Histone H3 as a novel substrate for MAP kinase phosphatase-1

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Mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) is a nuclear, dual-specificity phosphatase that has been shown to dephosphorylate MAP kinases. We used a “substrate-trap” technique involving a mutation in MKP-1 of the catalytically critical cysteine to a serine residue (“CS” mutant) to capture novel MKP-1 substrates. We transfected the MKP-1 (CS) mutant and control (wild-type, WT) constructs into phorbol 12-myristate 13-acetate (PMA)-activated COS-1 cells. MKP-1-substrate complexes were immunoprecipitated, which yielded four bands of 17, 15, 14, and 10 kDa with the CS MKP-1 mutant but not the WT MKP-1. The bands were identified by mass spectrometry as histones H3, H2B, H2A, and H4, respectively. Histone H3 was phosphorylated, and purified MKP-1 dephosphorylated histone H3 (phospho-Ser-10) in vitro; whereas, histone H3 (phospho-Thr-3) was unaffected. We have previously shown that thrombin and vascular endothelial growth factor (VEGF) upregulated MKP-1 in human endothelial cells (EC). We now show that both thrombin and VEGF caused dephosphorylation of histone H3 (phospho-Ser-10) and histone H3 (phospho-Thr-3) in EC with kinetics consistent with MKP-1 induction. Furthermore, MKP-1-specific small interfering RNA (siRNA) prevented VEGF- and thrombin-induced H3 (phospho-Ser-10) dephosphorylation but had no effect on H3 (phospho-Thr-3 or Thr-11) dephosphorylation. In summary, histone H3 is a novel substrate of MKP-1. We propose that MKP-1-mediated H3 (phospho-Ser-10) dephosphorylation is a key regulatory step in EC activation by VEGF and thrombin.

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bin receptor activating peptide (TRAP) with the peptide sequence SFLLRN-NH₂ was custom synthesized by PepTide International (Louisville, KY), and purified phosphorylated histones were purchased from Millipore-Upstate (Billerica, MA). Other reagents and suppliers included Myc antibody (Millipore-Upstate), histone H3 antibody (Cell Signaling, Danvers, MA), histone H3 (Ser-10) antibody (Cell Signaling), histone H3 (Thr-3) antibody (Millipore-Upstate), histone H3 (Thr-11) antibody (Millipore-Upstate), phospho-serine antibody (Biodesign), phospho-tyrosine antibody PY20 (Transduction Laboratories, Lexington, KY), phospho-threonine antibody (Santa Cruz Biotechnology, Santa Cruz, CA), MKP-1 antibody (M-18AC, Santa Cruz Biotechnology), human embryonic kidney (HEK) 293, HEK 293T, and COS-1 cells. All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and transfection. Human EC were isolated by trypsin digestion of umbilical veins as previously described (47). EC were maintained on fibronectin-coated plates in MCDB/F12 medium (Sigma) containing 15% fetal bovine serum, 90 µg/ml heparin, and 150 µg/ml EC growth supplement. The cells were used between passages two and five.

COS-1 cells were transfected with the MKP-1 (WT) and MKP-1 (CS) mutant using Targefect F-2 plus the peptide enhancer from Targeting Systems (Santee, CA), according to the manufacturer’s protocol as described before (27). Experiments were performed 24-36 h posttransfection.

Western blot analysis. Cell extract from 10⁵ EC was electrophoresed on a 12% SDS-PAGE gel. Proteins were transferred from the gel to nitrocellulose using a Trans-blot semidyv transfer cell (Bio-Rad, Hercules, CA). The blot was blocked by TBST (150 mM NaCl, 50 mM Tris·HCl, pH 7.5, 0.1% Triton X-100) with 5% bovine serum albumin. Blots were incubated for 1 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody at room temperature. Protein signals were detected using an ECL Western blotting exposure kit, according to the manufacturer’s instructions (Amer sham Biosciences).

Construction and validation of MKP-1 siRNA. siRNA was constructed that targeted the following mRNA sequences: MKP-1 600, AAG AGA CGT GTA CCA GAG CAG and MKP-1 1838, AAG CTG GAC GAG GCC TTT GAG. MKP-2 siRNA were purchased from Ambion (4372 ID:4392420, Austin, TX). The siRNAs were transfected as previously described (27). Real-time PCR was performed as previously described (27) using specific primers from SuperArray Bioscience (Frederick, MD).

Substrate trapping of MKP-1. The substrate-trapping technique was performed as described by others (48), with minor modifications. To identify potential MKP-1 substrates, we mutated the catalytically critical cysteine residue of the phosphatase to serine, using site-directed mutagenesis. We transfected WT MKP-1 (provided by Dr. Jack Dixon) and CS mutant (constructed using site-directed mutagenesis kit) into 10⁵ COS-1 cells. After 24 h, cells were stimulated with 80 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 10 min. The cell pellet was lysed with 2 ml of 1× RIPA buffer (50 mM Tris·Cl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM Na₂VO₃, 1 mM NaF, 0.1% SDS, 1× Complete protease inhibitor cocktail) from Roche (Indianapolis, IN) on ice and centrifuged to remove debris. The enzyme-substrate complex was immunoprecipitated from the total cell extract using an anti-MKP-1 antibody (M18AC, Santa Cruz Biotechnology) conjugated to agarose beads. The protein complex beads were pelleted and washed three times for 5 min with RIPA lysis buffer. Bound proteins were subjected to 1× SDS lamelli sample buffer, and proteins were separated by SDS-PAGE. The gel was stained with Coomassie blue (Bio-Rad), which is compatible with subsequent mass spectrometric peptide identification.

Mass spectrometry. Protein bands were excised from the Coomassie blue-stained gel, destained, and dehydrated in acetonitrile. Gel pieces were digested with trypsin (20 µg; Promega, Madison, WI) overnight at room temperature. The eluted tryptic peptides were concentrated to 5 µl using a SpeedVac and reconstituted to 30 µl in 1% acetic acid. The tryptic peptides were analyzed by liquid chromatography/mass spectrometry using a Finnigan LTQ-Decca ion trap mass spectrometry system incorporating a 10 cm × 75 µm inner diameter Phenomenex Jupiter C18 reversed-phase capillary chromatography column (Phenomenex, Torrance, CA). Protein identification was performed by a database search using the Mascot search engine from Matrix Sciences (London, UK).

Cloning of human MKP-1 (WT) and (CS) in lentiviral vector. MKP-1 (WT) and (CS) mutant cDNAs were used as templates to polymerase chain reaction (PCR) amplification with primer sequences obtained from Integrated DNA Technologies (Coralville, IA): MKP-1 BamHI: sense, 5'-AATTTTGATCCGAGACCCAAGCTTGGTAC-3'; MKP-1 XhoI antisense, 5'-AAATTTCTGAGCTTGAATCA-CAAAGCTTCTTCAG-3'. MKP-1 (WT) and (CS) amplification products were digested with BamHI-XhoI, and the product was cloned into the lentivirus cloning vector pLenti-(XB/SC)6.9 (provided by Dr. Andrei Gudkov) using the Rapid Ligation Kit, according to the manufacturer’s protocol (Roche). Packaging plasmids CMVΔ48.2 (4 µg), VSV-G (2 µg), and lentiviral plasmids MKP-1 (WT) or (CS) (4 µg) were transfected into HEK 293T cells in 100-mm plates using lipofectamine 2000, according to the manufacturer’s instructions. Lentiviral particles were prepared as previously described (17).

Immunoprecipitation of MKP-1 substrates in threbinin- and VEGF-activated EC. For coimmunoprecipitation of MKP-1 with histones, lentiviral MKP-1 (WT) and (CS) mutant constructs were infected into 10⁶ human umbilical vein endothelial cells. EC were treated with VEGF (10 ng/ml) and TRAP (100 µM) for 15 min. EC were harvested and lysed 48 h postinfection with 1× RIPA lysis buffer (50 mM Tris·Cl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM Na₂VO₃, 1 mM NaF, 0.1% SDS, 1× Complete protease inhibitor mixture) from Roche. Cell lysates for MKP-1 and histones were precleared using agarose and control IgG pulldowns, respectively. Immunoprecipitation was performed using an anti-myc-antibody for myc-tagged MKP-1 and an anti-histone H3 antibody for histones.

In vitro dephosphorylation of histones. To determine phosphatase activity in cell extracts, myc-tagged MKP-1 (WT) and (CS) were transfected in two 150-mm dishes of COS-1 cells. Cells were harvested and lysed 48 h after transfection using lysis buffer [50 mM Tris·HCl, pH 7.6, 150 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 1% Triton X-100 (vol/vol)] containing a protease inhibitor cocktail. COS-1 cells were treated with 10 µM MAPK inhibitors: PD-98059 (ERK inhibitor), SP-600125 (JNK inhibitor), and SB-203580 (p38 inhibitor) from Calbiochem, San Diego, CA). MAPK inhibitors were added to prevent activated MAPK from binding to the active site and coprecipitating with MKP-1. Lysates were clarified by centrifugation to remove debris. The lysate was precleared using anti-mouse IgG and protein A/G agarose. Immunoprecipitation was performed using agarose-conjugated anti-myc antibody. The c-Myc Tag IP/Co-IP Kit from (Pierce) was used to isolate the immunoglobulin-bound proteins, according to the manufacturer’s instructions. For the determination of protein concentration, bound proteins were eluted from the column using a 0.1 M glycine-HCl buffer (pH 2.6) and immediately neutralized with 1 M Tris (pH 9.5). Total protein was analyzed by the Bradford method. Dephosphorylation reactions were performed using 1 µg equivalent of agarose-bound total protein incubated with 5 µg of phosphorylated acid-extracted histones (Millipore-Upstate) for 30 min at 37°C. JNK-1 (2 µg) (Millipore-Upstate) was added to the reaction to enhance the activity of MKP-1 (48). Samples were analyzed by SDS-polyacrylamide gel electrophoresis. Detection of specific phosphorylated histone bands was determined using a phospho-histone H3 (Ser 10) antibody by immunoblot.
RESULTS

Coimmunoprecipitation of histones with catalytically inactive MKP-1. We used the substrate-trapping technique to identify novel phosphorylated substrates of MKP-1. The catalytically critical cysteine of MKP-1 was mutated to serine, denoted as (CS) mutant, allowing MKP-1 substrates to bind stably in the active site without dephosphorylation. We transfected the myc-tagged MKP-1 (CS) mutant or WT constructs into COS-1 cells and then stimulated with PMA to generate phosphorylated substrates. The enzyme-substrate complex was immunoprecipitated from the total cell extract using an anti-MKP-1 antibody. As shown in Fig. 1A, immuno-blot analysis showed the MKP-1 (CS) mutant, but not MKP-1 (WT), immunoprecipitated MAPK (ERK). We performed Western blot analysis using a combination of anti-phosphotyrosine, -threonine, and -serine antibodies. Immunoprecipitate from the MKP-1 (CS) mutant-transfected cell extract contained a unique ~17-kDa protein band that was not present in the MKP-1 (WT) lane (Fig. 1B).

The immunoprecipitated protein complex from the COS-1 cells was subjected to SDS-PAGE analysis to separate the enzyme from the phosphorylated substrates. After Coomassie blue staining of the gel, we detected several specific low molecular mass bands of 17, 15, 14, and 10 kDa in the MKP-1 (CS) mutant-immunoprecipitate (IP) lane but not in the MKP-1 (WT)-IP lane (Fig. 2). The specific bands that were “trapped” using the MKP-1 (CS) mutant were excised from the Coomassie blue-stained gel, tryptic digestion was performed, and the peptide fragments were identified by mass spectrometry as histones H3, H2B, H2A, and H4.

MKP-1 and histones are localized in the nucleus, and both are known to be involved in agonist-induced transcriptional gene regulation. We have previously shown that VEGF and thrombin transiently induce MKP-1 in cultured EC (27). The identification of the histone complex using the substrate trap and mass spectrometry experiments was confirmed in EC. Immuno-blot analysis using anti-histone H3 antibody showed that MKP-1 (CS) mutant pulled down histone H3 from VEGF-treated EC extract (Fig. 3A). In the reverse experiment, we immunoprecipitated histone H3 in VEGF-stimulated EC expressing either recombinant myc-tagged MKP-1 (WT) or (CS) mutant. As shown in Fig. 3B, histone H3 antibody immunoprecipitated MKP-1 (CS) mutant but not MKP-1 (WT). MKP-1 (WT) and MKP-1 (CS) were present at comparable protein levels (data not shown). These results indicate a potential role for MKP-1 in the modulation of histone phosphorylation.

Dephosphorylation of histone H3 (Ser-10) by MKP-1 in vitro. We confirmed a direct interaction between MKP-1 and histone substrates using an in vitro assay system. In this assay, we reconstituted the dephosphorylation reaction using immunoprecipitated recombinant MKP-1 (WT) or (CS) mutant and histone H3 isolated from EC. We transfected myc-tagged MKP-1 (WT) or MKP-1 (CS) mutant plasmid constructs into COS-1 cells and immunoprecipitated MKP-1 protein using sepharose-conjugated anti-myc antibody (Fig. 4A). The core histone substrate complex was prepared by acid extraction and acetone precipitation. Phosphorylation of histone H3 (Thr-3) and histone H3 (Thr-3) was detected by immunoblot in EC under basal conditions (Fig. 4B). Purified MKP-1 (CS) dephosphorylated histone H3 (Ser-10); however, histone H3 (Thr-3) was not dephosphorylated (Fig. 4B). The catalytically inactive MKP-1 (CS) mutant did not dephosphorylate histone H3 (Ser-10) or histone H3 (Thr-3) in vitro. Furthermore, the phosphatase inhibitor sanguinarine (SA) at 50 μM for 30 min (54) blocked MKP-1 dephosphorylation of histone H3 (Ser-10) (Fig. 4B).

VEGF and thrombin cause dephosphorylation of histone H3 (Ser-10) in EC. The impact of VEGF and thrombin on histone phosphorylation in EC has not been previously reported. VEGF- and thrombin-mediated EC activation have many similar signaling mediators, target genes, and phenotypic responses (33). Our previous studies showed that MKP-1 induction by VEGF and thrombin in EC begins as early as 10 min and peaked at 1 h (7, 27). Here, we treated EC with either VEGF or TRAP, the six-amino acid peptide that mimics the action of thrombin (SFLRN), and examined histone phosphorylation state by immuno-blot analysis using specific histone antibodies. VEGF- and thrombin-stimulated EC extracts showed the dephosphorylation of histone H3 (Ser-10) at 30 min and 1 h and the phosphorylation reappeared at 3 h (Fig. 5). There was no change in the total histone H3 levels (Fig. 5).

We next asked whether the depletion of MKP-1 activity would prevent VEGF- and thrombin-induced histone H3 (Ser-10) dephosphorylation. To deplete MKP-1, we synthesized
MKP-1-specific siRNA and transiently transfected it into EC for 4 h. Thirty-two hours posttransfection the cells were treated with VEGF for 1 h. Figure 6A demonstrates that MKP-1-specific siRNA, not the scrambled siRNA, effectively depleted VEGF-induced MKP-1 mRNA. Furthermore, scrambled siRNA did not alter VEGF-induced dephosphorylation of histone H3 (Ser-10) (Fig. 6B). In addition, VEGF induced the dephosphorylation of histone H3 (Thr-3), while histone H3 (Thr-11) phosphorylation remained unaltered. MKP-1-specific siRNA prevented VEGF-induced histone H3 (Ser-10) dephosphorylation in EC but did not alter histone H3 (Thr-3) dephosphorylation (Fig. 6C).

We also investigated the role of thrombin-induced MKP-1 dephosphorylation of histone H3 (Ser-10) in EC. Similar to VEGF, MKP-1-specific siRNA blocked thrombin-stimulated histone H3 (Ser-10) dephosphorylation (Fig. 6, D and E). However, there was no effect on histone H3 (Thr-3 and Thr-11) phosphorylation profiles (Fig. 6, D and E). Therefore, MKP-1 specifically dephosphorylated phospho-histone H3 (Ser-10), but not phospho-histone H3 (Thr-3 or Thr-11), in VEGF- or thrombin-activated EC.

We recently observed that VEGF and thrombin also induced MAPK phosphatase-2 (MKP-2) with similar temporal kinetics as MKP-1 in EC (data not shown). MKP-2 is localized in the nucleus and participates in cellular responses to inflammatory agonists (1, 20, 22). MKP-2 siRNA or MKP-2 scrambled siRNA was transfected into human EC, which were then stimulated with VEGF. MKP-2 mRNA levels were significantly reduced in VEGF-stimulated EC (Fig. 7A). Furthermore, VEGF- and thrombin-treated EC were transfected with MKP-2 siRNA and analyzed for phosphorylation of histone H3 (Ser-10). No change was observed in agonist-induced dephosphorylation of histone H3 (Ser-10) in EC depleted of MKP-2, compared with EC transfected with MKP-2-scrambled siRNA (Fig. 7, B–E). We propose that thrombin and VEGF induce...
MKP-1 through the ERK and JNK pathways, respectively (27), leading to the specific dephosphorylation of histone H3 (Ser-10).

**DISCUSSION**

VEGF and thrombin via the activation of their respective receptors trigger multiple signaling cascades in EC that are critical in inflammation and blood vessel growth. In this study, we used the substrate-trap technique (15, 51) to identify histone H3 as a novel MKP-1 substrate. Furthermore, using multiple biochemical approaches, we have demonstrated for the first time that MKP-1 induced by VEGF and thrombin efficiently dephosphorylated the Ser-10 residue of histone H3 in EC.

We have showed that histone H3 (Ser-10) is transiently dephosphorylated between 30 and 60 min in EC treated with VEGF and thrombin. Though many reports have described the action of histone kinases, little is known about histone phosphatases. In this study, we also demonstrated both VEGF and thrombin cause dephosphorylation of histone H3 (Thr-3). Unlike histone H3 (Ser-10) dephosphorylation, depletion of MKP-1...
did not prevent the dephosphorylation of histone H3 (Thr-3), suggesting the involvement of alternate phosphatases in the posttranslational modification of histones in EC upon VEGF and thrombin stimulation.

The role of histone posttranslational modifications in agonist-mediated gene induction is well documented (2, 37). Epidermal growth factor (EGF)-induced CREB and histone H3 (Ser-10) phosphorylation were activated via the Ras-MAPK-ERK pathway and have been proposed to modulate aberrant gene expression in oncogene-transformed mouse fibroblast. MSK1 has also been reported to phosphorylate histone H3 (Ser-10), leading to immediate early gene induction. In addition, reports show histone H3 (Ser-10) phosphorylation correlates with immediate early gene induction of c-fos, c-jun, and MKP-1 (9, 24, 29). Phosphatases are key regulatory modulators in maintaining a tight balance over the phosphorylation status of these signaling molecules. However, only a few nonmammalian phosphatases have been identified to dephosphorylate histone H3 (Ser-10). In Drosophila melanogaster, protein phosphatase 2A (PP2A) has been linked to genome-wide dephosphorylation of histone H3 (Ser-10) and the transcriptional regulation of heat shock genes (38). Furthermore, dephosphorylation of histone H3 (Ser-10) during mitotic chromosome condensation (57) correlates with MKP-1 upregulation during the first growth phase (G1) of the cell cycle (34). These studies were limited by their reliance on pharmacological phosphatase inhibitors to identify histone phosphatases. Their limited “target” proteins focused only on protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), which were subsequently confirmed using Drosophila mutants. Our substrate-trap approach enabled us to directly identify a potential pool of substrates for MKP-1 by mass spectrometry. Although, substrate-trapping mutants can vary in their efficiencies, the MKP-1 (CS) mutant has been shown to effectively bind to substrates with low catalytic turnover (40). Our data were confirmed in EC treated with MKP-1 siRNA by immunoblot analysis of phosphorylated histones.

In eukaryote models, Ipl1/aurora family kinases, Ipl1p in Saccharomyces cerevisiae and AIR-2 in Caenorhabditis elegans regulate mitotic histone H3 (Ser-10) phosphorylation (23, 35, 42). Furthermore, two related type 1 phosphatases Glc7p in S. cerevisiae and CeGLC-7 in C. elegans are required for regulation of mitotic histone H3 dephosphorylation (23, 35, 42). Our results show agonist-mediated dephosphorylation of histone H3 (Ser-10) in human EC by MKP-1. Interestingly, MKP-1 dephosphorylated histone H3 in a site-specific manner that is consistent with previous mitosis-focused studies. Histone H3 (Ser-10) phosphorylation is critical in numerous cel-
lular functions, but it appears that this protein site may be controlled by a cohort of function-specific kinases and phosphatases (41).

Histone H3 phosphorylation and subsequent acetylation are important in the regulation of gene transcription. Histone H3 phosphorylation has been linked to the regulation of numerous genes including VEGF- and thrombin-induced genes, such as urokinase plasminogen activator, VCAM-1, and E selection (14, 24, 28, 50). We have previously reported that MKP-1 activity is critically important in the negative regulation of some thrombin-induced EC genes, such as E-selectin and VCAM-1 (7). We also demonstrated an important role for MKP-1 induction in VEGF-stimulated EC migration (27). VEGF- and thrombin-stimulated dephosphorylation of histone H3 (Ser-10) may represent a causative link in the modulation of E-selectin gene induction in EC. In agreement with our results, TNF-α-mediated MKP-1 induction via the JNK/p38 signaling pathway has been shown to be a negative regulator of E-selectin expression in EC (55). Equally, TNF-α-induced histone H3 (Ser-10) dephosphorylation correlates with MKP-1 induction in EC (55). Our results suggest that histone H3 (Ser-10) dephosphorylation by MKP-1 in VEGF- and thrombin-stimulated EC may act as a temporal repressor or regulator, to control access of the transcriptional machinery to the promoters of inflammatory genes.

Using MkII-null mice and cells, numerous reports have shown that MKP-1 is physiologically important in the regulation of innate/adaptive immunity and metabolic homeostasis. Recent studies revealed that MkII-deficient mice have inducible nitric oxide synthase-mediated hypotension when challenged with low doses of endotoxin (3). In correlative studies, MkII-1 was shown to be important in switching arginine metabolism from nitric oxide synthase to arginase following LPS challenge of peritoneal macrophages derived from WT or MkII-null mice (36). Other investigators reported increased mortality in MkII-null mice challenged with Gram-positive bacterial infection, through the inactivation of JNK and p38 (56). MkII-null mice have shown enhanced susceptibility to anaphylaxis, while maintaining sensitivity to glucocorticoids in bone marrow-derived mast cells from the same mice (32). However, MkII-1 in EC was presented as a novel and crucial inflammatory modulating switch that is finely balanced in physiological homeostasis depending on the cell-type and disease state.

Posttranslational modifications of proteins are critical for regulating VEGF and thrombin-mediated gene induction in EC. Multiple studies have shown histone H3 acetylation by p300 is critical for gene induction (6, 44). More recent reports have shown acetylation of MKP-1 by p300 inhibits innate immune signaling by blocking LPS-activated Toll-like receptor signaling (5). These results correlate with our data and support the case for the “histone code hypothesis” in regulating VEGF- and thrombin-induced genes in EC (25). The dephosphorylation of histone H3 (Ser-10) in the nucleus by the dual specificity phosphatase MKP-1 led us to consider the potential role of this mechanism in the induction of specific inflammatory genes. Chromatin immunoprecipitation-based approaches will be required to address which VEGF and thrombin-regulated EC genes are modulated by MKP-1. Our studies should help to identify new mechanisms and therapies related to MKP-1 in this newly discovered MKP-1-histone pathway.

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