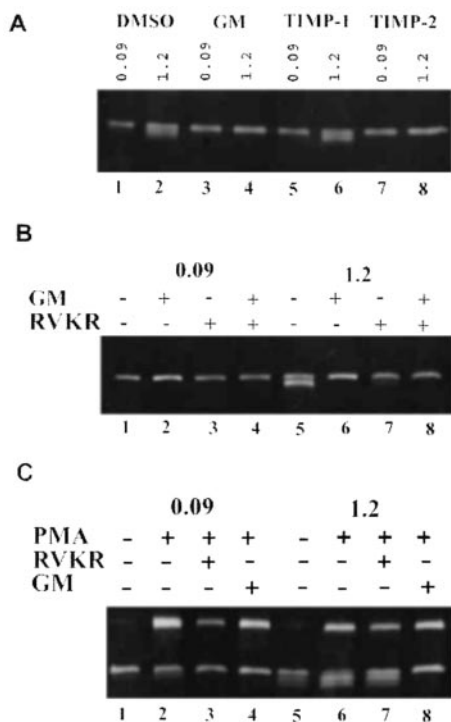


FIG. 2. Calcium-induced pro-MMP-2 activation involves MT1-MMP. A, SCC25 cells were plated on plastic in medium containing 0.09 mM calcium, subjected to overnight serum starvation, and transferred to fresh medium at the indicated calcium concentration. At the time of calcium switch, the cells were treated with Me₂SO (*DMSO*, vehicle control), MMP inhibitor GM6001 (*GM*, 10 μ M), 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. B and C, SCC25 cells were plated on plastic in medium containing 0.09 mM calcium, subjected to overnight serum starvation, and transferred to fresh medium at the indicated calcium concentration. At the time of calcium switch, the cells were treated with PMA (20 nM), MMP inhibitor GM6001 (10 μ M), and/or furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (RVKR, 20 μ M). The conditioned media were collected at 24 h and analyzed for MMP activity by gelatin zymography. The results are representative of three independent experiments.

3B). Activation was inhibited by the broad spectrum MMP inhibitor GM6001 (Fig. 3C) and by TIMP-2 (Fig. 3D).

Effect of Extracellular Calcium on MT1-MMP—To determine whether the calcium-induced increases in pro-MMP-2 activation are the result of enhanced MT1-MMP expression, the MT1-MMP mRNA levels from SCC25 and SCC25-MT cells were analyzed by RT-PCR. Changes in the message levels for GAPDH were used as internal control. SCC25-MT cells expressed higher levels of MT1-MMP message compared with SCC25 cells; however, calcium did not alter the steady state levels of MT1-MMP mRNA (Fig. 4A).

As the RT-PCR data indicated that MT1-MMP expression levels were unaffected by calcium, post-translational mechanisms of MT1-MMP regulation were investigated. To evaluate the effect of calcium on MT1-MMP processing, SCC25-MT cells were plated in low calcium, serum-starved, and incubated in fresh medium containing low (0.09 mM) or high (1.2 mM) calcium concentration in the presence or absence of GM6001 to prevent autocatalytic processing of MT1-MMP to the 43-kDa species. After 24 h, the cell lysates were probed for MT1-MMP by Western blotting. In agreement with the RT-PCR data, no significant change in overall MT1-MMP protein expression was observed (Fig. 4B, upper panel). However, increased processing of MT1-MMP to the 43-kDa autolysis product was observed in high calcium, indicative of enhanced MT1-MMP activity, as further supported by zymogram data showing increased pro-

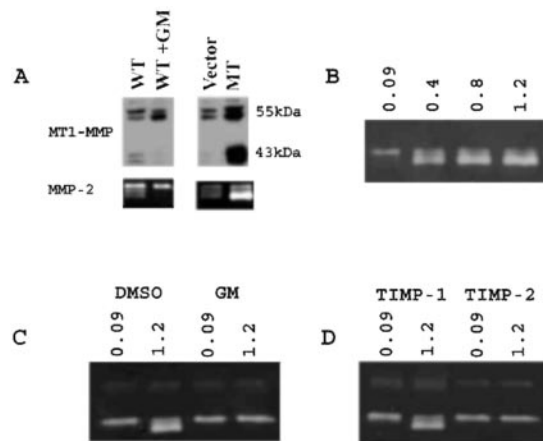


FIG. 3. Overexpression of MT1-MMP in SCC25 cells. A, SCC25 cells overexpressing MT1-MMP (SCC25-MT) were generated as described under "Experimental Procedures." At 24 h the cell lysates from wild-type (WT) SCC25 and SCC25-MT cells were analyzed for MT1-MMP expression by Western blotting and the conditioned media for MMP-2 activity by gelatin zymography. The membranes were immunoblotted with anti-MT1-MMP antibody followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. B, SCC25-MT cells were plated on plastic in medium containing 0.09 mM calcium. Following overnight serum starvation, cells were transferred to medium containing the indicated calcium concentration (0.09–1.2 mM). Conditioned media were collected at 24 h and analyzed for MMP activity by gelatin zymography. C and D, SCC25-MT cells were plated on plastic in medium containing 0.09 mM calcium, and following overnight serum starvation, the media were changed to either 0.09 or 1.2 mM calcium concentration. At the time of calcium switch, the cells were treated with Me₂SO (*DMSO*, vehicle control), MMP inhibitor GM6001 (*GM*, 10 μ M), 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. The results are representative of two independent experiments.

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). Enhanced surface levels of 43-kDa autolysis product were also observed, providing additional evidence for increased cell surface MT1-MMP activity.

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).

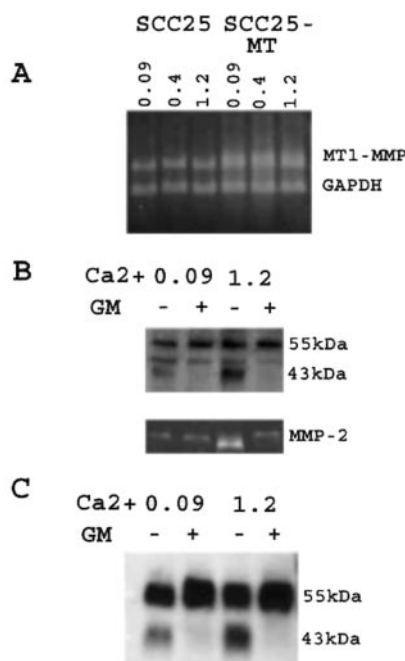


FIG. 4. Effect of extracellular calcium on MT1-MMP. A, SCC25 (1st to 3rd lanes) and SCC25-MT (4th to 6th lanes) cells were cultured for 24 h in medium at the indicated calcium concentration. Total RNA was isolated using Trizol reagent and quantified, and RT-PCR for MT1-MMP message was performed using primers as detailed under "Experimental Procedures." As loading control, amplification primers for GAPDH were used. PCR products were visualized by UV transillumination of 1.5% agarose gels stained with ethidium bromide. B, SCC25-MT cells were cultured in calcium-containing medium as indicated in the presence or absence of GM6001 (GM, 10 μ M). Cells were lysed at 24 h in modified RIPA buffer containing proteinase inhibitors. Equal amounts of cell lysates were separated by SDS-PAGE, and the membranes were immunoblotted with anti-MT1-MMP antibody followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection (upper panel). The conditioned media were analyzed for MMP-2 activation using gelatin zymography (lower panel). C, SCC25-MT cells were cultured in calcium-containing medium as indicated in the presence or absence of GM6001 (10 μ M). After 24 h the cells were surface-biotinylated and lysed. Samples were immunoprecipitated with streptavidin beads at 4 $^{\circ}$ C for 14 h to isolate cell surface (biotinylated) proteins and electrophoresed on a 9% SDS-polyacrylamide 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.

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 beads 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).

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 $^{\circ}$ C to block endocytosis and then transferred to 37 $^{\circ}$ C in 0.09 or 1.2 mM calcium-containing

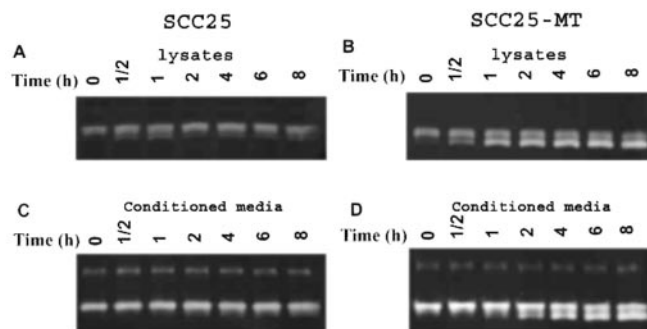


FIG. 5. Calcium induces rapid surface activation of pro-MMP-2. SCC25 and SCC25-MT cells were serum-starved overnight and incubated for an additional 24 h in medium containing 0.09 mM calcium. Calcium from a concentrated solution was added to the conditioned media to a final concentration of 1.2 mM, and at the indicated times (in hours) the cell lysates and conditioned media (CM) from SCC25 (A and C) and SCC25-MT (B and D) cells were analyzed for MMP-2 activation by gelatin zymography. The results are representative of four independent experiments.

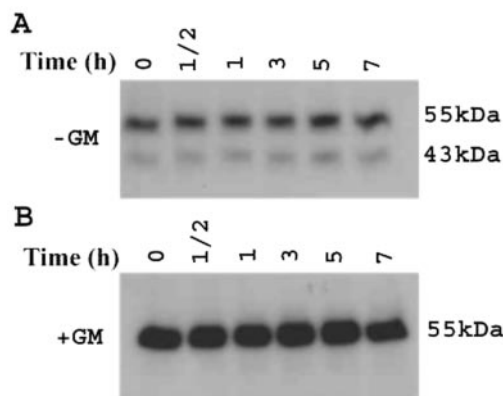


FIG. 6. Calcium does not affect cell surface MT1-MMP levels. SCC25-MT cells were serum-starved overnight and then incubated for an additional 24 h with Me₂SO or GM6001 (GM, 10 μ M) in medium containing 0.09 mM calcium. Calcium from a concentrated solution was added to the conditioned media to a final concentration of 1.2 mM, and at the indicated times the samples were surface-labeled with biotin, immunoprecipitated with streptavidin, and electrophoresed on a 9% SDS-polyacrylamide gel as described under "Experimental Procedures." The membranes were immunoblotted with anti-MT1-MMP antibody, followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The results are representative of three independent experiments.

medium to allow for internalization (Fig. 7A, [1]). Control cells were maintained at 4 $^{\circ}$ C to prevent internalization (Fig. 7A, [2]). After a 40-min incubation at 37 $^{\circ}$ C, the cells were returned to 4 $^{\circ}$ 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. In cells not subject to the temperature shift prior to reduction (Fig. 7A, (a)), no MT1-MMP was detected, demonstrating the efficacy of MESNA in removing the biotin from surface-labeled MT1-MMP (Fig. 7B, lanes 1 and 4). In contrast, cells incubated at 37 $^{\circ}$ C contained a protected, MESNA-resistant pool of MT1-MMP (Fig. 7A, (b)), demonstrating internalization of MT1-MMP in SCC25-MT cells (Fig. 7B, lanes 2 and 5). Treatment of cells with GM6001 for 40 min did not affect internalization of the

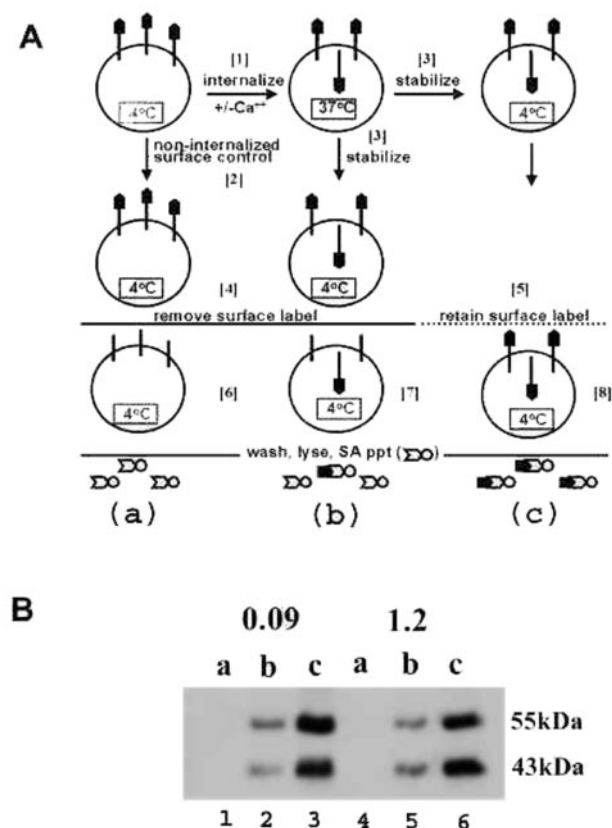


FIG. 7. Calcium does not affect MT1-MMP endocytosis. *A*, schematic of endocytosis protocol. [1], SCC25-MT cells were surface-biotinylated on ice to block endocytosis with cleavable sulfo-NHS-SS-biotin and transferred to 37 °C in medium containing 0.09 or 1.2 mM calcium. [2], control cells were maintained at 4 °C. [3], after a 40-min incubation, cells were returned to 4 °C to block further internalization. [4], surface biotin was removed with 100 mM MESNA. [5], in control experiments, MESNA was omitted to assess total labeled protein (surface and internalized). [6–8], cells were washed with ice-cold PBS, lysed, and labeled proteins precipitated at 4 °C for 14 h with streptavidin beads to isolate labeled proteins. *B*, samples prepared as described above were boiled, electrophoresed on a 9% SDS-polyacrylamide gel, and immunoblotted with anti-MT1-MMP antibody, followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The results are representative of three independent experiments.

55-kDa MT1-MMP species (data not shown). To evaluate the effect of calcium on MT1-MMP endocytosis, calcium levels in the medium were modulated at the time of temperature shift. MT1-MMP was effectively internalized in both low (0.09) and high (1.2 mM) calcium conditions (Fig. 7*B*, lanes 2 and 5). Analysis of total cellular MT1-MMP (Fig. 7*A*, (c); surface and internalized) confirmed previous results and indicated no change in the overall expression levels (Fig. 7*B*, lanes 3 and 6). Together, these data demonstrate that MT1-MMP is regulated by endocytosis under both low and high calcium conditions. However, the rapid calcium-induced changes in pro-MMP-2 activation kinetics are not mirrored by corresponding changes in surface MT1-MMP expression or endocytosis.

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

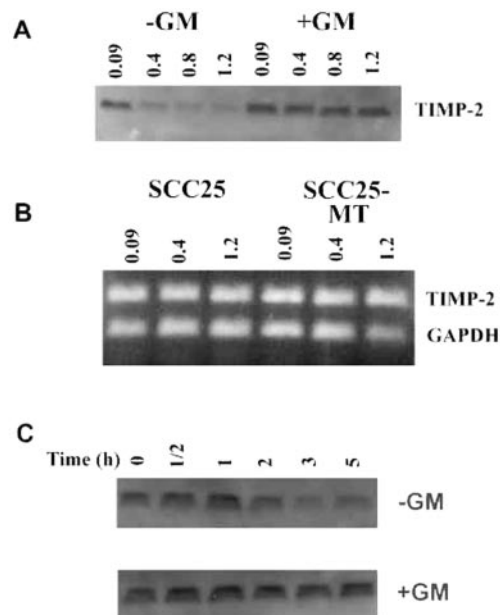


FIG. 8. Calcium decreases soluble TIMP-2. *A*, SCC25-MT cells were cultured for 24 h in medium at the indicated calcium concentration in the presence or absence of GM6001 (GM, 10 μ M). The conditioned media were collected at 24 h, concentrated 15–20-fold using Micron 10 microconcentrators, electrophoresed on a 15% SDS-polyacrylamide gel, and immunoblotted with anti-TIMP-2 antibody followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. *B*, SCC25 (1st to 3rd lanes) and SCC25-MT (4th to 6th lanes) cells were cultured for 24 h in medium at the indicated calcium concentrations. Total RNA was isolated using Trizol reagent and quantified, and RT-PCR for TIMP-2 message was performed using primers described under “Experimental Procedures.” As loading control, amplification primers for GAPDH were used. The samples were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV transillumination. *C*, SCC25-MT cells were serum-starved overnight and incubated for an additional 24 h in medium containing 0.09 mM calcium in the presence or absence of GM6001 (10 μ M). Calcium from a concentrated solution was added to the conditioned media to a final concentration of 1.2 mM, and at the indicated times the conditioned medium was harvested, concentrated 15–20-fold, electrophoresed on a 15% SDS-polyacrylamide gel, and immunoblotted with anti-TIMP-2 antibody followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The results are representative of at least three independent experiments.

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. 8*A*, 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. 8*B*). 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. 8*A*, 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 accu-

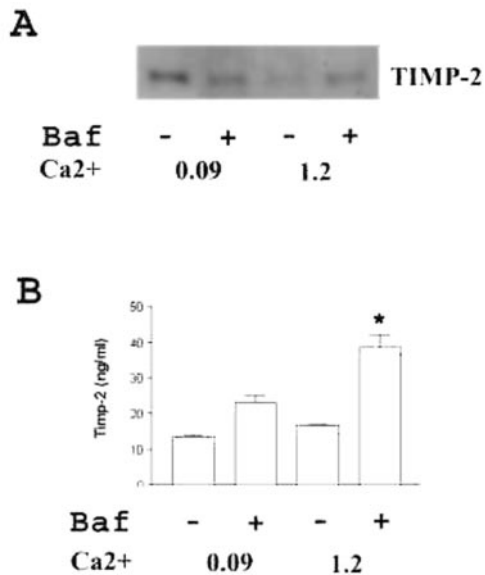


FIG. 9. Calcium promotes degradation of TIMP-2. SCC25-MT cells were serum-starved overnight and incubated for an additional 24 h at the indicated calcium concentration in the presence or absence of bafilomycin A1 (75 nM). *A*, conditioned media were concentrated 15–20-fold using Micron 10 microconcentrators 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. *, 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. 8C, upper panel). The presence of GM6001 blocked the decline (Fig. 8C, lower panel), further supporting the hypothesis that calcium promotes MT1-MMP dependent recruitment of TIMP-2 to the cell surface intramolecular 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-

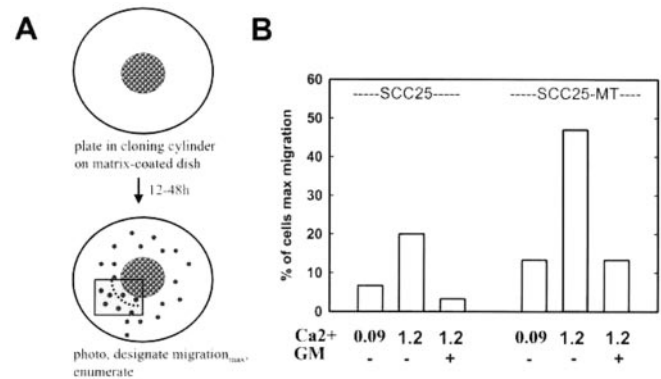


FIG. 10. Calcium promotes cell migration. *A*, schematic of migration protocol. Cells were plated in medium containing 0.09 mM calcium on laminin-5-enriched matrix inside a glass ring. After removal of the ring, the cells were serum-starved for 3 h, and then fresh serum-free medium containing either 0.09 or 1.2 mM calcium supplemented with 20 ng/ml EGF was added. Cells were allowed to migrate for 12–48 h. 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. *B*, SCC25 and SCC25-MT cells were allowed to migrate as described above in the presence or absence of GM6001 (GM, 10 μ M). In a representative experiment, the relative motility at 24 h was quantified by determining the percentage of the SCC25 and SCC25-MT cells crossing the migratory max line. The results are representative of three independent experiments.

tially blocked the calcium-mediated decline in soluble TIMP-2 (Fig. 9A, 4th lane). Because bafilomycin A1 blocks TIMP-2 degradation (58), these data support the hypothesis that the decline in soluble TIMP-2 observed under high calcium conditions reflects increased degradation of TIMP-2.

Calcium Promotes SCC25 Cell Migration—To evaluate the functional consequences of calcium-induced MMP-2 activation, the effect of calcium on SCC25 and SCC25-MT cell migration on laminin-5-rich matrix was examined. Migration was quantified using an *in vitro* cell dispersion assay (39), in which cells are plated at high density in a glass ring and allowed to migrate after removal of the ring (Fig. 10A). After the cells have attached and spread, the ring is removed, serum-starved for 3 h, and the media switched to either low (0.09 mM) or high (1.2 mM) calcium supplemented with 20 ng/ml EGF, and migration is quantified at 12–48 h. In a representative experiment quantified at 24 h, SCC25 cells migrated on laminin 5-rich matrix, with SCC25-MT cells displaying increased migration relative to wild-type cells (Fig. 10B). Calcium enhanced the motility of both wild-type and MT1-MMP-overexpressing SCC25 cells. Calcium-induced migration was blocked by GM6001, indicating that the enhanced migration observed can be attributed to increased MMP activity.

DISCUSSION

Studies using multiple cancer models have shown that MMP-2 activation is important in cellular behavior (59–62). The activation of MMP-2 *in vitro* is associated with increased migration and invasiveness of cancer cells (63–65). Furthermore, there is increased MMP-2 activation with lymph node metastasis in a number of different cancers, including OSCC (3, 8, 66). Hence, the regulation of MMP-2 activation has been studied extensively. Previously, it was shown that MMP-2 activation could be promoted with non-physiological agents like PMA (11, 52) and concanavalin A (67, 68) and also by proteins of the extracellular matrix such as collagen and fibronectin (42–48, 69, 70). In this study, we show that calcium also regulates pro-MMP-2 activation without altering expression of the zymogen. Increasing extracellular calcium resulted in a dose-dependent activation of pro-MMP-2, accompanied by enhanced

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.

REFERENCES

- Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Shafie, S. (1980) *Nature* **284**, 67–68
- Forastiere, A., Koch, W., Trotti, A., and Sidransky, D. (2001) *N. Engl. J. Med.* **345**, 1890–1900
- Kurahara, S., Shinohara, M., Ikebe, T., Nakamura, S., Beppu, M., Hiraki, A., Takeuchi, H., and Shirasuna, K. (1999) *Head Neck* **21**, 627–638
- Werb, Z. (1997) *Cell* **91**, 439–442
- Nagase, H., and Woessner, J. F., Jr. (1999) *J. Biol. Chem.* **274**, 21491–21494
- Sternlicht, M. D., and Werb, Z. (2001) *Annu. Rev. Cell Dev. Biol.* **17**, 463–516
- Egeblad, M., and Werb, Z. (2002) *Nat. Rev. Cancer* **2**, 161–174
- Shimada, T., Nakamura, H., Yamashita, K., Kawata, R., Murakami, Y., Fujimoto, N., Sato, H., Seiki, M., and Okada, Y. (2000) *Clin. Exp. Metastasis* **18**, 179–188
- Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **370**, 61–65
- Butler, G. S., Butler, M. J., Atkinson, S. J., Will, H., Tamura, T., van Westrum, S. S., Crabbe, T., Clements, J., d'Ortho, M. P., and Murphy, G. (1998) *J. Biol. Chem.* **273**, 871–880
- Lehti, K., Lohi, J., Valtanen, H., and Keski-Oja, J. (1998) *Biochem. J.* **334**, 345–353
- Sato, H., Kinoshita, T., Takino, T., Nakayama, K., and Seiki, M. (1996) *FEBS Lett.* **393**, 101–104
- Maquoi, E., Noel, A., Frankenke, F., Angliker, H., Murphy, G., and Foidart, J. M. (1998) *FEBS Lett.* **424**, 262–266
- Yana, I., and Weiss, S. J. (2000) *Mol. Biol. Cell* **11**, 2387–2401
- Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) *J. Biol. Chem.* **270**, 5331–5338
- Itoh, Y., Ito, A., Iwata, K., Tanzawa, K., Mori, Y., and Nagase, H. (1998) *J. Biol. Chem.* **273**, 24360–24367
- Zucker, S., Drews, M., Conner, C., Foda, H. D., DeClerck, Y. A., Langley, K. E., Bahou, W. F., Docherty, A. J., and Cao, J. (1998) *J. Biol. Chem.* **273**, 1216–1222
- Jo, Y., Yeon, J., Kim, H. J., and Lee, S. T. (2000) *Biochem. J.* **345**, 511–519
- Overall, C. M., King, A. E., Sam, D. K., Ong, A. D., Lau, T. T., Wallon, U. M., DeClerck, Y. A., and Atherstone, J. (1999) *J. Biol. Chem.* **274**, 4421–4429
- Toth, M., Bernardo, M. M., Gervasi, D. C., Soloway, P. D., Wang, Z., Bigg, H. F., Overall, C. M., DeClerck, Y. A., Tschesche, H., Cher, M. L., Brown, S., Mobashery, S., and Fridman, R. (2000) *J. Biol. Chem.* **275**, 41415–41423
- Madlener, M., Parks, W. C., and Werner, S. (1998) *Exp. Cell Res.* **242**, 201–210
- Baumann, P., Zigrino, P., Mauch, C., Breitkreutz, D., and Nischt, R. (2000) *Br. J. Cancer* **83**, 1387–1393
- Nagavarapu, U., Relloma, K., and Herron, G. S. (2002) *J. Invest. Dermatol.* **118**, 573–581
- Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980) *Cell* **19**, 245–254
- Hennings, H., and Holbrook, K. A. (1983) *Exp. Cell Res.* **143**, 127–142
- Yuspa, S. H., Kilkenny, A. E., Steinert, P. M., and Roop, D. R. (1989) *J. Cell Biol.* **109**, 1207–1217
- Bikle, D. D., Ng, D., Tu, C. L., Oda, Y., and Xie, Z. (2001) *Mol. Cell. Endocrinol.* **177**, 161–171
- Menon, G. K., Grayson, S., and Elias, P. M. (1985) *J. Invest. Dermatol.* **84**, 508–512
- Menon, G. K., Price, L. F., Bommannan, B., Elias, P. M., and Feingold, K. R. (1994) *J. Invest. Dermatol.* **102**, 789–795
- Mauro, T., Bench, G., Siddaras-Haddad, E., Feingold, K., Elias, P., and Cullander, C. (1998) *J. Invest. Dermatol.* **111**, 1198–1201
- Kobayashi, T., Hattori, S., Nagai, Y., Tajima, S., and Nishikawa, T. (1998) *Dermatology* **197**, 1–5
- Kobayashi, T., Hattori, S., Nagai, Y., and Tajima, S. (2000) *IUBMB Life* **50**, 221–226
- Kobayashi, T., Kishimoto, J., Ge, Y., Jin, W., Hudson, D. L., Ouahes, N., Ehama, R., Shinkai, H., and Burgeson, R. E. (2001) *EMBO Rep.* **2**, 604–608
- Munshi, H. G., and Stack, M. S. (2002) *Methods Cell Biol.* **69**, 195–205
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Wong, H., Muzik, H., Groft, L. L., Lafleur, M. A., Matouk, C., Forsyth, P. A., Schultz, G. A., Wall, S. J., and Edwards, D. R. (2001) *Methods Mol. Biol.* **151**, 305–320
- Neuhaus, E. M., and Soldati, T. (2000) *J. Cell Biol.* **150**, 1013–1026
- Gospodarowicz, D. (1984) in *Methods for Preparation of Media Supplements and Substrata* (Barnes, D. W., Sirbasku, D. A., and Stao, G. H., eds) pp. 275–293, Alan R. Liss, Inc., New York
- Gilles, C., Polette, M., Coraux, C., Tournier, J. M., Meneguzzi, G., Munaut, C., Volders, L., Rousselle, P., Birembaut, P., and Foidart, J. M. (2001) *J. Cell Sci.* **114**, 2967–2976
- Hernandez-Barrantes, S., Bernardo, M., Toth, M., and Fridman, R. (2002) *Semin. Cancer Biol.* **12**, 131–138
- Toth, M., Hernandez-Barrantes, S., Osenkowski, P., Bernardo, M. M., Gervasi, D. C., Shimura, Y., Meroueh, O., Kotra, L. P., Galvez, B. G., Arroyo, A. G., Mobashery, S., and Fridman, R. (2002) *J. Biol. Chem.* **277**, 26340–26350
- Azzam, H. S., and Thompson, E. W. (1992) *Cancer Res.* **52**, 4540–4544
- Seltzer, J. L., Lee, A. Y., Akers, K. T., Sudbeck, B., Southon, E. A., Wayner, E. A., and Eisen, A. Z. (1994) *Exp. Cell Res.* **213**, 365–374
- Gilles, C., Polette, M., Seiki, M., Birembaut, P., and Thompson, E. W. (1997) *Lab. Invest.* **76**, 651–660
- Haas, T. L., Davis, S. J., and Madri, J. A. (1998) *J. Biol. Chem.* **273**, 3604–3610
- Ellerbroek, S. M., Fishman, D. A., Kearns, A. S., Bafetti, L. M., and Stack, M. S. (1999) *Cancer Res.* **59**, 1635–1641
- Ellerbroek, S. M., Wu, Y. I., Overall, C. M., and Stack, M. S. (2001) *J. Biol. Chem.* **276**, 24833–24842
- Aznavoorian, S., Moore, B. A., Alexander-Lister, L. D., Hallit, S. L., Windsor, L. J., and Engler, J. A. (2001) *Cancer Res.* **61**, 6264–6275
- Strongin, A. Y., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1993) *J. Biol. Chem.* **268**, 14033–14039
- Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) *J. Biol. Chem.* **271**, 17119–17123
- Kurschat, P., Zigrino, P., Nischt, R., Breitkopf, K., Steurer, P., Klein, C. E., Krieg, T., and Mauch, C. (1999) *J. Biol. Chem.* **274**, 21056–21062
- Lohi, J., and Keski-Oja, J. (1995) *J. Biol. Chem.* **270**, 17602–17609
- Simon, C., Goeppfert, H., and Boyd, D. (1998) *Cancer Res.* **58**, 1135–1139
- Yamamoto, M., Tsujishita, H., Hori, N., Ohishi, Y., Inoue, S., Ikeda, S., and Okada, Y. (1998) *J. Med. Chem.* **41**, 1209–1217
- Rozanov, D. V., Deryugina, E. I., Ratnikov, B. I., Monosov, E. Z., Marchenko, G. N., Quigley, J. P., and Strongin, A. Y. (2001) *J. Biol. Chem.* **276**, 25705–25714
- Jiang, A., Lehti, K., Wang, X., Weiss, S. J., Keski-Oja, J., and Pei, D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 13693–13698
- Uekita, T., Itoh, Y., Yana, I., Ohno, H., and Seiki, M. (2001) *J. Cell Biol.* **155**, 1345–1356
- Maquoi, E., Frankenke, F., Baramova, E., Munaut, C., Soumni, N. E., Remacle, A., Noel, A., Murphy, G., and Foidart, J. M. (2000) *J. Biol. Chem.* **275**, 11368–11378

59. Brown, P. D., Bloxidge, R. E., Stuart, N. S., Gatter, K. C., and Carmichael, J. (1993) *J. Natl. Cancer Inst.* **85**, 574–578
60. Nomura, H., Fujimoto, N., Seiki, M., Mai, M., and Okada, Y. (1996) *Int. J. Cancer* **69**, 9–16
61. Liabakk, N. B., Talbot, I., Smith, R. A., Wilkinson, K., and Balkwill, F. (1996) *Cancer Res.* **56**, 190–196
62. Crescimanno, C., Foidart, J. M., Noel, A., Polette, M., Maquoi, E., Birembaut, P., Baramova, E., Kaufmann, P., and Castellucci, M. (1996) *Exp. Cell Res.* **227**, 240–251
63. Deryugina, E. I., Bourdon, M. A., Luo, G. X., Reisfeld, R. A., and Strongin, A. (1997) *J. Cell Sci.* **110**, 2473–2482
64. Makela, M., Larjava, H., Pirila, E., Maisi, P., Salo, T., Sorsa, T., and Uitto, V. J. (1999) *Exp. Cell Res.* **251**, 67–78
65. Itoh, Y., Takamura, A., Ito, N., Maru, Y., Sato, H., Suenaga, N., Aoki, T., and Seiki, M. (2001) *EMBO J.* **20**, 4782–4793
66. Kawata, R., Shimada, T., Maruyama, S., Hisa, Y., Takenaka, H., and Murakami, Y. (2002) *Acta Oto-Laryngol.* **122**, 101–106
67. Overall, C. M., and Sodek, J. (1990) *J. Biol. Chem.* **265**, 21141–21151
68. Yu, M., Sato, H., Seiki, M., and Thompson, E. W. (1995) *Cancer Res.* **55**, 3272–3277
69. Maquoi, E., Frankenne, F., Noel, A., Krell, H. W., Grams, F., and Foidart, J. M. (2000) *Exp. Cell Res.* **261**, 348–359
70. Stanton, H., Gavrilovic, J., Atkinson, S. J., d'Ortho, M. P., Yamada, K. M., Zardi, L., and Murphy, G. (1998) *J. Cell Sci.* **111**, 2789–2798
71. Gilles, C., Bassuk, J. A., Pulyaeva, H., Sage, E. H., Foidart, J. M., and Thompson, E. W. (1998) *Cancer Res.* **58**, 5529–5536
72. Shofuda, K., Moriyama, K., Nishihashi, A., Higashi, S., Mizushima, H., Yasumitsu, H., Miki, K., Sato, H., Seiki, M., and Miyazaki, K. (1998) *J. Biochem. (Tokyo)* **124**, 462–470
73. Koshikawa, N., Giannelli, G., Cirulli, V., Miyazaki, K., and Quaranta, V. (2000) *J. Cell Biol.* **148**, 615–624
74. Nawrocki Raby, B., Polette, M., Gilles, C., Clavel, C., Strumane, K., Matos, M., Zahm, J. M., Van Roy, F., Bonnet, N., and Birembaut, P. (2001) *Int. J. Cancer* **93**, 644–652
75. Takahashi, K., Eto, H., and Tanabe, K. K. (1999) *Int. J. Cancer* **80**, 387–395
76. Giannelli, G., Falk-Marzillier, J., Schiraldi, O., Stetler-Stevenson, W. G., and Quaranta, V. (1997) *Science* **277**, 225–228