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Shedding of the Urinary Biomarker Kidney Injury Molecule-1 (KIM-1) Is Regulated by MAP Kinases and Juxtamembrane Region

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ABSTRACT

Kidney injury molecule-1 (KIM-1) is markedly upregulated in renal proximal tubule cells by stimuli that promote dedifferentiation, including ischemic or toxic injury, as well as in cases of tubulointerstitial disease, polycystic kidney disease, and renal cell carcinoma. Structurally, KIM-1 possesses a single transmembrane domain and undergoes membrane-proximal cleavage, which leads to the release of soluble KIM-1 ectodomain into the urine. Urinary KIM-1 ectodomain is a promising sensitive and specific biomarker for acute kidney injury in humans, and therefore it is important to determine what regulates KIM-1 shedding. We found that constitutive cleavage of KIM-1 is mediated by ERK activation, and that cleavage is accelerated by p38 MAP kinase activation. After cleavage, a 14-kD membrane-bound fragment of KIM-1, which contains two highly conserved tyrosine residues, was tyrosine-phosphorylated. Mutagenesis studies demonstrated that the juxtamembrane secondary structure, not the primary amino acid sequence, was critical to the cleavage of KIM-1.

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Kidney injury molecule-1 (KIM-1) is a type 1 membrane protein that is not expressed in normal kidney but is markedly upregulated in the injured proximal tubular epithelial cells of the human and rodent kidney in ischemic[1] and toxic[2] acute kidney injury. KIM-1, also known as T cell Ig mucin (TIM-1)[3] and hepatitis A virus cellular receptor-1,[4] is also expressed in other conditions where proximal tubules are dedifferentiated, including renal cell carcinoma,[5] chronic cyclosporine nephrotoxicity,[6] a protein-overload model of tubulointerstitial disease,[7] and polycystic kidney disease.[8] Extrarenal functions for KIM-1 are described in the immune system, where the mouse *kim-1* gene[3] is a susceptibility locus for experimental allergic asthma,[9] and human KIM-1 (TIM-1) has been implicated in the regulation of T_H2 cytokine production. KIM-1 contains a six-cysteine Ig-like domain and a mucin domain in its extracellular region and three tyrosine residues including a predicted tyrosine kinase phosphorylation motif in the cytosolic domain. Two splice variants of the cytoplasmic domain are expressed.[10] The KIM-1a variant is mainly expressed by human liver and lacks the tyrosine-kinase phosphorylation motif, whereas the KIM-1b variant (referred to as KIM-1 hereafter) is mainly expressed by human kidney and contains two conserved tyrosine residues, including a predicted tyrosine kinase phosphorylation motif, QAEDNIY. A ligand for mouse TIM-1 has

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been reported to be TIM-4.[11] The regulated shedding of KIM-1 ectodomain should contribute to regulation of ligand binding during the reparative response of the injured proximal tubule and potentially during the T_H2 immune response as well.[10]

Many transmembrane proteins undergo proteolytic cleavage, releasing soluble extracellular domain (ectodomain), which may have autocrine and paracrine signaling functions.[12,13] Shedding is generally enhanced by phorbol ester (PMA)-mediated activation of protein kinase C, which occurs in close proximity to the cell membrane and is blocked by hydroxamate-based metalloproteinase inhibitors.[12,13] There is no apparent sequence similarity at the cleavage site of different proteins, and no minimal consensus shedding sequence has been identified.[14,15] It has been hypothesized that structural changes may allow access of a protease to a membrane-proximal region of cleaved proteins.[16]

KIM-1 is shed constitutively into the culture medium of cell lines expressing endogenous or recombinant KIM-1 by membrane-proximal cleavage in a metalloproteinase-dependent manner.[10] Soluble KIM-1 is a very sensitive urinary biomarker of human tubular injury[17,18] and is under investigation as a tissue and urinary biomarker of renal cell carcinoma.[5,19] Despite the mounting evidence of its clinical utility, the regulation and underlying mechanism for KIM-1 cleavage are poorly characterized. We undertook this study to understand the molecular mechanisms that regulate urinary levels of this important biomarker. Here, we demonstrate that KIM-1 shedding is enhanced dramatically by pervanadate, a potent inhibitor of protein tyrosine phosphatases. We show that separate mitogen-activated protein kinases (MAPKs) regulate constitutive and pervanadate-induced shedding and investigate the structural requirements for KIM-1 cleavage. We also show that a 14-kDa tyrosine-phosphorylated cell membrane–associated fragment of KIM-1 can be detected after cleavage of the KIM-1 ectodomain.

RESULTS

Pervanadate Enhances Ectodomain Shedding of KIM-1

769-P cells (human renal cell adenocarcinoma cell line) express high levels of endogenous KIM-1.[10] Pervanadate, a potent inhibitor of protein tyrosine phosphatases, enhances tyrosine phosphorylation of cellular proteins whose steady state of phosphorylation is normally reduced by phosphatases. Pervanadate was freshly prepared for each experiment by mixing H_2O_2 (0.5 M) and orthovanadate (0.5 M) and was used within 20 min of preparation.[20] Pervanadate treatment of 769-P cells results in the rapid appearance of a large amount of a 90-kDa molecule in the culture medium that is immunoreactive with antibody directed to the ectodomain of KIM-1, AKG7 (Figure 1A). Induction of KIM-1 shedding does not occur when cells are exposed to similar concentrations of either orthovanadate or H_2O_2 alone (Figure 1A). Pervanadate-induced ectodomain shedding of KIM-1 is time dependent, with increased amounts in the culture medium apparent within 15 min of exposure and reaching a peak at 30 min of incubation (Figure 1B, top). In a dosage-response study, 50 μM pervanadate achieved a maximal effect (Figure 1B, bottom).

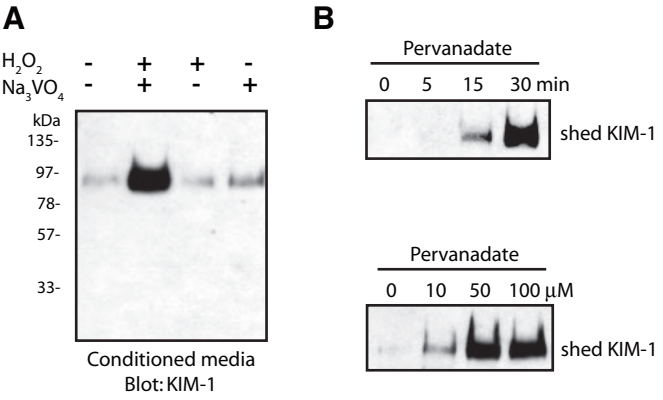

Figure 1. Pervanadate enhances ectodomain shedding of KIM-1. (A) Confluent 769-P cells were incubated in serum-free medium in the presence of either 50 μM H_2O_2 or orthovanadate (Na_3VO_4) or a combination of both (pervanadate) for 30 min. Shed KIM-1 was detected in conditioned medium using an antibody against the ectodomain of KIM-1 (AKG7). (B) Cells were incubated in serum-free medium in the presence of 50 μM pervanadate for various times (top) or incubated for 30 min with various concentrations of pervanadate (bottom), and conditioned media were subjected to Western blot analysis for assessment of shed KIM-1 using AKG7.

Pervanadate-Induced Ectodomain Shedding of KIM-1 Is Inhibited by an Oxygen Free Radical Scavenger and a Metalloproteinase Inhibitor

The underlying molecular mechanism for the inhibitory action of pervanadate on protein tyrosine phosphatases is thought to involve oxidation of an essential thiol at the active site of the enzyme, explaining why the oxygen radical scavenger pyrrolidine dithiocarbamate blocks the effect of pervanadate on tyrosine phosphorylation of cellular proteins.[21] Pyrrolidine dithiocarbamate pretreatment of 769-P cells prevented pervanadate-induced ectodomain shedding of KIM-1 in a dose-dependent manner (Figure 2A), indicating that oxidation is required for pervanadate-stimulated KIM-1 shedding and supporting the conclusion that pervanadate regulates KIM-1 shedding *via* inhibition of protein phosphatase activity. In our previous study, we reported that GM6001, a hydroxamate-based broad-spectrum metalloproteinase inhibitor, completely blocked the constitutive shedding of KIM-1 ectodomain in 769-P cells.[10] We found that GM6001 also inhibited the accelerated shedding stimulated by pervanadate in a dose-dependent manner (Figure 2B). Thus, the accelerated

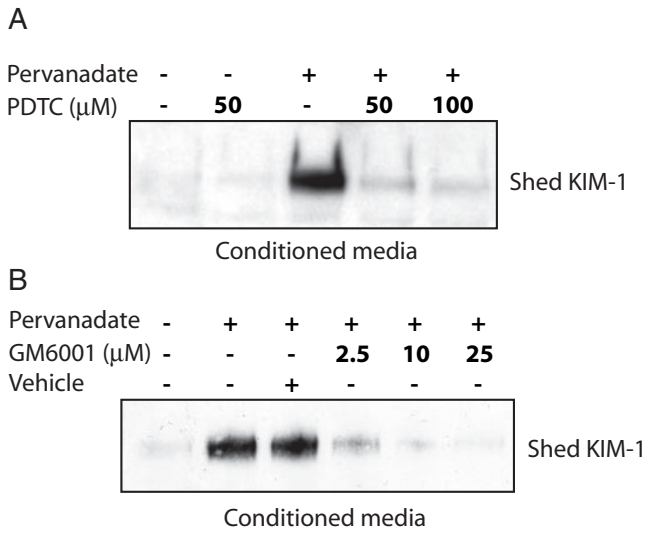

Figure 2. Pervanadate-induced ectodomain shedding of KIM-1 is inhibited by an oxygen free radical scavenger and a metalloproteinase inhibitor. Confluent 769-P cells were preincubated in serum-free medium with various concentrations of pyrrolidine dithiocarbamate (PDTC), an oxygen radical scavenger (A), or GM6001, a metalloproteinase inhibitor (B), for 30 min before the addition of pervanadate (50 μM final concentration for 30 min). Shed KIM-1 was detected as in Figure 1.

shedding induced by pervanadate is also mediated by a metalloproteinase.

p-Aminophenylmercuric Acid Induces Ectodomain Shedding of KIM-1

Because KIM-1 shedding is mediated by a metalloproteinase(s), we tested whether direct metalloproteinase activation could stimulate KIM-1 shedding. Incubation of 769-P cells with *p*-aminophenylmercuric acid (APMA), a metalloproteinase activator,[22] significantly increased the amount of shed KIM-1 present in the culture medium both time (Figure 3A, left) and dosage (Figure 3A, right) dependently. GM6001 attenuated APMA-induced ectodomain shedding (Figure 3B), supporting the conclusion that the shedding of KIM-1 ectodomain was induced by APMA *via* activation of a member of the metalloproteinase family.

A KIM-1 Splice Variant Is Also Shed

As an initial step toward defining the structural requirements of shedding, the splice variant KIM-1a, which encodes a KIM-1 polypeptide with identical extracellular and transmembrane domains to KIM-1 but a different cytosolic domain (Figure 4A), was expressed in LLC-PK$_1$ cells (porcine renal tubular epithelial cell line) that do not express detectable endogenous KIM-1 (B.D.H. and J.V.B., unpublished observations, 2002). Cells were infected with adenovirus encoding either *LacZ* (Ad*LacZ*) or KIM-1a (AdKIM-1a). The ectodomain of KIM-1a was constitutively shed into the culture medium (Figure 4B) and pervanadate

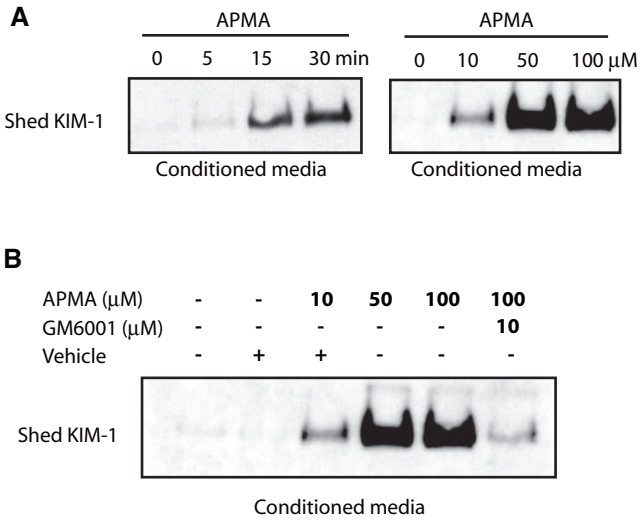

Figure 3. Activation of metalloproteinase-induced ectodomain shedding of KIM-1. (A) Confluent 769-P cells were incubated in serum-free medium in the presence of the metalloproteinase activator APMA (50 μM) for various times (left) or incubated for 30 min with various concentrations of APMA (right), and conditioned media were collected. (B) Effect of metalloproteinase inhibitor on APMA-induced ectodomain shedding of KIM-1. 769-P cells were preincubated in serum-free medium with 10 μM of GM6001 or vehicle (DMSO) for 30 min and then exposed to various concentrations of APMA for an additional 30 min. Shed KIM-1 was detected as in Figure 1.

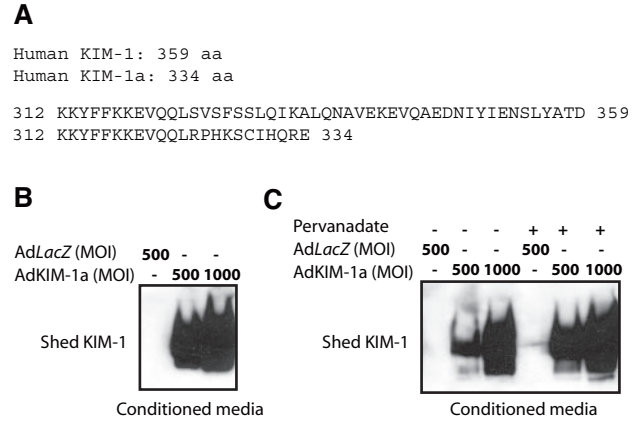

Figure 4. Constitutive and accelerated ectodomain shedding of KIM-1a, a splice variant of KIM-1. (A) Schematic representation of the cytosolic domain of human KIM-1 (359 amino acids) and the splice variant KIM-1a (334 amino acids). (B) Subconfluent LLC-PK$_1$ cells were infected for 48 h with a recombinant adenoviral vector expressing KIM-1a (AdKIM-1a) in culture medium supplemented with 2% FBS. Ad*LacZ* served as a control for infection. The multiplicity of infection (MOI) is indicated. At the end of infection, the conditioned media were collected for analysis. (C) After infection with AdKIM-1a or Ad*LacZ* for 48 h, cells were incubated in serum-free medium with 50 μM pervanadate for 30 min, and the conditioned media were assessed for shed KIM-1 as in Figure 1.

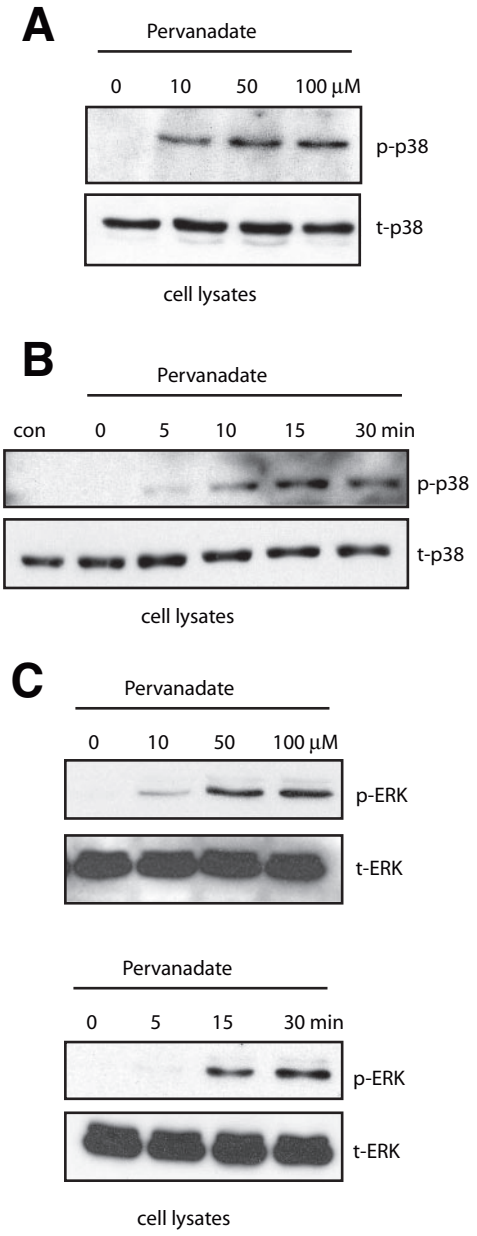

Figure 5. Pervanadate activates p38 and ERK MAPK. (A) 769-P cells were incubated in serum-free medium for 30 min with various concentrations of pervanadate, and activation of p38 in cell lysates was determined by Western blot analysis using a p38 phospho-specific antibody (top lane). The same blot was stripped and reprobed with an antibody against total p38 (bottom lane) to control for protein loading. (B) For determination of the time course for p38 activation, cells were incubated in serum-free medium in the absence (Con) or presence of 50 μM pervanadate for various times, and both activated p38 and total p38 were determined as in A. (C) Cells were incubated in serum-free medium for 30 min with various concentrations of pervanadate (top) or in the presence of 50 μM pervanadate for various times (bottom), and the activation of ERK in cell lysates was determined by Western blot analysis using a phospho-specific antibody (top lane). The same blot was stripped and reprobed with an antibody against total ERK (bottom lane).

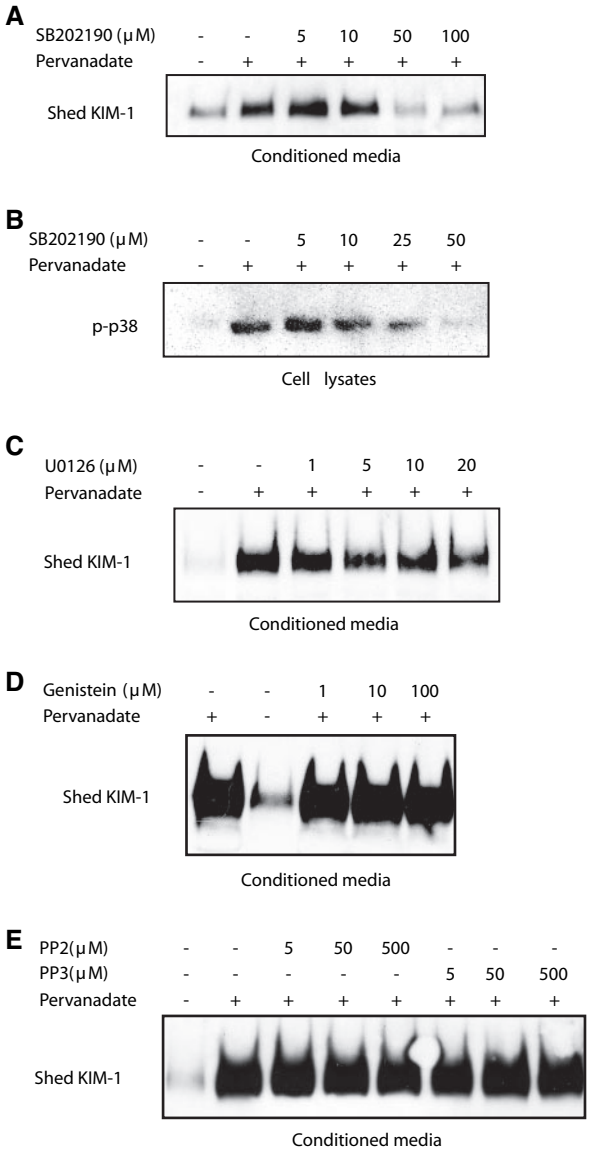

Figure 6. p38 MAPK signaling pathway regulates pervanadate-induced ectodomain shedding of KIM-1. (A) Confluent 769-P cells were preincubated in serum-free medium with various concentrations of SB202190, a p38 MAPK inhibitor, for 1 h before the addition of pervanadate (50 μM for 30 min). The conditioned media were analyzed for shed KIM-1 as in Figure 1. (B) SB202190 was added to cells in serum-free medium for 1 h and then exposed to pervanadate (50 μM) for an additional 30 min. The activation of p38 in cell lysates was determined using a phospho-specific antibody. (C through E) Cells were preincubated in serum-free medium with various concentrations of U0126 (C), genistein (D), the Src kinase inhibitor PP2 or its control PP3 (E) for 1 h before the addition of pervanadate (50 μM for 30 min), and KIM-1 shedding was assessed.

accelerated the shedding pathway (Figure 4C). Despite different cytosolic domains, shedding of KIM-1a is regulated in a similar manner to KIM-1.

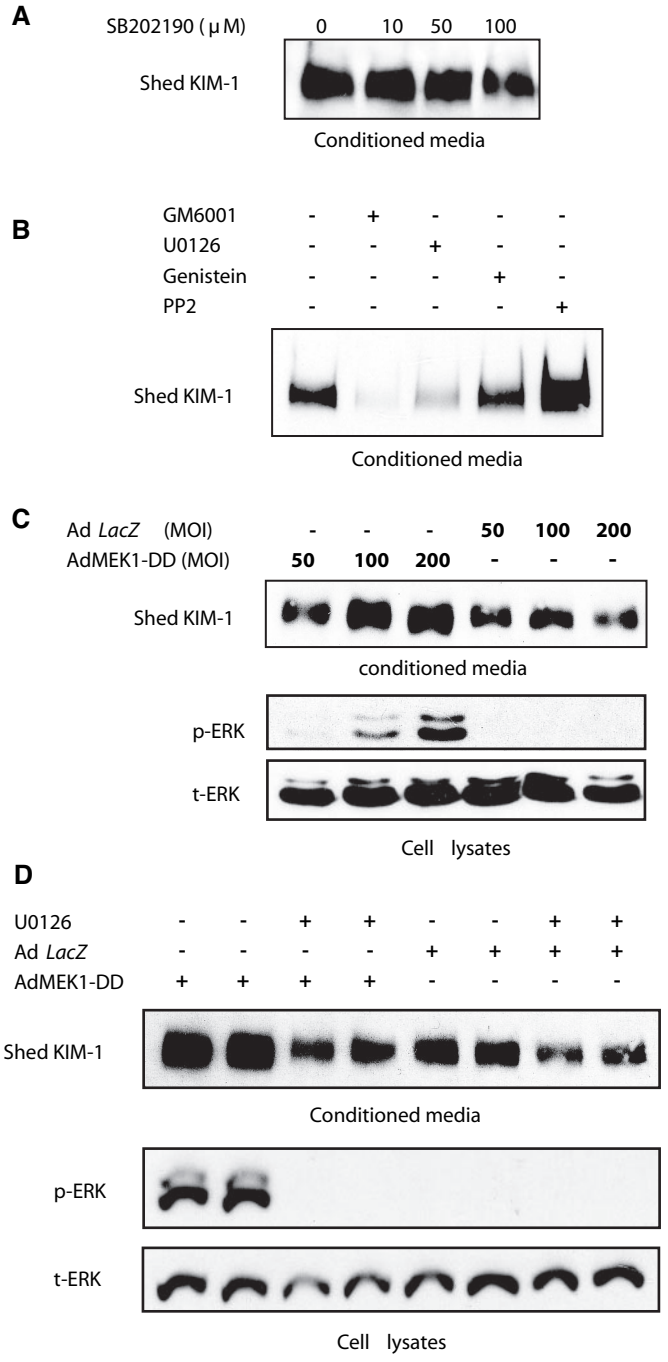

Figure 7. ERK MAPK signaling pathway regulates the constitutive shedding of KIM-1 ectodomain. (A and B) Confluent 769-P cells were maintained in medium supplemented with 2% FBS in the presence of SB202190 (A), at various concentrations, or other inhibitors (B), including GM6001 (25 μM), U0126 (20 μM), genistein (100 μM), and PP2 (100 μM). The conditioned media were collected for 24 h and then subjected to Western blot analysis for assessment of shed KIM-1. (C) For determination of whether constitutive activation of ERK MAPK would stimulate shedding, subconfluent 769-P cells were infected for 48 h with a recombinant adenoviral vector expressing MEK1-DD (AdMEK-DD; the constitutively activated MEK1 mutant) in culture medium supplemented with 2% FBS. Ad*LacZ* served as a control for infection. The MOI is indicated. After infection, cells were maintained in serum-free medium for 24 h, and the conditioned media were assessed for shed KIM-1 (top). Activated ERK (p-ERK, top lane of bottom) and total ERK (t-ERK; bottom lane of bottom) were determined as described in Figure 5C. (D) After infection with AdMEK-DD or Ad*LacZ* for 48 h, medium was changed to serum-free medium for an additional 24 h in the presence or absence of U0126 (20 μM). The conditioned media were analyzed for shed KIM-1 (top). p-ERK (top lane of bottom) and t-ERK (bottom lane of bottom) were determined as described in Figure 5C.

MAPK Signaling Pathway Regulates Ectodomain Shedding of KIM-1

The rapid increase in KIM-1 ectodomain shedding after pervanadate treatment suggests that this effect is not dependent on gene expression. Indeed, we observed that pretreatment of 769-P cells with cycloheximide, a protein synthesis inhibitor, had no effect on pervanadate-induced KIM-1 shedding (data not shown). Because the MAPK family regulates shedding in other systems, we investigated whether such signaling pathways regulate pervanadate-induced KIM-1 ectodomain shedding. Extracellular signal–regulated kinase (ERK) and p38 MAPK activation were assessed using phospho-specific antibodies that recognize only activated ERK or p38. Pervanadate treatment induced dosage-dependent (Figure 5A) and rapid p38 phosphorylation with activation by 5 min and peaking by 15 min (Figure 5B). This is well before the peak of activation of KIM-1 ectodomain cleavage at this dosage (Figure 1B). ERK activation was also observed upon pervanadate treatment (50 μM), occurring within 15 min and reaching a peak at 30 min (Figure 5C). The rapid activation of P38 and ERK suggested that these kinases might be involved in pervanadate-induced KIM-1 shedding.

SB202190, a potent and specific inhibitor of p38 MAPK, efficiently blocked pervanadate-induced ectodomain shedding of KIM-1 (Figure 6A). The inhibitory effect was observed at dosages of 10 μM and greater. The dosage-response relationship of p38 MAPK phosphorylation paralleled that of inhibition of KIM-1 ectodomain shedding with SB202190 upon treatment with pervanadate (Figure 6B). A similar dosage of this inhibitor was used in other studies to block pervanadate-mediated tyrosine phosphorylation of keratins 8 and 19 *via* a p38 MAPK pathway.[23] By contrast, the MEK inhibitor U0126 at various concentrations showed only minimal inhibition of pervanadate-induced KIM-1 shedding (Figure 6C). These experiments suggest that the pervanadate-induced ectodomain shedding of KIM-1 is mediated by the p38 MAPK pathway. Although shedding of certain transmembrane proteins is sensitive to the receptor tyrosine kinase inhibitor genistein and the Src kinase inhibitor PP2, neither of these agents had any effect on pervanadate-induced ectodomain shedding of KIM-1 (Figure 6, D and E).[24,25]

769-P cells exhibit constitutive shedding in the absence of pervanadate that is detectable with longer supernatant collection periods. Unlike its ability to block pervanadate-induced

KIM-1 shedding, basal KIM-1 shedding was unaffected by p38 inhibition (Figure 7A). Like pervanadate-induced shedding, the constitutive shedding pathway is inhibited by the metalloproteinase inhibitor GM6001 (Figure 7B). In contrast to its lack of an effect on pervanadate-induced shedding of KIM-1 ectodomain, U0126, a specific inhibitor of MEK, significantly blocked the constitutive shedding of KIM-1 ectodomain (Figure 7B), suggesting the involvement of the ERK MAPK pathway in this process. Genistein was also without effect (Figure 7B). By contrast, the Src kinase inhibitor PP2 enhanced constitutive shedding of KIM-1 ectodomain (Figure 7B). These experiments indicate that the constitutive and accelerated shedding pathways are differentially regulated.

For further examination of the role of the ERK MAPK pathway on the regulation of constitutive shedding of KIM-1 ectodomain, cells were infected with adenovirus encoding MEK1-DD (AdMEK-DD), a constitutively active upstream kinase of ERK. 769-P cells infected with AdMEK-DD had increased levels of constitutive shedding of KIM-1 compared with Ad*LacZ*-infected control cells (Figure 7C). The activation of ERK MAPK after viral infection was confirmed using anti–phospho-ERK antibody (Figure 7C). Enhanced constitutive shedding of KIM-1 with MEK1-DD is abolished in the presence of U0126 (Figure 7D, top), suggesting that the effect of MEK1-DD on constitutive shedding of KIM-1 was indeed due to activation of ERK MAPK pathway. The detection of KIM-1 shedding in conditions without ERK activation indicates that other signaling pathways are also involved in the constitutive shedding of KIM-1 ectodomain (Figure 7D).

Protein Secondary Structure in the Juxtamembrane Region Is Important for the Cleavage of KIM-1 Ectodomain

One mAb to KIM-1 (Figure 8A), ABE3, blocks constitutive cleavage, indicating that the cleavage site may overlap the ABE3 binding site.[10] We investigated whether ABE3 also blocked pervanadate-induced KIM-1 shedding, and indeed ABE3 did inhibit the accelerated shedding pathway dosage dependently (Figure 8B). A different KIM-1 mAb, ACA12, whose epitope is farther from the KIM-1 transmembrane domain than ABE3, did not inhibit KIM-1 shedding (Figure 8B).

For further characterization of the structural requirements for KIM-1 shedding, KIM-1 protein secondary structure was predicted with the NNPREDIT program (http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html). The model predicts a helical polypeptide structure in the juxtamembrane region, partially overlapping with the ABE3 binding site (Figure 9A). To distinguish whether the primary amino acid sequence and/or the secondary structure at the cleavage site is important for the cleavage of KIM-1 ectodomain, we constructed several deletion mutants as depicted in Figure 9B. These mutants, along with the wild-type KIM-1 construct, were transiently transfected into COS-7 cells that do not express endogenous KIM-1.[1] Mutant Δ246 to 273, in which a region between the end of the ACA12 binding site and part of the ABE3 binding

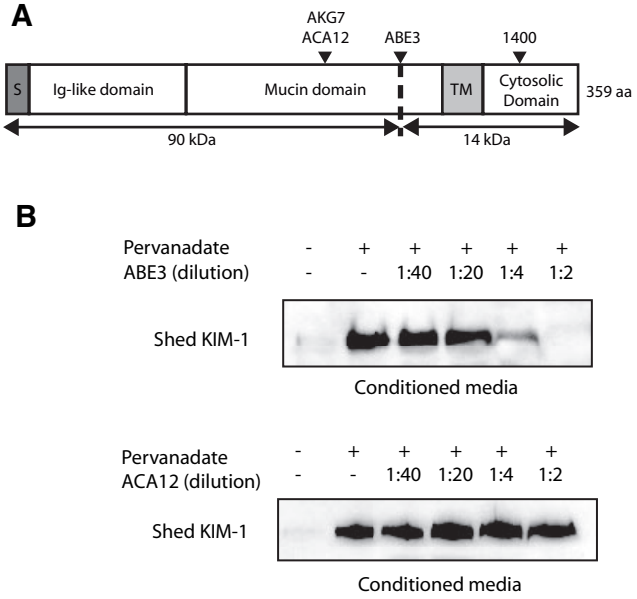

Figure 8. Antibody directed to the juxtamembrane region of KIM-1 blocks the cleavage of KIM-1 ectodomain. (A) Schematic representation of the 359–amino acid human KIM-1 protein structural domains and the relative site of KIM-1 cleavage. The approximate sites of antibody (AKG7, ACA12, ABE3, and 1400) recognition are indicated. (B) Effect of mAb against various regions of KIM-1 ectodomain on pervanadate-induced ectodomain of KIM-1. Confluent 769-P cells were preincubated in serum-free medium with various dilutions of ABE3 (top) or ACA12 (bottom) for 30 min and then exposed to pervanadate (50 μM, final concentration) for an additional 30 min. The conditioned media were subjected to Western blot analysis for assessment of shed KIM-1 using AKG7Ab. S, signal peptide; TM, transmembrane domain.

site is deleted, was robustly shed in response to pervanadate, generating an appropriately smaller shed fragment compared with the wild-type protein (Figure 9C, left). Another mutant, Δ264 to 281, in which the entire ABE3 binding site is deleted, had a similar shedding response to pervanadate (Figure 9C, left). Of note, the juxtamembrane helical structure is predicted to remain intact for both mutants (Figure 9B). By contrast, a mutant in which the helical structure is predicted to be disrupted by deletion of six amino acids just outside the ABE3 binding site (Δ278 to 283) exhibited dramatically reduced shedding in response to pervanadate with shed KIM-1 virtually absent in the conditioned medium (Figure 9C, left). The expression of KIM-1 mutants in transfected cells was confirmed in cell lysates by Western blot analysis (Figure 9C, right). The higher molecular mass mature KIM-1 band for Δ246 to 273 is reduced in comparison with the wild-type KIM-1 (Figure 9C, right), despite robust shedding of the Δ246 to 273 ectodomain (Figure 9C, left). This suggests that either the Δ246 to 273 mutant is shed more efficiently than the wild-type KIM-1 or the Δ246 to 273 mature protein is unstable. Taken together, we conclude that the secondary structure in the juxtamembrane region is critical for cleavage. Because the mutant with the least pervanadate-induced shedding also was predicted to disrupt

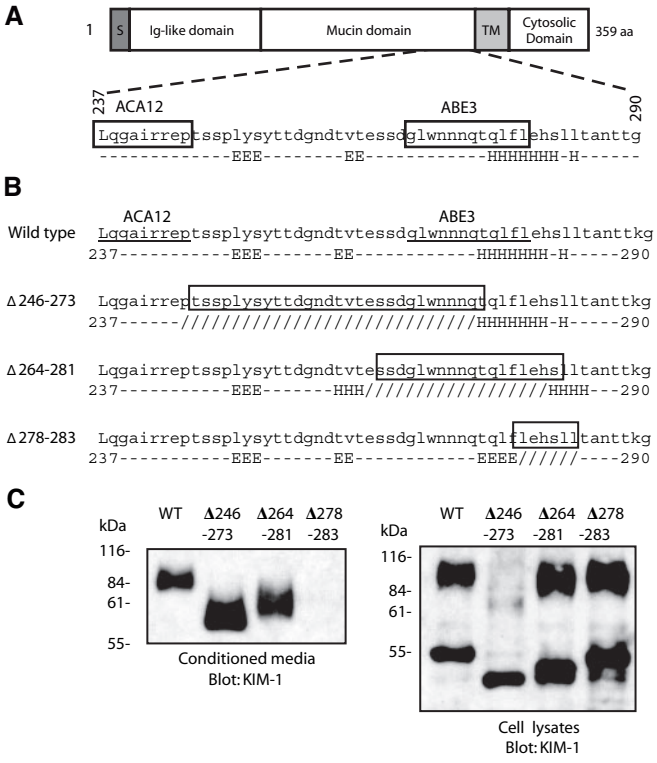

Figure 9. Protein secondary structure in the juxtamembrane region is critical for the cleavage of KIM-1 ectodomain. (A) Schematic representation of the juxtamembrane region of KIM-1 ectodomain. The sequences at the approximate sites of antibody recognition are underlined. Protein secondary structure was predicted using the NNPREDICT program and predicted helix and strand regions marked. (B) The predicted protein secondary structure in the juxtamembrane region of wild-type KIM-1 and its deletion mutants. The deleted amino acids are boxed. (C) Effect of pervanadate on ectodomain shedding of KIM-1 in KIM-1 deletion mutants. Subconfluent COS-7 cells were transfected with cDNA of either wild-type KIM-1 (WT) or its deletion mutants (Δ246 to 273, Δ264 to 281, and Δ278 to 283) for 48 h and then incubated with pervanadate (50 μM) in serum-free medium for 30 min. The conditioned media were analyzed for shed KIM-1 as in Figure 1 (left). Total KIM-1 expression was evaluated by Western blot analysis in cell lysates using AKG7 (right). *H*, helix; *E*, strand.

the juxtamembrane helical structure the most, we conclude that KIM-1 secondary structure determines the accessibility of the protease to its substrate. This conclusion is supported by the lack of effect of Δ264 to 281 on shedding even though the ABE3 antibody, which recognizes an epitope in this region, did inhibit shedding.

A 14-kDa Tyrosine-Phosphorylated and Cell Membrane–Associated Fragment of KIM-1 Is Generated after Ectodomain Cleavage of KIM-1

As shown in Figure 10A, pervanadate treatment of 769-P cells results in an increased amount of a 14-kDa fragment of KIM-1, representing the remnant transmembrane and cytosolic domain that remain after ectodomain cleavage. This fragment is

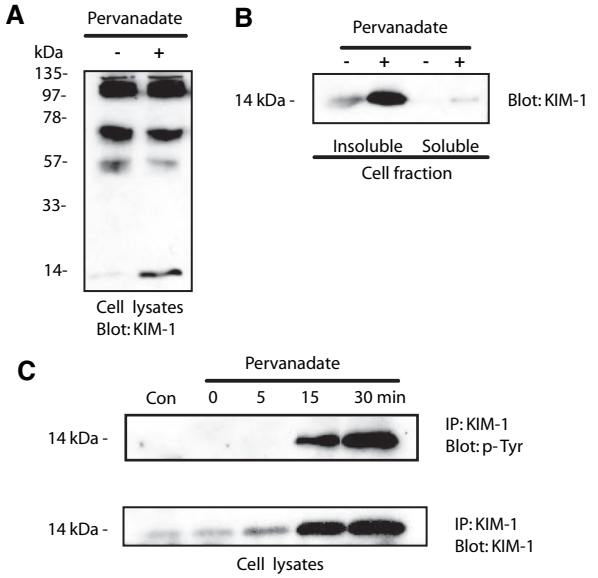

Figure 10. A tyrosine-phosphorylated and cell membrane–associated fragment of KIM-1 is generated after pervanadate treatment. (A) A 14-kDa fragment of KIM-1 is generated after pervanadate treatment. Confluent 769-P cells were incubated in serum-free medium in the presence or absence of pervanadate (50 μM) for 30 min, and total KIM-1 in cell lysates was determined by Western blot analysis using an antibody against the cytosolic domain of KIM-1 (1400). (B) The 14-kDa fragment of KIM-1 is associated with cell membranes. After incubation with or without pervanadate, confluent 769-P cells were mechanically disrupted in isotonic buffer solution without detergents and separated into soluble (cytosolic) and insoluble (membrane) fractions. Aliquots of each fraction were examined for KIM-1 protein by Western blot analysis using 1400. (C) The 14-kDa fragment of KIM-1 is tyrosine phosphorylated. Confluent 769-P cells were incubated in serum-free medium in the presence or absence of pervanadate (50 μM of final concentration) for various times. The cell lysates were immunoprecipitated using 1400 followed by Western blot analysis with an antibody against phosphotyrosine (top). The same blot was stripped and reprobed with the 1400 antibody (bottom). Control cells were kept for 30 min in serum-free medium in the absence of pervanadate.

recognized by polyclonal sera directed against the cytosolic domain of KIM-1 (1400) but not by the mAb recognizing the ectodomain of KIM-1 (data not shown). The 14-kDa fragment is associated primarily with the insoluble fraction of the cells (Figure 10B), suggesting that it remains membrane bound.

For examination of whether the 14-kDa truncated fragment is tyrosine-phosphorylated, cell lysates were immunoprecipitated with antibody 1400, followed by Western blotting with anti-phosphotyrosine antibodies. A tyrosine-phosphorylated band at 14 kDa was apparent after pervanadate treatment, and it was confirmed to be the 14-kDa fragment of KIM-1 by stripping and reprobing the same blot with 1400 (Figure 10C).

Generation of the tyrosine-phosphorylated 14-kDa fragment was stimulated by APMA, and GM6001 completely blocked formation of the tyrosine-phosphorylated 14-kDa

fragment (data not shown). Taken together, these experiments indicate that generation of the 14-kDa, tyrosine-phosphorylated C-terminal fragment is the consequence of metalloproteinase-mediated KIM-1 ectodomain cleavage. Tyrosine phosphorylation does not seem to be required for cleavage, however, because the KIM-1a splice variant, which lacks Y350 and Y356, still underwent pervanadate-induced ectodomain cleavage (Figure 4).

DISCUSSION

Although KIM-1 shedding is not stimulated by the typical sheddase activator PMA, we have demonstrated that pervanadate, a potent inhibitor of protein tyrosine phosphatases, induces a striking increase in the shedding of KIM-1 ectodomain. We speculate that physiologic stimuli, such as growth factors, may also regulate shedding *in vivo* and that pervanadate mimics this process; however, we have not identified a more physiologic stimulus to date. Pervanadate activates the shedding of a variety of cell surface proteins. Some of them, such as HER4,[26] MUC1,[27] and L-adhesion molecule,[24] are also shed in response to PMA. By contrast, the shedding of HER2,[28] betaglycan,[29] and TNF-related activation-induced cytokine[30] can be stimulated by pervanadate but not by PMA, although protein kinase C–activated shedding of other proteins was found in the cell lines tested.[28] In our previous study,[22] we reported that hydroxamate metalloproteinase inhibitors, including BB-94 and GM6001, blocked the constitutive shedding of KIM-1 ectodomain.[10] This study demonstrates that pervanadate-induced ectodomain shedding of KIM-1 is inhibited by GM6001 and activated by APMA, a general activator of metalloproteinases. Thus, both accelerated and constitutive KIM-1 shedding is mediated by a metalloproteinase.

Protein tyrosine phosphorylation is controlled by coordinate actions of protein tyrosine kinases and protein tyrosine phosphatases.[31] Growth factors, hormones, and cytokines shift the balance by rapidly stimulating protein tyrosine kinase activity, thereby inducing tyrosine phosphorylation and initiating signal transduction. However, inhibition of protein tyrosine phosphatases mimics certain aspects of signal transduction that are normally triggered by tyrosine kinase activation. Cross-talk between protein tyrosine kinase and MAPKs has been reported.[32] Pervanadate, which is a potent inhibitor of protein tyrosine phosphatases and therefore is able to sustain protein tyrosine phosphorylation-dependent events,[33] also leads to activation of MAPKs.[34,35]

MAPKs are integral to many signal transduction pathways, and pervanadate activates both ERK and p38 MAPK in 769-P cells. Because pervanadate activates p38 before KIM-1 shedding is detected and because inhibition of p38 also strongly attenuates pervanadate-induced shedding, we conclude that the p38 pathway mediates pervanadate-induced, accelerated KIM-1 shedding. Although ERK is also activated by pervanadate, the lack of effect seen with the MEK inhibitor suggests that this pathway is less important for regulation of accelerated shedding. We did find evidence that the ERK (but not p38) pathway regulates constitutive shedding of KIM-1 ectodomain. The differential regulation of constitutive KIM-1 shedding by the ERK pathway and accelerated shedding by the P38 pathway suggest that KIM-1 shedding is a physiologically important process under tight regulation. Our data contrast with TGF-α in which the basal and growth factor–accelerated shedding of TGF-α are regulated by p38 and ERK pathways, respectively,[36] suggesting that no simple rule can account for the regulation of constitutive and accelerated shedding activities of unrelated membrane proteins.

The mechanism linking MAPK pathways to the regulation of ectodomain shedding of membrane proteins has not been elucidated. TNF-α–converting enzyme, a prototype sheddase, is phosphorylated at threonine 735 by the ERK pathway,[37] and MAPKs have also been shown to mediate the expression and activation of a variety of metalloproteinases, including matrix metalloproteinases 1, 2, 3, 9, 10, and 13; membrane type 1 (MT1)-matrix metalloproteinase; and a disintegrin-like metalloproteinase domain with thrombospondin type 1 motifs.[38–43] Thus, it is possible that MAPK pathways may lead to direct activation of the metalloproteinases that mediate KIM-1 ectodomain shedding.

A mAb directed to the juxtamembrane region of KIM-1 (ABE3) effectively blocked the ectodomain shedding of KIM-1 that either occurs constitutively or is induced by pervanadate. Deleting the ABE3 epitope had no effect on shedding, however, suggesting that a mechanism other than peptide sequence might regulate metalloproteinase recognition of KIM-1. Our mutagenesis studies, based on prediction of KIM-1 secondary structure, indicate that the protein structure in the juxtamembrane region is the most important factor determining the cleavage of KIM-1. Specifically, maintenance of the helical structure in the juxtamembrane region of KIM-1 may be the critical variable for cleavage to occur, probably by regulating accessibility of the protease to its substrate. Our findings are consistent with other reports in which no apparent primary sequence similarity at the cleavage site of various transmembrane proteins or minimal consensus shedding sequence were identified.[14,15]

The functional significance of KIM-1 ectodomain shedding is unknown. There are three tyrosine residues in the cytosolic domain of human KIM-1, and two of them are conserved in mouse and rat. In this study, a 14-kDa truncated membrane-bound fragment of KIM-1, in which the tyrosine residues are phosphorylated, was generated after pervanadate treatment. The generation of this tyrosine-phosphorylated 14-kDa fragment also occurs after APMA treatment, which has no independent effect on tyrosine phosphorylation. One interpretation is that phosphorylation of tyrosine residues in the 14-kDa fragment of KIM-1 is a consequence of KIM-1 ectodomain cleavage by metalloproteinases. Previous studies have shown that ectodomain cleavage by metalloproteinases generates tyrosine-phosphorylated membrane-associated fragments

that retain tyrosine kinase activities in the Heregulin receptor ErbB-2, ErbB-4, and TrkA neurotrophin receptors.[20,44,45] Phosphorylation is an important means of propagating intracellular signals. Although KIM-1 itself does not have a kinase domain that could initiate a phosphorylation cascade, it does have a predicted tyrosine kinase phosphorylation motif, QAEDNIY, in its cytosolic domain. Tyrosine phosphorylation of KIM-1 may provide docking sites for downstream transducers and effectors to regulate cellular functions. The regulated shedding of KIM-1 may represent a mechanism to limit cell exposure to a KIM-1 ligand, and soluble extracellular KIM-1 could bind and neutralize KIM-1 ligand. Testing this hypothesis requires identification of a KIM-1 ligand in kidney.

The shedding of KIM-1 into the urine of patients with acute kidney injury is clinically significant, and elevated urinary KIM-1 levels are associated with adverse outcomes in this population.[18] In both preclinical and clinical studies using several mechanistically different models of kidney injury, urinary KIM-1 serves as an earlier and more specific diagnostic indicator of kidney injury when compared with any of the conventional biomarkers (plasma creatinine, blood urea nitrogen, glycosuria, proteinuria, urinary N-acetyl-β-D-glucosaminidase, γ-glutamyltransferase, or alkaline phosphatase).[17,46] Recently, a consortium of pharmaceutical companies the Preventive Safety Testing Consortium (PSTC), has been working on identifying and qualifying protein biomarkers for predictive nephrotoxicity in preclinical drug development. The Consortium has concluded that KIM-1 is an excellent urinary marker of nephrotoxicity when correlated with histopathology in the rodent.[47] Understanding the pathways that regulate the appearance of KIM-1 ectodomain in human urine will be informative as we further qualify KIM-1 as a useful biomarker of nephrotoxicity in humans.

We report that ectodomain shedding of KIM-1 is stimulated by pervanadate, a potent inhibitor of protein tyrosine phosphatases. The constitutive and pervanadate-induced shedding of KIM-1 is mediated by metalloproteinases and regulated by ERK and p38 MAPK, respectively. We provide evidence that the protein secondary structure in the juxtamembrane region of KIM-1 is important for its cleavage. We also demonstrate that ectodomain cleavage of KIM-1 results in generation of a truncated 14-kDa cell membrane–associated and tyrosine-phosphorylated KIM-1 fragment. Because shed KIM-1 is a sensitive urinary biomarker for kidney injury, understanding the regulation of KIM-1 shedding at the cellular level will ultimately inform our interpretation of elevated urinary KIM-1 levels in human disease.

CONCISE METHODS

Cells and Reagents

769-P cells (human renal cell adenocarcinoma, CRL-1933) and COS-7 cells were grown in RPMI supplemented with 10% FBS and maintained at 37°C in 5% CO_2. LLC-PK_1 cells (porcine renal tubular epithelial cell line, CRL-1390) were grown in DMEM supplemented with 10% FBS and maintained at 37°C in 5% CO_2. MAb against phosphotyrosine was purchased from Upstate Biotechnology (Upstate, NY). Murine mAb against the extracellular domain of KIM-1 (AKG7, ACA12, and ABE3) and rabbit polyclonal antibody against the cytosolic domain of KIM-1 (1400) have been described.[10] GM6001 (Ilomastat) was from Chemicon Int. (Temecula, CA), and APMA was from Sigma Chemical Co. (St. Louis, MO).

Western Blot Analysis

Conditioned media were collected, and cell extracts from confluent cell monolayers were prepared on ice in lysis buffer (1% Triton X-100, 20 mM HEPES [pH 7.4], 2 mM EGTA, 1 mM DTT, 50 mM β-glycerophosphate, 10% glycerol, 1 mM $NaVO_4$, 2 μM leupeptin, and 400 μM PMSF). Samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to Western analysis using standard procedures. Each experiment was repeated independently at least twice with similar results.

Construction of Recombinant Adenoviral Vectors

A recombinant adenoviral vector carrying a constitutive active mutant of MEK-1 (MEK1-DD) cDNA (AdMEK-DD) was constructed as described previously,[48] and the activation of ERK was confirmed in LLC-PK_1 cells infected with AdMEK-DD in our previous study.[49] The adenovirus carrying the *Escherichia coli LacZ* gene (Ad*LacZ*) was provided by Dr. Roger Hajjar (Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA).

For creation of an adenovirus for expression of human KIM-1a, a KIM-1a cDNA was subcloned into the shuttle vector pAdTrack-CMV. This was linearized with *Pme*I and transformed together with supercoiled adenoviral backbone vector (pAdEasy-1) into *E. coli* strain BJ5183. The recombinant adenoviral construct was amplified in DH 5α cells and plasmid purified by CsCl banding. Virus was created by transfecting *Pac*I linearized adenoviral construct into 293 cells using Lipofectamine and OptiMEM (Life Technologies, Gaithersburg, MD).

Immunoprecipitation and Phosphotyrosine Detection

Cell lysates were prepared as described previously, immunoprecipitated with antibody against the cytosolic domain of KIM-1 (1400) for 2 h on ice, separated with protein A–agarose beads (Boehringer Mannheim, Mannheim, Germany), and incubated for 1 h at 4°C with end-over-end rotation. Beads were washed with lysis buffer, resuspended in 2× SDS-PAGE sample buffer, and boiled for 5 min. Aliquots of the boiled samples were fractionated on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore, MA), and subjected to Western analysis using standard techniques.

Fractionation of Soluble and Insoluble KIM-1 Proteins

Confluent cell monolayers were washed once with ice-cold PBS; scraped; and sonicated briefly in isotonic buffer solution without detergents containing 130 mM KCL, 20 mM NaCl, 2 mM EDTA, 1 mM EGTA, 50 mM Tris-HCl (pH 7.4), 1 mM PMSF, and 10 μg/ml leupeptin on ice. The homogenates were centrifuged at 100,000 × g for 10 min at 4°C to recover the soluble and insoluble fractions in the

supernatant and pellet, respectively. Equivalent amounts of supernatant and pellet were subjected to Western blot analysis using antibody directed against the cytosolic domain of KIM-1 (1400).

Construction of KIM-1 cDNA and Mutagenesis

The coding region for human KIM-1 (amino acids 1 to 359) was generated by PCR using phKIM1.2 as template and then subcloned into the *Bam*H1 and XhoI sites of eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Deletions in the juxtamembrane region of the KIM-1 ectodomain were produced using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of all constructs were confirmed by DNA sequencing.

Mammalian Cell Transfection

COS-7 cells were seeded in 6-cm tissue culture dishes at a density of 4×10^5 cells per dish and cultured for 24 h in complete medium. The expression plasmids containing the cDNA encoding for wild-type KIM-1 or its mutants (5 μg each) were transfected into COS-7 cells using Superfect reagent (Qiagen, Valencia, CA). Empty vector (pcDNA3) served as a control for transfection. Experiments were performed 48 h after transfection.

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DISCLOSURES

Therapeutic use of KIM-1 has been very recently licensed to Biogen Idec Corp. JVB holds patents on KIM-1.

REFERENCES

1. Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, Sanicola M: Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *J Biol Chem* 273: 4135–4142, 1998
2. Ichimura T, Hung CC, Yang SA, Stevens JL, Bonventre JV: Kidney injury molecule-1: A tissue and urinary biomarker for nephrotoxicant-induced renal injury. *Am J Physiol Renal Physiol* 286: F552–F563, 2004
3. Kuchroo VK, Umetsu DT, DeKruyff RH, Freeman GJ: The TIM gene family: Emerging roles in immunity and disease. *Nat Rev Immunol* 3: 454–462, 2003
4. Kaplan G, Totsuka A, Thompson P, Akatsuka T, Moritsugu Y, Feinstone SM: Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus. *EMBO J* 15: 4282–4296, 1996
5. Han WK, Alinani A, Wu CL, Michaelson D, Loda M, McGovern FJ, Thadhani R, Bonventre JV: Human kidney injury molecule-1 is a tissue and urinary tumor marker of renal cell carcinoma. *J Am Soc Nephrol* 16: 1126–1134, 2005
6. Perez-Rojas J, Blanco JA, Cruz C, Trujillo J, Vaidya VS, Uribe N, Bonventre JV, Gamba G, Bobadilla NA: Mineralocorticoid receptor blockade confers renoprotection in preexisting chronic cyclosporine nephrotoxicity. *Am J Physiol Renal Physiol* 292: F131–F139, 2007
7. van Timmeren MM, Bakker SJ, Vaidya VS, Bailly V, Schuurs TA, Damman J, Stegeman CA, Bonventre JV, van Goor H: Tubular kidney injury molecule-1 in protein-overload nephropathy. *Am J Physiol Renal Physiol* 291: F456–F464, 2006
8. Kuehn EW, Park KM, Somlo S, Bonventre JV: Kidney injury molecule-1 expression in murine polycystic kidney disease. *Am J Physiol Renal Physiol* 283: F1326–F1336, 2002
9. McIntire JJ, Umetsu SE, Akbari O, Potter M, Kuchroo VK, Barsh GS, Freeman GJ, Umetsu DT, DeKruyff RH: Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. *Nat Immunol* 2: 1109–1116, 2001
10. Bailly V, Zhang Z, Meier W, Cate R, Sanicola M, Bonventre JV: Shedding of kidney injury molecule-1, a putative adhesion protein involved in renal regeneration. *J Biol Chem* 277: 39739–39748, 2002
11. Meyers JH, Chakravarti S, Schlesinger D, Illes Z, Waldner H, Umetsu SE, Kenny J, Zheng XX, Umetsu DT, DeKruyff RH, Strom TB, Kuchroo VK: TIM-4 is the ligand for TIM-1, and the TIM-1-TIM-4 interaction regulates T cell proliferation. *Nat Immunol* 6: 455–464, 2005
12. Hooper NM, Karran EH, Turner AJ: Membrane protein secretases. *Biochem J* 321: 265–279, 1997
13. Werb Z, Yan Y: A cellular striptease act. *Science* 282: 1279–1280, 1998
14. Pang S, Chubb AJ, Schwager SL, Ehlers MR, Sturrock ED, Hooper NM: Roles of the juxtamembrane and extracellular domains of angiotensin-converting enzyme in ectodomain shedding. *Biochem J* 358: 185–192, 2001
15. Schwager SL, Chubb AJ, Scholle RR, Brandt WF, Mentele R, Riordan JF, Sturrock ED, Ehlers MR: Modulation of juxtamembrane cleavage ("shedding") of angiotensin-converting enzyme by stalk glycosylation: Evidence for an alternative shedding protease. *Biochemistry* 38: 10388–10397, 1999
16. Althoff K, Mullberg J, Aasland D, Voltz N, Kallen K, Grotzinger J, Rose-John S: Recognition sequences and structural elements contribute to shedding susceptibility of membrane proteins. *Biochem J* 353: 663–672, 2001
17. Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV: Kidney Injury Molecule-1 (KIM-1): A novel biomarker for human renal proximal tubule injury. *Kidney Int* 62: 237–244, 2002
18. Liangos O, Perianayagam MC, Vaidya VS, Han WK, Wald R, Tighiouart H, Mackinnon RW, Li L, Balakrishnan VS, Pereira BJ, Bonventre JV, Jaber BL: Urinary N-acetyl-beta-(D)-glucosaminidase activity and kidney injury molecule-1 level are associated with adverse outcomes in acute renal failure. *J Am Soc Nephrol* 18: 904–912, 2007
19. Lin F, Zhang PL, Yang XJ, Shi J, Blasick T, Han WK, Wang HL, Shen SS, Teh BT, Bonventre JV: Human kidney injury molecule-1 (hKIM-1): A useful immunohistochemical marker for diagnosing renal cell carcinoma and ovarian clear cell carcinoma. *Am J Surg Pathol* 31: 371–381, 2007
20. Vecchi M, Rudolph-Owen LA, Brown CL, Dempsey PJ, Carpenter G: Tyrosine phosphorylation and proteolysis. Pervanadate-induced, metalloprotease-dependent cleavage of the ErbB-4 receptor and amphiregulin. *J Biol Chem* 273: 20589–20595, 1998
21. Krejsa CM, Nadler SG, Esselstyn JM, Kavanagh TJ, Ledbetter JA, Schieven GL: Role of oxidative stress in the action of vanadium phosphotyrosine phosphatase inhibitors. Redox independent activation of NF-kappaB. *J Biol Chem* 272: 11541–11549, 1997
22. Housley TJ, Baumann AP, Braun ID, Davis G, Seperack PK, Wilhelm

SM: Recombinant Chinese hamster ovary cell matrix metalloprotease-3 (MMP-3, stromelysin-1). Role of calcium in promatrix metalloprotease-3 (pro-MMP-3, prostromelysin-1) activation and thermostability of the low mass catalytic domain of MMP-3. *J Biol Chem* 268: 4481–4487, 1993
23. Feng L, Zhou X, Liao J, Omary MB: Pervanadate-mediated tyrosine phosphorylation of keratins 8 and 19 via a p38 mitogen-activated protein kinase-dependent pathway. *J Cell Sci* 112: 2081–2090, 1999
24. Gutwein P, Oleszewski M, Mechtersheimer S, Agmon-Levin N, Krauss K, Altevogt P: Role of Src kinases in the ADAM-mediated release of L1 adhesion molecule from human tumor cells. *J Biol Chem* 275: 15490–15497, 2000
25. Subramanian SV, Fitzgerald ML, Bernfield M: Regulated shedding of syndecan-1 and -4 ectodomains by thrombin and growth factor receptor activation. *J Biol Chem* 272: 14713–14720, 1997
26. Vecchi M, Baulida J, Carpenter G: Selective cleavage of the heregulin receptor ErbB-4 by protein kinase C activation. *J Biol Chem* 271: 18989–18995, 1996
27. Thathiah A, Carson DD: MT1-MMP mediates MUC1 shedding independently of TACE/ADAM17. *Biochem J* 382: 363–373, 2004
28. Codony SJ, Albanell J, Lopez TJ, Arribas J, Baselga J: Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. *Cancer Res* 59: 1196–1201, 1999
29. Velasco-Loyden G, Arribas J, Lopez-Casillas F: The shedding of betaglycan is regulated by pervanadate and mediated by membrane type matrix metalloprotease-1. *J Biol Chem* 279: 7721–7733, 2004
30. Schlondorff J, Lum L, Blobel CP: Biochemical and pharmacological criteria define two shedding activities for TRANCE/OPGL that are distinct from the tumor necrosis factor alpha convertase. *J Biol Chem* 276: 14665–14674, 2001
31. Hunter T: Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* 80: 225–236, 1995
32. Crews CM, Erikson RL: Extracellular signals and reversible protein phosphorylation: What to Mek of it all. *Cell* 74: 215–217, 1993
33. Huyer G, Liu S, Kelly J, Moffat J, Payette P, Kennedy B, Tsaprailis G, Gresser MJ, Ramachandran C: Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. *J Biol Chem* 272: 843–851, 1997
34. Daum G, Kalmes A, Levkau B, Wang Y, Davies MG, Clowes AW: Pervanadate inhibits mitogen-activated protein kinase kinase-1 in a p38MAPK-dependent manner. *FEBS Lett* 427: 271–274, 1998
35. Zhao Z, Tan Z, Diltz CD, You M, Fischer EH: Activation of mitogen-activated protein (MAP) kinase pathway by pervanadate, a potent inhibitor of tyrosine phosphatases. *J Biol Chem* 271: 22251–22255, 1996
36. Fan H, Derynck R: Ectodomain shedding of TGF-alpha and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. *EMBO J* 18: 6962–6972, 1999
37. Diaz-Rodriguez E, Montero JC, Esparis-Ogando A, Yuste L, Pandiella A: Extracellular signal-regulated kinase phosphorylates tumor necrosis factor alpha-converting enzyme at threonine 735: A potential role in regulated shedding. *Mol Biol Cell* 13: 2031–2044, 2002
38. Park MJ, Park IC, Hur JH, Kim MS, Lee HC, Woo SH, Lee KH, Rhee CH, Hong SI, Lee SH: Modulation of phorbol ester-induced regulation of matrix metalloproteinases and tissue inhibitors of metalloproteinases by SB203580, a specific inhibitor of p38 mitogen-activated protein kinase. *J Neurosurg* 97: 112–118, 2002
39. Ramos MC, Steinbrenner H, Stuhlmann D, Sies H, Brenneisen P: Induction of MMP-10 and MMP-1 in a squamous cell carcinoma cell line by ultraviolet radiation. *Biol Chem* 385: 75–86, 2004
40. Ravanti L, Hakkinen L, Larjava H, Saarialho-Kere U, Foschi M, Han J, Kahari VM: Transforming growth factor-beta induces collagenase-3 expression by human gingival fibroblasts via p38 mitogen-activated protein kinase. *J Biol Chem* 274: 37292–37300, 1999
41. Reunanen N, Li SP, Ahonen M, Foschi M, Han J, Kahari VM: Activation of p38 alpha MAPK enhances collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by mRNA stabilization. *J Biol Chem* 277: 32360–32368, 2002
42. Ruhul Amin AR, Oo ML, Senga T, Suzuki N, Feng GS, Hamaguchi M: SH2 domain containing protein tyrosine phosphatase 2 regulates concanavalin A-dependent secretion and activation of matrix metalloproteinase 2 via the extracellular signal-regulated kinase and p38 pathways. *Cancer Res* 63: 6334–6339, 2003
43. Sylvester J, Liacini A, Li WQ, Zafarullah M: Interleukin-17 signal transduction pathways implicated in inducing matrix metalloproteinase-3, -13 and aggrecanase-1 genes in articular chondrocytes. *Cell Signal* 16: 469–476, 2004
44. Cabrera N, Diaz-Rodriguez E, Becker E, Martin-Zanca D, Pandiella A: TrkA receptor ectodomain cleavage generates a tyrosine-phosphorylated cell-associated fragment. *J Cell Biol* 132: 427–436, 1996
45. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J: Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res* 61: 4744–4749, 2001
46. Vaidya VS, Ramirez V, Ichimura T, Bobadilla NA, Bonventre JV: Urinary kidney injury molecule-1: A sensitive quantitative biomarker for early detection of kidney tubular injury. *Am J Physiol Renal Physiol* 290: F517–F529, 2006
47. Dieterle F, Straedtler E, Grener O, Cordier A, Perentes E, Roth D, Mahl A, Papoian R, Grass P, Kammueller M, Legay F, Wahl D, Vonderscher J, Chibout S, Mauter G: Qualification of biomarkers for regulatory decision making: A kidney safety biomarker project [Abstract]. *Toxicologist* 96: 381, 2007
48. Choukroun G, Hajjar R, Kyriakis JM, Bonventre JV, Rosenzweig A, Force T: Role of the stress-activated protein kinases in endothelin-induced cardiomyocyte hypertrophy. *J Clin Invest* 102: 1311–1320, 1998
49. Hung CC, Ichimura T, Stevens JL, Bonventre JV: Protection of renal epithelial cells against oxidative injury by endoplasmic reticulum stress preconditioning is mediated by ERK1/2 activation. *J Biol Chem* 278: 29317–29326, 2003