ABSTRACT

The regulation of matrix metalloproteinases (MMPs) has been studied extensively due to the fundamental roles these zinc-endopeptidases play in diverse physiological and pathological processes. However, phosphorylation has not previously been considered as a potential modulator of MMP activity. The ubiquitously expressed MMP-2 contains 29 potential phosphorylation sites. Mass spectrometry reveals that at least five of these sites are phosphorylated in hrMMP-2 expressed in mammalian cells. Treatment of HT1080 cells with an activator of protein kinase C results in a change in MMP-2 immunoreactivity on 2D immunoblots consistent with phosphorylation, and purified MMP-2 is phosphorylated by protein kinase C in vitro. Furthermore, MMP-2 from HT1080 cell-conditioned medium is immunoreactive with antibodies directed against phosphothreonine and phosphoserine, which suggests that it is phosphorylated. Analysis of MMP-2 activity by zymography, gelatin dequenching assays, and measurement of kinetic parameters shows that the enzymatic activity of the enzyme is affected by phosphorylation. Consistent with this, dephosphorylation of MMP-2 immunoprecipitated from HT1080 conditioned medium with alkaline phosphatase significantly increases its enzymatic properties. Consistent with this, dephosphorylation of MMP-2 activity by phosphorylation. The FASEB J. 21, 2486–2495 (2007)

Key Words: dephosphorylation • gelatinase • alkaline phosphatase • protein kinase C • mass spectrometry

The matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases best known for their roles in physiological and pathological remodeling of the extracellular matrix (ECM) during angiogenesis, wound healing, embryogenesis, tumor metastasis, and various cardiovascular and inflammatory diseases (1, 2). The activation of MMPs requires proteolytic removal of the N-terminal propeptide, or disruption of the bond between the cysteine sulfhydryl moiety in the autoinhibitory propeptide domain and the zinc ion in the catalytic site of the enzyme (3). Proteolytic removal of the propeptide is catalyzed by proteases such as plasminogen, trypsin, or kallikrein (4, 5) as well as by membrane-type-1 MMP activity (6). Alternatively, proMMPs can be activated post-translationally either by S-glutathiolation of the propeptide cysteine sulfhydryl, as was shown for MMP-1, -8, and -9 (7) or by S-nitrosylation as shown for MMP-9 (8). This results in an active, full-length MMP, without proteolytic removal of the propeptide. Thus, MMP activity is regulated at several levels, including by transcriptional and post-translational mechanisms, and by the inhibition of enzymatic activity by endogenous tissue inhibitors of metalloproteinases (9).

MMP-2, also known as gelatinase A or type IV collagenase, is the most widely expressed of all the MMPs and is found in most tissues and cells. Although MMP-2 is well known as an ECM degrading enzyme, it also acts on several nonmatrix substrates, including interleukin-1β precursor (10), big endothelin-1 (11), monocyte chemoattractant protein-3 (12), and stromal cell-derived factor 1 (13), which have expanded the biological roles of this important protease. We discovered novel intracellular localization and actions for MMP-2 in the proteolytic degradation of the sarcomeric proteins tropomycin I and myosin light chain 1 in myocardial ischemia-reperfusion injury (14, 15). Recently we found that MMP-2 also associates with caveolin-1 in the membrane of cardiac myocytes, where caveolin-1 appears to help maintain MMP-2 in a membrane-bound and inhibited state (16). Caveolin-1 also binds to other regulatory proteins including protein kinase C (PKC) (17). MMP-2 also has a nuclear localization sequence and its
presence was verified in the nuclei of heart and liver cells (18).

Because of its unexpected intracellular localization and actions, we reasoned that there may also be novel regulatory mechanisms modulating MMP-2 activity within the cell. Since phosphorylation is often involved in regulating the activities of intracellular proteins, we examined the possibility that MMP-2 is phosphorylated. In particular, we explored the role of phosphorylation in regulating MMP-2 activity. Here we show that MMP-2 is phosphorylated and that its phosphorylation status significantly affects its activity. Furthermore, we show that MMP-2 is phosphorylated by PKC in vitro and that the PKC activator phorbol 12-myristate 13-acetate (PMA) induces the phosphorylation of MMP-2 in cell culture. Our data suggest that phosphorylation is an important and previously unrecognized regulator of MMP-2 activity.

MATERIALS AND METHODS

Materials

Unless otherwise specified, the reagents used were obtained from Sigma-Aldrich (Oakville, ON, Canada) or Fisher Scientific (Ottawa, ON, Canada). Polyclonal antiphosphothreonine, antiphosphoserine and monoclonal MMP-2 antibodies were obtained from Chemicon (Temecula, CA, USA). Monoclonal antiphosphotyrosine was purchased from Cell Signaling Technology (Danvers, MA, USA). The polyclonal MMP-2 antibody was a gift from Dr. Mieczyslaw Wozniak (Department of Clinical Chemistry, Medical University, Wroclaw, Poland). The following were purchased from the sources indicated: 72 kDa and 64 kDa hrMMP-2 (purified from mammalian cells) and bisindolylmaleimide (Calbiochem, San Diego, CA, USA); the catalytic fragment of PKC and Omniphotenase fluorogenic peptide substrate, BIOMOL, Plymouth Meeting, PA; γ-S33P-ATP and γ-32P-H2P4O7 (Perkin-Elmer, Wellesley, MA, USA); histone H1 protein (Stressgen, San Diego, CA, USA); goat anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA); DQ gelatin (Molecular Probes, Eugene, OR, USA); molecular weight markers, Coomassie Brilliant Blue R-250, Silver Stain Plus kit, Bradford Protein Assay and PVDF membrane (Bio-Rad, Hercules, CA, USA); protein A- and G-Sepharose beads and ECL plus (Amersham, Piscataway, NJ, USA).

Prediction of phosphorylation sites within human MMP-2

The primary sequence of human MMP-2 (accession number P08253) was obtained from the Swiss-Prot protein database (http://us.expasy.org/sprot/). NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) is based on a neural network method, which predicts serine, threonine, and tyrosine phosphorylation sites in eukaryotic proteins (19). NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/) was used to predict kinases that may act on MMP-2.

Cell culture

HT1080 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% (v/v) fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD, USA) and were passaged twice per week with a 0.05% trypsin/1 mM EDTA wash (Life Technologies, Inc.). Cells were maintained at 37°C under 5% CO2 atmosphere. The medium was removed and replaced by serum-free medium 24 h before experimentation.

Cell homogenates

Untreated or PMA-treated (100 nM, 24 h) cells were collected and homogenized by sonication in 50 mmol/L Tris-HCl (pH 7.4) containing 3.1 mmol/L sucrose, 1 mmol/L DTT, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, and 0.1% Triton X-100. The homogenate was centrifuged at 10,000 g (4°C for 10 min), and the supernatant was collected. Protein content in homogenates was analyzed using the Bradford Protein Assay, and bovine serum albumin was used as the standard.

Two-dimensional polyacrylamide gel electrophoresis and mass spectrometry

hrMMP-2 (72 kDa, 3.2 μg) or HT1080 cell homogenates (200 μg) was applied to an 11 cm immobilized pH gradient (pH 5–8) strip (Bio-Rad) and equilibrated for 16–18 h at 20°C in rehydration buffer (Bio-Rad). Isoelectric focusing was performed using a Bio-Rad Protein isoelectric focusing cell as described previously (15). Two-dimensional electrophoresis was performed using Criterion precast gradient gels (4–20% acrylamide, Bio-Rad). After separation, proteins were detected by Coomassie blue or silver staining or by Western blot using anti-MMP-2 antibody. The best resolved of the 72 kDa acidic protein spots of MMP-2 (pl 5.405 and, hence, potentially phosphorylated) was excised from the gel. Subsequently, a peptide mass fingerprint was obtained by in-gel digestion with trypsin and analyzed with a Bruker Daltonics Ultraflex MALDI TOF/TOF mass spectrometer. Analysis of experimentally manipulated human recombinant MMP-2 was performed similarly but using only 400 ng of protein (necessitating silver staining) and mass fingerprinting of a spot which resolved at pl 5.64.

Calculation of the theoretical masses of MMP-2 peptides generated by trypsin cleavage

Peptide-Mass (http://expasy.org/tools/peptide-mass.html) was used to predict the mass of trypsin digested peptides from the sequence of human MMP-2. For calculation of theoretical peptide masses we assumed: a) 1 missed cleavage level and ≤0.2 Da mass tolerance; b) mandatory alkylation and reduction of cysteines with iodoacetamide; and c) variable oxidation of methionines. Only peptides bigger than 500 Da were considered.

Examination of experimental peptide mass fingerprint for phosphorylation

FindMod tool was used to find potential phosphorylation (http://expasy.org/tools/findmod/) in tryptic peptides of MMP-2.

MMP-2 sequence alignment

We aligned the complete amino acid sequences of MMP-2 homologues from rat (NM_031054), mouse (NM_008610), human (NM_004530), and pig (NM_214192) using ClustalW.
In vitro kinase assay

Human recombinant 64 kDa MMP-2 was diluted in reaction buffer (50 mM Tris pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween-20 with 5 μg/ml sodium azide) to 20 ng/μl and incubated with 14 U/μl alkaline phosphatase for 1 h at 37°C. After dephosphorylation, AP activity was abolished by heating to 80°C for 15 min. Aliquots of dephosphorylated MMP-2 were then diluted to 10 ng/μl in PKC reaction buffer (100 μM ATP, 40 mM MES pH 6.0, 1 mM EGTA, 10 mM MgCl₂) and incubated with 6 μCi γ³²P-ATP (specific activity 3000 Ci/mmol) in the presence or absence of 10 ng of the catalytic fragment of PKC. Purified bovine histone H1 was used as a positive control for PKC activity. The reaction was stopped by adding an equal volume of 2× SDS-PAGE sample-loading buffer (50 mM Tris pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 7.5, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, and 0.1% SDS). The immunoprecipitates from conditioned medium of HT1080 cells was incubated with 14 U/μl alkaline phosphatase for 1 h at 37°C. The reaction mixture was split and stopped by adding an equal volume of 2× SDS-PAGE sample-loading buffer (for AP treatment) or bisindolylmaleimide (100 nM, for PKC treatment).

Kinetic analysis of MMP-2 activity

The hydrolysis of OmniMMP fluorogenic substrate (0–66 μM, prepared in 1.8% (v/v) DMEM) by either untreated, AP-treated, or PKC-treated samples of human recombinant 64 kDa MMP-2 (0.2 nM in 50 mM Tris pH 7.6, 10 mM CaCl₂, 0.05% Brij-35, 10 μM ZnSO₄) was measured at 37°C in a continuous plate reader-based protocol. Assays were made in a total volume of 120 μl in black polystyrene half-area plates (Corning, Corning, NY, USA), and contained MMP-2 (60 μl in 2× reaction buffer) and substrate (60 μl) or DMSO (1.8%, v/v, 60 μl in blank wells). Fluorescence associated with a (7-methoxycoumarin-4-yl)acetyl-labeled cleavage product was measured every 30 s for 1 h (λₚₑ 328 nm, λₘₐₓ 393 nm) in a Molecular Devices SPECTRAMax Gemini XPS fluorescence microplate reader. Neither AP nor PKC interfered with the hydrolysis of OmniMMP or had any effect on fluorescence (results not shown). The rate of product formation in each well was determined through linear regression of the fluorescence-time data by the plate reader software (SOFTmax Pro, v 4.8; Molecular Devices Inc., Sunnyvale, CA, USA). Appropriate lag times, to preclude data obtained prior to equilibration at 37°C, and end times, to preclude data obtained following a loss of linearity, were entered manually prior to linear regression of data to obtain slopes. Rate values were then corrected for loss of signal due to absorption by substrate at 393 nm, using a measured substrate extinction coefficient of 7627 M⁻¹cm⁻¹ and a measured path length of 0.672 cm. Linear reaction rates (r²>0.98 for all data) were fitted to the Michaelis-Menten equation using the nonlinear curve fitting facility of GraphPad Prism v 4.03 (GraphPad Software) in order to obtain Kₘ and Vₘₐₓ values.

Fluorescent gelatin dequenching assay

Untreated, AP-treated, or PKC-treated samples of 64 kDa human recombinant MMP-2 (50 ng) were added to 190 μl reaction buffer (150 mM NaCl, 10 mM HEPES pH 7.5, 0.25 mM DTT, 5 mM CaCl₂, 0.1% Triton X-100 with 5 μg/ml sodium azide) with 50 ng DQ gelatin in 96-well microplates, and incubated at 37°C for 30 min. FITC fluorescence generated by the cleavage of DQ gelatin was measured using a Thermo Labsystems Fluoroskan Ascent microplate reader fitted with FITC excitation and emission filters. Data are reported as the percent increase above background fluorescence observed in substrate-only blanks. Neither AP nor PKC interfered with fluorescent dequenching (results not shown). All assays were performed in triplicate.

Gelatin zymography

Gelatin zymography for MMP activity was performed according to Sawicki et al. (20). Briefly, nonreduced immunoprecipitated proteins using antiphospho antibodies were loaded onto an 8% polyacrylamide gel containing 2 mg/ml gelatin and electrophoresed for 60–90 min (150 V, 4°C). After washing the gels with Triton X-100 (2.5% v/v, 3×20 min), the gels were incubated at 37°C for 18–24 h, stained with 0.05% Coomassie Brilliant Blue G, and destained. Gelatinolytic activities were detected as transparent bands against the protein for 20 min at 37°C. For PKC treatment, hrMMP-2 was treated with 1 ng of the catalytic fragment of PKC per nanogram of MMP-2 in PKC reaction buffer containing 200 μM ATP and incubated for 1 h at 37°C. The reaction mixture was split and stopped by adding an equal volume of 2× SDS-PAGE sample-loading buffer (for AP treatment) or bisindolylmaleimide (100 nM, for PKC treatment).
to quantify the activities of the detected enzymes, zymograms were imaged using a GS-800 Calibrated Densitometer (Bio-Rad). The intensities of the separate bands were analyzed using Quantity1 measurement software (Bio-Rad) and reported as such or expressed as a specific activity per mg protein. Conditioned medium from untreated HT1080 cells was used as a MMP-2 reference standard.

Western blotting

Phospho-MMP-2 levels were determined by Western blot analysis. Briefly, immunoprecipitated proteins using anti-MMP-2 antibody were loaded and separated on 8% polyacrylamide SDS-PAGE gels under reducing conditions (21). After electrophoresis (150 V, 20°C), samples were electroblotted onto a PVDF membrane by semidry technique (25 V, 30 min, Bio-Rad). Phospho-MMP-2 content in immunoprecipitated samples was identified using antiphosphoserine, antiphosphothreonine, or antiphosphotyrosine antibodies. Immunoreactive protein bands were visualized using the ECL plus detection system.

Computer rendering

3D computer models of MMP-2 showing putative phosphorylation sites were generated using PyMol (http://pymol.sourceforge.net/) and the 2.8 Å X-ray diffraction-based structure of human MMP-2 (22).

Statistical analysis

Results are expressed as mean ± SEM or means with 95% confidence intervals. Statistical analyses were performed, where appropriate, using Student’s t test or one-way analysis of variance with Tukey’s post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Human proMMP-2 (72 kDa) contains 29 potential phosphorylation sites (Fig. 1). Three putative threonine phosphor-
ylation sites are located within the autoinhibitory propeptide domain. Three putative serine, two threonine, and two tyrosine phosphorylation sites are located within collagenase-like domains-1 and -2. One threonine or tyrosine and three serine phosphorylation sites are located in the hemopexin-like domain, which is involved in binding to TIMPs and certain substrates, membrane activation, and some proteolytic activities. The other predicted phosphorylation sites (4 on serine, 4 on threonine, and 6 on tyrosine) are located in the collagen-binding domain (Fig. 1).

We first examined the phosphorylation status of 72 kDa hrMMP-2 expressed in mammalian cells empirically by 2D electrophoresis and mass-spectroscopy. Purified recombinant MMP-2 (3.2 µg) was resolved by 2D electrophoresis, resulting in 14 spots with an apparent molecular weight consistent with MMP-2, and isoelectric points ranging from pH 5.29 to 6.07 (Fig. 2A). To maximize the probability of detecting phosphorylated peptides, we analyzed the best resolved of the more acidic spots (Fig. 2A, pI=5.405) by mass-spectrometry. Evidence of at least 5 phosphorylated peptides was present in this spectrum (data not shown). Sequence analysis of the detected peptides using NetPhos 2.0 showed very high probability scores for phosphorylation of serine 160 and 365, threonine 250, and tyrosine 271. Also serine 32 was indicated as a putative phosphorylated amino acid but with a lower probability score (Fig. 2B). NetPhosK 1.0 was used to determine the kinases most likely to phosphorylate these residues. PKC had the highest probability score (for T250), followed by protein kinase A (for S160) and glycogen synthase kinase 3β (for S32). The kinases likely to be acting on Y271 and S365 were not predicted. These identified phosphorylation sites on MMP-2 and predicted amino acid targets for known kinases are shown in Fig. 2B. All five of the putative phosphorylation sites are conserved in the MMP-2 sequences of all mammals.

Figure 1. Domain structure and theoretical phosphorylation sites for human MMP-2. Potential phosphorylation sites within human MMP-2 were identified using the NetPhos prediction program. Vertical lines that extend above the demarcated threshold indicate putative amino acid phosphorylation targets, whether serine, threonine, or tyrosine. The residues discussed in the text are indicated.
for which sequence data are available, suggesting that these sites may have functional significance (Fig. 3).

We tested the ability of PKC to phosphorylate hrMMP-2 in vitro in two ways. Firstly, we treated 400 ng 72 kDa hrMMP-2 expressed in mammalian cells with either PKC or AP, and resolved the MMP-2 by 2D electrophoresis (Fig. 4A inset). Since we had used only one-eighth of the material in this experiment compared to that shown in Fig. 2A, we picked the best resolved spot which occurred at pI 5.64. We then determined the phosphorylation status of the excised spots by mass spectrometry (Fig. 4A, inset). Untreated 72 kDa MMP-2 excised from gels at pI 5.64 shows evidence of phosphorylation on S160 and S365 (Fig. 4A, top panel, and B). After treatment with AP, no evidence of phosphorylation was detected in the mass fingerprints (Fig. 4A, middle panel, and B). Treatment with PKC yielded a mass fingerprint with evidence of phosphorylation on S160, T250, S365 and T377/8 (Fig. 4A, bottom panel, and B). Secondly, we assessed the ability of PKC to phosphorylate MMP-2 using an in vitro kinase assay. We find that 64 kDa hrMMP-2 is effectively phosphorylated under these conditions (Fig. 5A).

To determine whether PKC is likely acting on MMP-2 in a biological system, we used the phorbol ester PMA as a PKC activator, which activates diglyceride-dependent α-, β-, and δ-PKC, but not γ-PKC. We analyzed proteins in the homogenates from untreated or 100 nM PMA-treated HT1080 cells, by 2D electrophoresis followed by immunoblotting with anti-MMP-2. The MMP-2 detected in 2D immunoblots of untreated HT1080 homogenates resolves as a single broad spot, whereas two broad spots of MMP-2 immunoreactivity are detected in the PMA-treated homogenates, one with more acidic pI (as indicated by an arrow), consistent with PKC-mediated phosphorylation of MMP-2 (Fig. 5B). We next sought to determine the basal MMP-2 phosphorylation status in a biological system. HT1080 cells were metabolically labeled with 32P, followed by immunoprecipitation of MMP-2 and autoradiography. Figure 5C shows basal phosphorylation of MMP-2 that is effectively inhibited by PKC blockade using bisindoylmaleimide. Immunoprecipitation control using unrelated IgG did not show any similar band (Fig. 5C).

To investigate the effects of phosphorylation on the catalytic behavior of MMP-2, we measured the kinetics of hrMMP-2-catalyzed hydrolysis using a synthetic fluorogenic substrate under various conditions. Fluorescence of this substrate, (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-
Phosphorylation of MMP-2

Historically, the most widely utilized assay for MMP-2 activity has been gelatin zymography. This approach has the advantages of sensitivity and the ability to distinguish the relative electrophoretic mobility (and therefore the identity) of the protease activities being detected, making it very well suited to the analysis of cell homogenates and other complex protein mixtures. In an effort to verify the biological relevance of our observations, we performed gelatin zymography on material immunoprecipitated from HT1080-conditioned media using phospho-specific antibodies. Antiphosphothreonine, antiphosphoserine, and to a lesser extent antiphosphotyrosine antibodies are all able to immunoprecipitate MMP-2, suggesting that MMP-2 is phosphorylated on these residues (Fig. 7A). Consistent with this, both antiphosphoserine and antiphosphothreonine (but not antiphosphotyrosine) can detect material immunoprecipitated using anti-MMP-2 antibody on Western blots (Fig. 7B). Negative controls of immunoprecipitation experiments revealed no activity on gelatin zymography and no bands on Western blots (data not shown). As gelatin zymography for MMP-2 activity is at least 100 times more sensitive than Western blotting, it is not surprising that no band for MMP-2 was observed when using anti-phosphotyrosine antibody. Finally, we determined that dephosphorylation of immunoprecipitated MMP-2 from HT1080 cell conditioned media using AP increases its gelatinolytic activity by 25-fold in zymography, consistent with our kinetic analyses of MMP-2 activity in vitro (Fig. 7C).

**DISCUSSION**

The regulation of MMPs has been the focus of intensive investigation for decades. These efforts have yielded a sophisticated understanding of the complex regulation of these proteases by several signaling pathways (9), post-translational processing by various proteases (4), and inhibition by TIMPs and RECK (REversion-inducing-Cysteine-rich protein with Kazal motifs) (25). Despite all of these investigations, the role of phosphorylation in regulating MMP activity has not been...
considered, presumably due to the focus on the extracellular activities of MMPs. Given the existence of MMP-2 splice variants lacking an N-terminal secretory signal peptide (e.g., accession number AL832088), and empirical evidence of MMP-2 protein within cells (14, 15, 18), it seems clear that the intracellular roles of these proteases, and their regulation, are important research questions. Here we present for the first time evidence that MMP-2 is phosphorylated, that its phosphorylation state modulates its enzymatic activity, and that PKC may be involved in regulating MMP-2.

Autoradiography, mass spectrometry, immunoprecipitation, 2D gel electrophoresis, and Western blot analysis all confirm the bioinformatic inference that MMP-2 may be phosphorylated on multiple residues. Several lines of evidence show that the phosphorylation state of MMP-2 modulates its activity. We determined that the kinetics of MMP-2-mediated hydrolysis of a synthetic fluorogenic substrate are significantly affected by phosphorylation. Dephosphorylation of recombinant MMP-2 had only modest effects on the kinetics of substrate hydrolysis or the rate of fluorescent gelatin dequenching. However, PKC-mediated phosphorylation resulted in significant inhibitory effects in both of these assays, suggesting that our preparations of hrMMP-2 were not highly phosphorylated. Also consistent with this is the observation that dephosphorylation of immunoprecipitated MMP-2 from HT1080 cells after incubation with 32P-H3PO4 in the absence or presence of PKC inhibitor.

![Figure 5. MMP-2 phosphorylation by PKC.](image)

**Figure 5.** MMP-2 phosphorylation by PKC. A) Autoradiography of 64 kDa hrMMP-2 after incubation with 32P-ATP with or without PKC. Radioactive labeling is detected at appropriate molecular weights showing that MMP-2 is phosphorylated by PKC. Histone H1 is used as a positive control for phosphorylation. B) Immunoblots of HT1080 cell homogenates separated by 2D electrophoresis probed with MMP-2 specific antibody. Top panel: homogenates of untreated cells yield a single broad spot of MMP-2 immunoreactivity. Bottom panel: homogenates of cells treated with 100 nM PMA (a PKC activator) produced two broad spots of MMP-2 immunoreactivity. Arrow indicates putative phosphorylated MMP-2. C) Autoradiography of MMP-2 immunoprecipitated from unstimulated HT1080 cells after incubation with 32P-H3PO4 in the absence or presence of PKC inhibitor.

![Figure 6. Kinetic analysis of MMP-2-mediated hydrolysis of a fluorogenic peptide substrate as a function of phosphorylation status of MMP-2.](image)

**Figure 6.** Kinetic analysis of MMP-2-mediated hydrolysis of a fluorogenic peptide substrate as a function of phosphorylation status of MMP-2. Kinetic data for cleavage of OmniMMP fluorogenic peptide by 64 kDa hrMMP-2 (0.2 nM) after phosphorylation with PKC (A), dephosphorylation with AP (C), or no treatment (O), fitted to the Michaelis-Menten equation by least-squares nonlinear regression analysis. Data shown are mean ± SEM of 4 replicate determinations. K_M and V_max values obtained are listed in Table 1. For regression analysis of the PKC group, only the first 6 of the 12 data points shown were included (see text).
AP treatment. Furthermore, unlike the modest effect of dephosphorylation observed in our assays using purified recombinant enzyme, dephosphorylation of MMP-2 immunoprecipitated from HT1080 conditioned medium resulted in a 20-fold increase in activity. Therefore, it seems likely that MMP-2 may be phosphorylated on multiple sites, but either only a small fraction of these sites are relevant to the enzymatic properties measured in our studies or there are multiple sites with opposing effects. Consistent with this is the observation that MMP-2 is immunoreactive with antibodies specific for at least two different phosphorylated amino acids. Elucidating which sites are responsible for the changes we have observed in this study, as well as the kinases and phosphatases responsible for their phosphorylation status, is the focus of ongoing research.

All five of the phosphorylation sites that we could confirm by mass spectrometry occur on residues with side chains accessible at the surface of the protein (Fig. 8) and are conserved in the sequences of MMP-2 from other mammals, which suggests that these may be evolutionarily constrained and, therefore, functionally relevant modifications. Parameters that phosphorylation might modulate other than substrate affinity and turnover number include substrate specificity, intracellular trafficking, protein stability, protein-protein interactions (such as with endogenous inhibitors or activating proteases, phosphatases, kinases, and/or other effectors). The majority of the predicted phosphorylation sites are within the collagen binding domain, which is an essential element for substrate binding (26–28), consistent with regulation of substrate specificity. Nevertheless all of these possibilities warrant further investigation.

Previous studies have shown a link between kinases and the regulation of MMP-2 expression, activity and secretion (27–32), although none of these considered the possibility of MMP-2 phosphorylation. We suggest that one or more kinases and phosphatases may have a role in the direct regulation of MMP-2 activity. As alkaline phosphatase can dephosphorylate MMP-2, it is tempting to speculate that alkaline phosphatases, so characteristically abundant in vascular tissue (29), may be involved in activating MMP-2 during angiogenesis.

Pathological conditions affect many enzymes involved in either phosphorylation or dephosphorylation
events, which affect the regulation of cell function. For that reason, identification of the protein kinases and phosphatases involved in the regulation of MMP-2 activity will be important to better understand the pathologies in which this ubiquitous protease is suggested to contribute, such as cancer (30), inflammation (31), ischemia-reperfusion (14), and cytokine-mediated injury to the heart (32). The finding that secreted MMP-2 is phosphorylated suggests that this may occur somewhere along its secretory pathway. As PKC localizes to caveolin-1 of the cell membrane (17) where MMP-2 very recently has been found to associate with caveolin-1 (16), suggests a possible role for this kinase in the phosphorylation of secreted MMP-2. Since the effects of phosphorylation of MMP-2 may not only affect its activity but possibly also its substrate specificity and cellular localization, this may have major implications in the design of rational drug therapies to lessen the impact of MMP-2 activation in these pathologies.

In summary we have shown that MMP-2 is phosphorylated and that its phosphorylation status modulates its activity. It is hoped that further investigations will reveal the identities of the kinases and phosphatases involved in regulating MMP-2 activity by this novel mechanism.

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REFERENCES


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