Proteinase 3 is an IL-32 binding protein

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IL-32, a recently discovered proinflammatory cytokine with four isoforms, induces IL-1β, TNF-α, IL-6, and chemokines. Here, we used ligand (IL-32α) affinity chromatography in an attempt to isolate an IL-32α soluble receptor or binding protein. Recombinant IL-32α was covalently immobilized on agarose, and preparations of concentrated crude human urinary proteins were applied for chromatographic separation. A specific 30-kDa protein eluted from the column during acid washing and was identified by mass spectrometry as proteinase 3 (PR3) and confirmed by N-terminal microsequencing. PR3, a neutrophil granule serine protease, exists in a soluble or membrane form and is the major autoantigen for autoantibodies in the systemic vasculitic disease, Wegener's granulomatosis. The affinity of IL-32α to PR3 was determined by surface plasmon resonance. The dissociation constants were 2.65 ± 0.4 nM for urinary PR3 and 1.2 ± 0.05 nM for neutrophil-derived PR3. However, irreversible inactivation of PR3 enzymatic activity did not significantly change binding to the cytokine. Nevertheless, limited cleavage of IL-32 yielded products consistent with PR3 enzyme activity. Moreover, after limited cleavage by PR3, IL-32α was more active than intact IL-32α in inducing macrophage inflammatory protein-2 in mouse macrophages and IL-8 in human peripheral blood mononuclear cells. We suggest that PR3 is a specific IL-32α binding protein, independent of its enzymatic activity. However, limited cleavage of IL-32α by PR3 enhances activities of the cytokine. Therefore, specific inhibition of PR3 activity to process IL-32 or neutralization of IL-32 by inactive PR3 or its fragments may reduce the consequences of IL-32 in immune regulated diseases.

autoimmune | cytokine | inflammation | ligand affinity chromatography

Using an IL-18-induced microarray, a recent study revealed the induction of a cytokine-like molecule, IL-32 (1), originally termed natural killer cell transcript 4 (2). IL-32 induces TNF-α, IL-8, and macrophage inflammatory protein (MIP)-2 in various cells. The gene encoding IL-32 resides in chromosome 16 p13.3 and four mRNA transcripts resulting from mRNA splicing are presently known, IL-32α being the most abundant transcript in the A549 cell line. It was initially observed that T cells were the primary source of IL-32 because human peripheral blood mononuclear cells (PBMC), which contain mostly T cells, produced IL-32 after stimulation with ConA. Primary human blood monocytes stimulated with Toll-like receptor ligands synthesize the cytokine (3). After IFN-γ or IL-1β stimulation, epithelial cell lines also produce IL-32. The proinflammatory activity of IL-32 appears to take place after degradation of IκB, leading to activation of NF-κB as well as phosphorylation of mitogen-activated protein p38 (1).

Proteinase 3 (PR3), also known as myeloblastin, neutrophil PR3, and Wegener's autoantigen, is a granule serine protease present in neutrophils and monocytes capable of processing multiple biologic substrates (4). PR3 degrades a variety of extracellular matrix proteins, including elastin, fibronectin, type IV collagen, and laminin, and inactivates p65 NF-κB (5). Several prohormones and cytokines are cleaved or exhibit increased activity by PR3, including IL-8 (6), TGF-β1 (7), membrane-bound TNF-α (8), IL-1β (9), IL-18 (10), and angiotensinogen (11). PR3, which exists in a soluble and a membrane-bound form, is the major autoantigen in Wegener's granulomatosis, the most common autoimmune necrotizing systemic vasculitis in adults (12, 13). Autoantibodies to PR3, known as anti-neutrophil cytoplasmic autoantibodies, are often associated with small vessels vasculitis (14, 15).

In addition to the activity of PR3 on the cell surface and in the extracellular space, the protease also affects endothelial cells, in which it can mimic caspases by cleaving NF-κB and inducing sustained JNK activation (5). PR3 also cleaves and inactivates the major cell-cycle inhibitor $p21^{Waf1/Cip1/Sdi1}$ (16). High levels of PR3 and the p21 cleavage product were found in intestinal biopsies from patients with Crohn's disease or ulcerative colitis (16). It also appears that the role of PR3 in autoimmune disease extends beyond these functions. Patients exhibit autoantibodies to a peptide translated from a complementary (antisense) strand of the human cDNA of PR3 such that these anti-peptide antibodies in turn result in antiidiotypic antibodies. Because antiidiotypic antibodies mimic the peptide, therefore they crossreact with the autoantibody (17). These findings suggest a previously uncharacterized interpretation of autoimmune disease.

The frequency of the membrane PR3 is increased in patients with anti-neutrophil cytoplasmic autoantibody-associated vasculitis as well as in patients with rheumatoid arthritis and is, therefore, a risk factor in these diseases (18, 19). Dipeptidyl peptidase I (DPPI) is required for the full activation of neutrophil-derived serine proteases, such as PR3. PR3 knockout mice are not available, but DPPI-deficient mice have been successfully generated, and they mature normally (20). Importantly, DPPI-deficient mice are resistant to arthritis induced by anti-collagen antibodies, and joint neutrophil accumulation was not observed (20).

Gene expression profiles of peripheral neutrophils and monocytes from patients with anti-neutrophil cytoplasmic autoantibody-related kidney diseases showed increased levels of PR3 transcripts, and the expression correlated with disease activity and with glomerulonephritis (21–23). In patients with cystic fibrosis (CF), increased levels of PR3 mRNA have been reported in circulating monocytes upon exacerbation of pulmonary disease (24). Surfactant protein D is an innate host defense molecule present in the lung of CF-affected patients; it interacts with CF-associated pathogens and is a target for PR3 (25). One hypothesis is that impaired host defense against bacterial colonization in patients with CF may be due to increased proteolysis of surfactant protein D by PR3, thereby increasing the incidence of active lung infection. In patients with gingivitis and periodontitis, functional PR3 is expressed in oral epithelial cells, and anti-neutrophil cytoplasmic autoantibodies are found in the patient's serum (26).

Conflict of interest statement: No conflicts declared.

Abbreviations: PR3, proteinase 3; MIP-2, macrophage inflammatory protein; PBMC, peripheral blood mononuclear cell; PAR, proteinase activated receptors.

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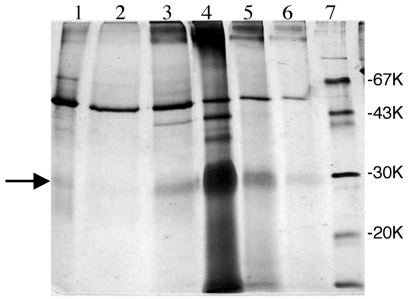

Fig. 1. Silver-stained SDS/PAGE (under nonreducing conditions) of elution fractions (60 μl) of urinary proteins from the IL-32α affinity chromatography column. Shown are the wash fraction (lane 1) representing crude urinary proteins and acid elution fractions 1–5 (lanes 2–6, respectively). Molecular mass (lane 7) is indicated on the right. The arrow indicates an IL-32 binding protein.

In the present study, we immobilized IL-32α and isolated an IL-32α binding molecule from urine of healthy humans. Unlike other urinary proteins isolated by ligand affinity chromatography, this binding protein is neither a soluble receptor nor a specific inhibitor, but it is the enzyme PR3. In addition, we describe the interaction of urinary and neutrophil-derived PR3 with IL-32α. The binding affinity between IL-32α and PR3 is high (K_d in the nM range), and it remains unclear whether this binding is due to the active site of PR3 or another site in the enzyme. Nevertheless, limited proteolysis of recombinant IL-32α by PR3 in the fluid phase enhances its biological activity modestly. Therefore, antagonizing PR3 enzymatic activity or alternatively blocking IL-32 by enzymatically inactive PR3 or fragments of PR3 may be a therapeutic strategy in diseases mediated by IL-32.

Results

Isolation of an IL-32α Binding Protein from Human Urinary Proteins. In an attempt to isolate a putative soluble IL-32 receptor, concentrated urine was applied to a column comprised of IL-32α covalently immobilized to agarose beads. After extensive washing with PBS, bound proteins were eluted at low pH. Aliquots of the various fractions were resolved by SDS/PAGE under nonreducing conditions, and the protein bands were visualized by silver staining. A broad band corresponding to a specific IL-32α binding protein with an apparent molecular mass between 28 and 32 kDa was detected mainly in the acid-eluted fraction 3 (Fig. 1). A band of this molecular mass was not observed in the wash fractions, which contain nonbinding urinary proteins.

Identification of IL-32α Binding Protein by Mass Spectrometry Analysis. Ligand affinity purified elution fraction 3 was concentrated and subjected to SDS/PAGE. Bands were visualized by silver staining and the 28- to 32-kDa protein band was excised and analyzed by liquid chromatography and tandem mass spectrometry. The sequence of three tryptic peptides was unequivocally determined as the following amino acid sequences: LFPDFFTR, VALYVDWIR, and LVNVVLGAHNVR. A search for homologous sequence using all three tryptic peptides as well as individual tryptic peptides in the protein database of the National Center for Biotechnology Information revealed that the isolated IL-32α binding protein was PR3 (SwissProt accession no. P24158).

N-terminal protein microsequence analysis further confirmed that the IL-32α binding protein is PR3 and that the N-terminal sequence Ile-Val-Gly-Gly was identical to that of PR3 purified from human neutrophils. This sequence is present in positions 28–31 of the PR3 precursor protein. In conclusion, using two

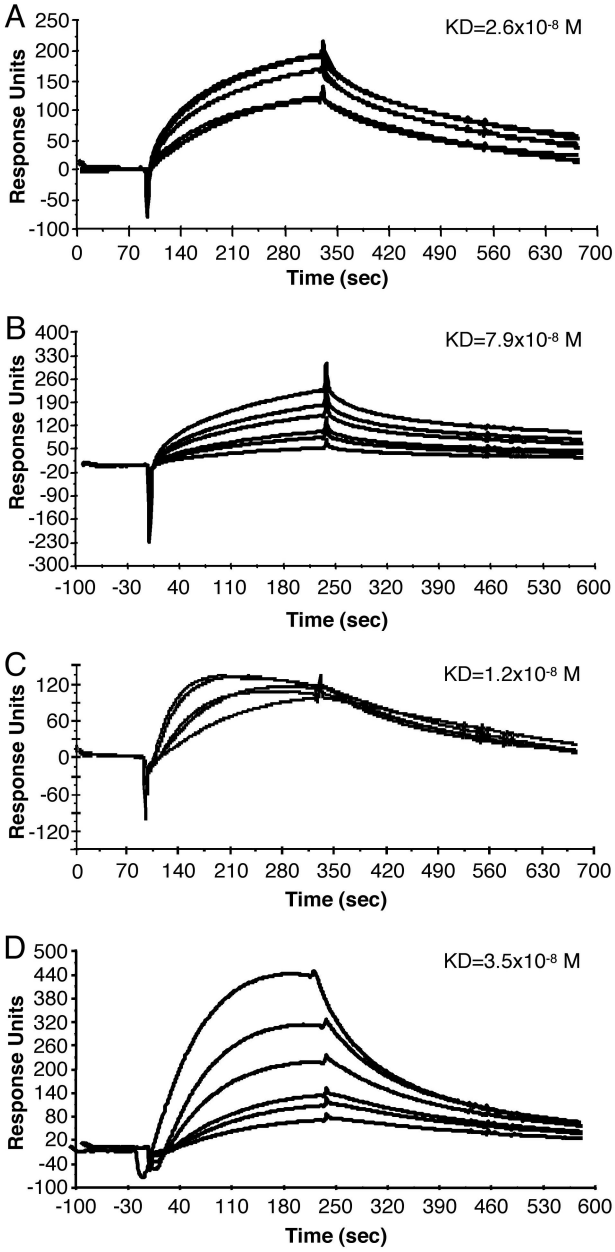

Fig. 2. Kinetics of binding of urinary and neutrophil-derived PR3 to IL-32α. (*A*) Binding of elution fraction 3 from the IL-32α affinity column at concentrations of 10, 20, 30, 40 and 80 nM to IL-32α immobilized to a BIAcore chip and analysis by the BIAcore apparatus. (*B*) The same experiment as in *A* but after inactivation of the urinary PR3 aliquot by pretreatment with PMSF. (*C*) Same analysis as *A* but with neutrophil-derived PR3. (*D*) Same as *C* but with neutrophil-derived PR3 inactivated by PMSF.

different criteria, it is clear that the urinary IL-32α binding protein of 28–32 kDa, which was isolated by ligand affinity purification, is PR3.

Binding Affinities of PR3 to IL-32α. The binding affinities of urinary or neutrophil-derived PR3 to IL-32α were measured by surface plasmon resonance. Samples containing various concentrations of affinity-purified urinary PR3 were passed over IL-32α immobilized to a BIAcore chip. The dissociation constant was determined to be 2.65×10^{-8} M (Fig. 2*A*). The experiment was repeated with PR3 that was rendered enzymatically inactive by pretreatment with PMSF. Unexpectedly, the dissociation con-

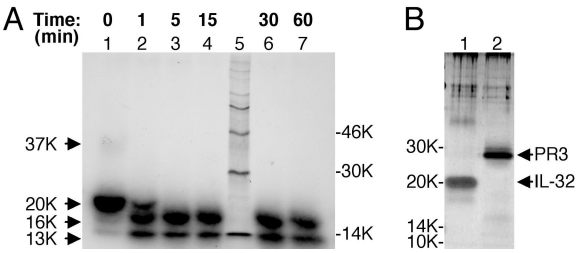

Fig. 3. Digestion of ^{125}I-IL-32α by urinary PR3. (*A*) Undigested ^{125}I-IL-32α (lane 1) and ^{125}I-IL-32α after incubation with PR3 for 1, 5, 15, 30, and 60 min at 37°C (lanes 2, 3, 4, 6, and 7, respectively). The molecular mass of cleaved products is shown by arrows on the left. Molecular mass (lane 5) is indicated on the right. (*B*) An overnight digestion of IL-32α by neutrophil-derived PR3 at 4°C. Intact IL-32α (lane 1) is compared with IL-32α incubated with neutrophil-derived PR3 (lane 2). Molecular mass is indicated on the left. Samples were resolved by 12% SDS/PAGE, and bands were visualized by silver staining.

stant did not change significantly ($K_d = 7.9 \times 10^{-8}$ M) (Fig. 2*B*). A similar analysis was performed with PR3 purified from neutrophils. The affinity was comparable with that of the urinary PR3 ($K_d = 1.2 \times 10^{-8}$ M) (Fig. 2*C*). As in the case of urinary PR3, the affinity of the neutrophil PR3 was not affected by pretreatment with PMSF ($K_d = 3.5 \times 10^{-8}$ M) (Fig. 2*D*). We conclude that PR3 derived from either urine or neutrophils have similar binding affinities to recombinant IL-32α and that binding of IL-32α to PR3 is independent of its enzymatic activity.

Kinetics of Cleavage of IL-32α by Urinary PR3. Radio-iodinated IL-32α was used to explore the catalytic activity of PR3. Affinity-purified urinary PR3 was added to ^{125}I-IL-32α and incubated for various times at 37°C. The reaction was terminated, and the samples were resolved by SDS/PAGE (Fig. 3*A*). A reduction of intact IL-32α (20 kDa) and increase in the level of IL-32α cleavage products (13 and 16 kDa) was observed as soon as after 1 min of incubation with PR3. After 5 min, the 20-kDa band was no longer visible and the appearance of the 13- and 16-kDa fragments increased and remained stable for up to 60 min. Incubation of IL-32α with PR3 at 4°C overnight resulted in a complete digestion of the 13- and 16-kDa fragments and stable bands higher than the 10-kDa marker were not observed (Fig. 3*B*). To control for the specificity of PR3 cleavage, IFN-α2 was used instead of IL-32. No cleavage products were observed (data not shown).

Comparison of Cleavage of ^{125}I-IL-32α by Urinary PR3 and Neutrophil-Derived PR3. Urinary and neutrophil-derived PR3 were each incubated with ^{125}I-IL-32α for 1 and 5 min. The reaction was terminated, and the samples were resolved by SDS/PAGE. The neutrophil-derived PR3 was more potent than the urinary PR3, because there was near complete disappearance of the 20-kDa IL-32α band within 1 min (Fig. 4, lanes 2 and 6). The performance of neutrophil-derived PR3 purchased from two sources was similar. The results of experiments performed with neutrophil PR3 from Sigma are not shown. The enhanced cleavage of IL-32α by the neutrophil-derived PR3 compared with urinary PR3 is likely due to partial inactivation of PR3 in the crude urine or during the acid elution from the IL-32α affinity column.

PMSF-Treated PR3 Does Not Cleave IL-32α. PR3 was preincubated with PMSF, a serine protease inhibitor, for 10 min at 37°C before incubation with ^{125}I-IL-32α. As shown in Fig. 5, in contrast to active PR3 (lanes 1/2 and 9/10 for urinary PR3 and neutrophil-derived PR3, respectively), no cleavage products of IL-32α were observed with PMSF-pretreated PR3 (lanes 3, 4, 7, and 8). Thus,

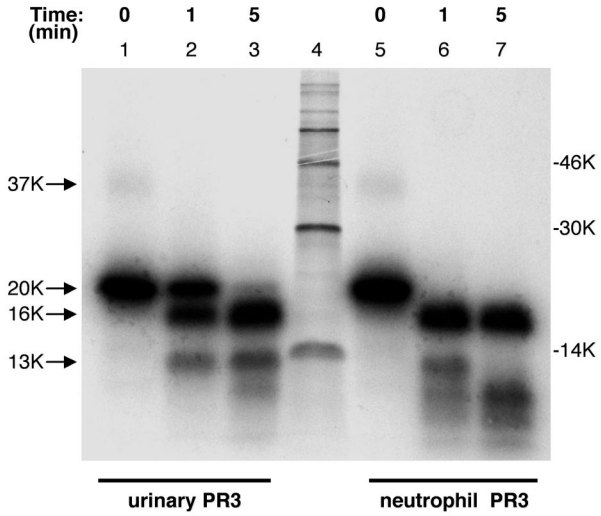

Fig. 4. Comparison of IL-32α cleavage by urinary PR3 and neutrophil-derived PR3. Urinary PR3 (lanes 1–3) or neutrophil-derived PR3 (lanes 5–7) was incubated with ^{125}I-IL-32α for 0, 1, and 5 min or undigested ^{125}I-IL-32 (lanes 1 and 5). The size of cleavage products is shown by arrows on the left. The molecular mass (lane 4) is shown on the right.

the digestion of IL-32α after incubation with PR3 is due to the proteolytic activity of the enzyme.

The Cleavage Site of PR3. The N-terminal sequence of PR3 from both neutrophils and urinary sources, the similar binding affinities to IL-32α, and the similar capacity to cleave IL-32α established that the urinary PR3 and the neutrophil-derived PR3 are identical. N-terminal amino acid sequencing of the IL-32α fragments derived by limited proteolysis revealed that PR3 cleaves between Thr-57 and Val-58 of the IL-32 α and β isoforms, but that PR3 cleaves between Thr-103 and Val-104 for IL-32γ and between Thr-47 and Val-48 for IL-32δ (Fig. 6). The IL-32γ cleavage site by PR3 is shifted to Val-104 because of insertion of 46 amino acids from mRNA splicing. The second exon contains the start codon but this exon is deleted in IL-32δ, resulting in a frame shift in the start codon to exon 3 (1). Thus, IL-32δ has a PR3 cleavage site in the position between Thr-47 and Val-48. The schematic drawing of the PR3 cleavage site of each of the IL-32 isoforms is shown in Fig. 6.

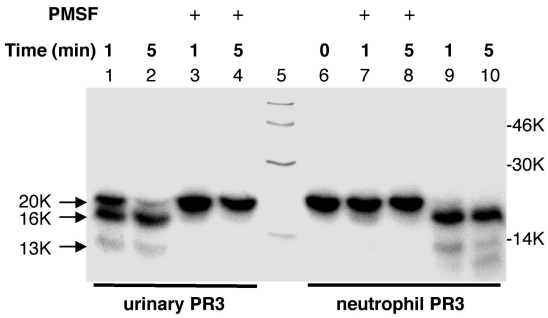

Fig. 5. PMSF-treated PR3 does not cleave IL-32α. Undigested ^{125}I-IL-32α is shown in lane 6. Lanes 1–4 show ^{125}I-IL-32α after incubation with urinary PR3 for 1 and 5 min. Lanes 1 and 2 show PR3 not pretreated with PMSF, and lanes 3 and 4 show PR3 pretreated with PMSF. Lanes 7–10 show ^{125}I-IL-32α after incubation with neutrophil-derived PR3 for 1 and 5 min. Lanes 7 and 8 show PR3 pretreated with PMSF, and, in lanes 9 and 10, PR3 is not pretreated. The sizes of the cleavage products is shown by arrows on the left. Molecular mass markers in kDa (lane 5) are indicated at the right.

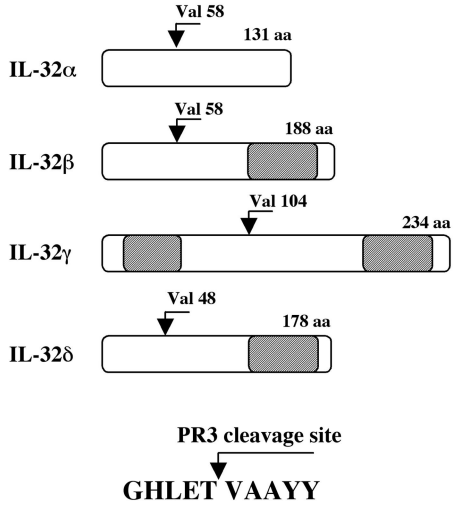

Fig. 6. PR3 cleavage sites in IL-32 isoforms. The arrows indicate the positions of the corresponding valine for each isoform shown above each IL-32 isoform. IL-32 isoforms β, γ, and δ have an additional 57 aa in the C terminus (hatched area) because of the lack of splicing between exons 7 and 8. The hatched area in the N terminus of isoform γ indicates the insertion of 46 aa by a similar splicing event between exons 3 and 4 (1). The arrow at the bottom indicates the specific cleavage site by PR3 between threonine and valine (the sequences of 10 amino acids are shown).

The Effect of PR3 on the Biological Activity of IL-32α. The effect of PR3 on recombinant IL-32α activity was assessed by using bioassays of IL-32α-induced MIP-2 in the mouse macrophage Raw cell line and IL-8 in PBMC (1). Recombinant IL-32α (20 ng/ml and 200 ng/ml) was mixed with PR3 (10 ng/ml and 100 ng/ml) in the presence of polymyxin B (1 unit/ml) at room temperature. After 30 min, all components were added to Raw cells or to PBMC. Treatment of the lower concentration of IL-32α with PR3 enhanced the ability of IL-32α to induce MIP-2 and IL-8 by 3-fold (Fig. 7) as compared with the induction of these cytokines by recombinant IL-32α incubated with saline and similarly treated. The addition of PR3 to Raw macrophages or PBMC without IL-32α did not affect the basal level of these cytokines after 24 h of incubation. In addition, a modest increase in the IL-32-induced TNF-α from Raw macrophages was observed after a pretreatment of IL-32 with PR3 (data not shown).

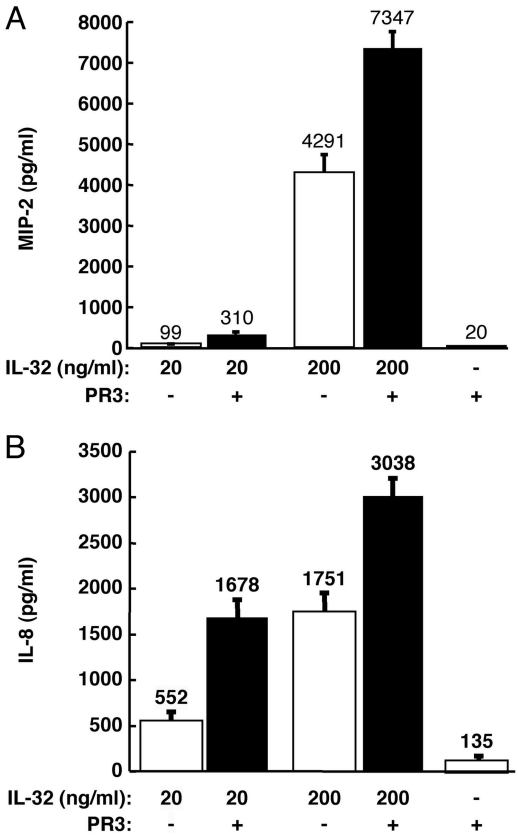

Fig. 7. Enhanced biological activity of IL-32α after incubation with PR3. (*A*) Induction of MIP-2 by mouse Raw 264.7 cells stimulated with intact recombinant IL-32α or IL-32α preincubated for 30 min with PR3. The basal level of MIP-2 (225 pg/ml) was subtracted. (*B*) Induction of IL-8 in PBMC incubated with IL-32α as described in *A*. The basal level of IL-8 (625 pg/ml) was subtracted.

Discussion

A putative cell surface or intracellular receptor for the cytokine IL-32 (1) is not known. Upon contact with macrophages, recombinant forms of IL-32 induce several proinflammatory cytokines through characteristic surface receptor-mediated signaling pathways, such as NF-κB and p38 mitogen-activated protein kinase. In the present study, we sought to isolate a soluble (extracellular) form(s) of the putative IL-32 receptor. Using a one-step isolation and purification procedure, IL-32 ligand-affinity chromatography, we isolated an IL-32α binding protein from a concentrated preparation of crude urinary proteins. Mass spectrometry analysis identified the isolated major IL-32α binding molecule as PR3. Although in the past the combination of a rich source of proteins (crude urine), together with a highly specific isolation method (ligand-affinity chromatography), yielded soluble cytokine receptors (27–30) or binding proteins (31), in the present study, we did not isolate a soluble receptor but rather a proteolytic enzyme that appears to cleave the ligand (IL-32) into a more active form.

A possible explanation that a putative soluble receptor for IL-32α was not isolated from the urine is that its size is too large to be filtered through the glomerulus. In fact, the soluble leptin receptor with a molecular mass of 130 $\pm$ 10 kDa is not found in the urine (32). Another explanation is that the affinity of the putative soluble receptor to IL-32 is low compared with the affinity of PR3 for IL-32. In addition, if the concentration of any soluble receptor in the crude urine preparations is low, the efficiency of ligand-affinity chromatography is reduced. Nevertheless, the binding of IL-32α to urine- or neutrophil-derived PR3, even in the presence of inhibitory concentration of PMSF, supports the specificity of PR3 as a binding protein for IL-32α. However, it is also clear that PR3 can enzymatically cleave IL-32α when exposed to the recombinant cytokine in the liquid phase. It is unclear, however, whether cell-associated PR3 functions primarily as a binding protein for endogenous IL-32α or cleaves IL-32α, resulting in the generation of biologically active fragments.

Cumulated data indicate that PR3 can have direct effects on intracellular processes in the absence of proteolytic activity. For example, PR3, rendered enzymatically inactive by α1-proteinase inhibitor, has been shown to induce IL-8 at the transcriptional and translational level (33). Also, a secreted, inactive form of PR3 (a complex of PR3 and the serine proteinase inhibitor α1-antitrypsin), as well as an enzymatically silent mutant of PR3, were shown to down-modulate DNA synthesis in normal hematopoietic progenitor cells (34). This effect was reversed by the presence of granulocyte–macrophage colony-stimulating factor, implying that PR3 can function as a counterbalance to regulators of proliferation (34). In addition, enzymatically inactive PR3 induced apoptosis of bovine pulmonary artery endothelial cells

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(35). PR3 fragments generated by deletion of the catalytic triad are enzymatically inactive but induce apoptosis in human umbilical vein endothelial cells (36). Therefore, PR3 seems to be a multifunctional protein influencing cell cycle, differentiation, and cell death.

In an attempt to dissect the binding capability of PR3 from its enzymatic activity, the BIAcore binding was performed with PMSF-treated PR3. PMSF, a serine protease inhibitor, inactivates PR3 (37). No significant change in the binding affinity of either urine- or neutrophil-derived PR3 to IL-32α was observed after inactivation by PMSF. The rapid turnover of substrates bound to the active site of enzymes exclude the possibility of isolating enzymes by binding to their immobilized substrates. In the present study, the enzyme PR3 was isolated because of its binding to its "substrate," IL-32α. Therefore, it is likely that the binding of PR3 to the immobilized IL-32α represents the nonenzymatic interaction of enzyme to substrate and supports the concept that the binding of IL-32α and the processing of IL-32α are two separate properties of PR3. The nonenzymatic role of PR3 as an inhibitor of IL-32α activity similar to other cytokine-binding proteins (31) is yet to be established.

A role for PR3 in cytokine-mediated disease may be due to its enzymatic property, by which PR3 activates the proteinase-activated receptor-2 (PAR-2). PAR-2 is found in many tissues where it participates in proinflammatory and pathologic roles. For example, in mice deficient for PAR-2, surgical trauma-induced, leukocyte-mediated endothelial inflammation is reduced (38). PAR-2 appears to play an essential role in models of arthritis. Inflammatory arthritis is significantly decreased in PAR-2-deficient mice, and PAR-2 agonists induce joint inflammation (39). PAR-2-specific activating peptides induce colonic granulocyte infiltration and elevated T helper type 1 cytokines but not in PAR-2-deficient mice (40). Other investigators have reported a significant participation of PAR-2 in airway secretion and inflammation (41) and in infectious colitis (42). As its name connotes, PAR-2 is activated by proteases: Trypsin, mast cell chymase, and PR3 each activate PAR-2 and result in downstream inflammation.

Cytokine-induced inflammation is also linked to PR3 and PAR-2 activation. For example, in cells activated by agonistic anti-PR3 antibodies, chemokines IL-8 and monocyte chemoattractant protein-1 are readily produced, but transfection with small interfering RNA specific for PAR-2 markedly reduced the production of these inflammatory chemokines (26). In addition to agonist anti-PR3, several proinflammatory cytokines, such as IL-1 and TNF, increase membrane PR3 and soluble PR3 (26). The cytokine-induction of PR3 results in cleavage and activation of PAR-2 with its downstream proinflammatory effects. IFN-γ is a particularly potent inducer of PR3 activity in epithelial cells (26) and IFN-γ-induced epithelial cell PR3 also cleaves the inactive IL-18 precursor into an active cytokine (10). Therefore, it is possible that the induction of IL-32 in IFN-γ-stimulated epithelial cells (1) includes the induction of active PR3 (26), with subsequent limited proteolysis resulting in increased IL-32 activity.

Because most of the IFN-γ-induced PR3 is membrane bound (26), it is an attractive hypothesis that cleavage and increased activity of IL-32 described in this report take place on the cell membrane. It is likely that IL-32 is first inserted into the plasma membrane via three putative myristoylated side groups (1), followed by PR3-mediated cleavage. Lacking signal peptides, myristoylated cytokines, such as IL-1α and TNF-α, are found as membrane-inserted proteins, where they are biologically active (43–45). Membrane IL-1α is cleaved by a calcium-activated calpain (46), and membrane TNF-α is cleaved by serine proteases, both resulting in the release of the cytokines into the extracellular space. In the present study, limited proteolysis of soluble recombinant IL-32α resulted in the formation of two peptides of 16 and 13 kDa. When these fragments of IL-32 were assessed for biological activity, there was an increase in the induction of MIP-2 and IL-8 from mouse and human monocytes, respectively. Extended cleavage of IL-32 by PR3 destroyed the cytokine. It is possible that membrane IL-32 is oriented so that membrane PR3 cleaves the cytokine by limited proteolysis. A similar limited proteolysis by serine proteases of membrane TNF-α also exists (8).

Materials and Methods

Reagents. PR3 was purchased from two different sources: Athens Research and Technology (Athens, GA) and Sigma. IFN-α2 was from PeproTech (Rocky Hill, NJ). Tobacco etch virus enzyme was purchased from Invitrogen. Anti-PR3 monoclonal antibody was purchased from Biocompare (Foster City, CA).

Recombinant IL-32α. Human recombinant IL-32α and AIL-32γ were prepared as reported in ref. 1. Briefly, recombinant IL-32α and IL-32γ were expressed in *Escherichia coli* (DH5α) with an N-terminal His tag and purified by a three-step purification procedure as described in ref. 1.

Isolation of a Urinary IL-32α Binding Protein. Recombinant IL-32α (3 mg) was immobilized by coupling to Affigel-15 beads according to manufacturer's instructions (Bio-Rad). Batches of 500 ml of crude urinary proteins concentrated 1,000-fold were passed over the IL-32α-bound beads at 4°C. The column was washed with 250 ml of phosphate buffer containing 0.65 M NaCl, pH 7.4. Bound proteins were eluted in 1-ml fractions with 25 mM citric acid, pH 2.2, solution containing 1 mM benzamidine, and each fraction was immediately neutralized.

Mass Spectrometry. Elution fraction 3 from the IL-32α ligand-affinity chromatography was concentrated with an Amicon Ultrafree-MC 10,000 NWML filter unit (Millipore) and subjected to SDS/PAGE, and the bands were visualized by silver staining. The protein band corresponding to 28–32 kDa was excised from the gel, and the proteins were electro-eluted and digested with trypsin. The resulting tryptic digest was subjected to liquid chromatography and tandem mass spectrometry (Smoler Protein Center, Technion, Haifa, Israel).

Surface Plasmon Resonance. Recombinant IL-32α (20 μg/ml) was immobilized on a single channel of a BIAcore chip as recommended by the manufacturer (Amersham Pharmacia). Aliquots of elution fraction 3 from the IL-32α affinity column containing urinary PR3 were brought to concentrations of 10, 20, 30, 40 and 80 nM and analyzed by the BIAcore system, and the binding constants were determined. The same analysis was performed with purified neutrophil-derived PR3. The binding was repeated with urine-derived PR3 and neutrophil-derived PR3 that were inactivated by 1 mM PMSF for 10 min at 37°C before binding to the chip-immobilized IL-32α.

Radioiodination of Recombinant IL-32α. Recombinant IL-32α (15 μg) was iodinated with the chloramine T method. Chloramine T (50 μl, 1 mg/ml in H_2O) and 1 mCi (1 Ci = 37 GBq) ^{125}I-Na (10 μl) was incubated for 20 s at 4°C. The mixture was added to a preparation of IL-32α for an additional 20 s at 4°C. The addition of 5 mg/ml sodium metabisulfite and 5 mg/ml potassium iodide terminated the reaction. Radioactively labeled IL-32α was separated from free iodine on a column of Sephadex G25 (Amersham Pharmacia) equilibrated with 0.25% gelatin in PBS. Peak fractions of labeled IL-32α with a specific activity of $\approx 2 \times 10^5$ cpm/ng were used.

Cleavage of Radioiodinated IL-32α by PR3. Either affinity-purified urinary PR3 (elution fraction no. 3, 50 μl) or neutrophil-

derived PR3 were added to ^{125}I-IL-32α (250,000 cpm in 10 μl of gelatin solution) and incubated at 37°C for increasing periods of time. In some experiments, PR3 was preincubated with 1 mM PMSF for 10 min at 37°C before the incubation with ^{125}I-IL-32α. The reaction was terminated by boiling for 10 min in SDS/PAGE loading buffer, and samples were resolved by 15% SDS/PAGE under reducing conditions. The gel was dried and autoradiographed.

Bioassays of IL-32. Mouse macrophage Raw 264.7 cells were used as described in ref. 1. For an experiment, 5×10^5 cells per milliliter were seeded in 96-well plates (0.1 ml per well) and cultured for 24–48 h. The medium was removed, and the cells were stimulated with fresh medium containing IL-32 in the presence of 1 unit/ml polymyxinB (Bedford Laboratories, Bedford, OH). After 16–20 h, the supernatants were harvested and assayed for cytokine levels (see below). PBMC were isolated from healthy donors (approved by the Combined Colorado Investigational Review Board) as described in ref. 3. Cells were seeded in 0.2-ml volumes at 5×10^5 cells per well in 96-well plates. The cells were stimulated with IL-32 for 24 h, and the supernatants were assayed for cytokines. Cytokine levels were determined as described in refs. 47–49 by using an M16 Analyzer (BioVeris, Gaithersburg, MD).

Cleavage of IL-32α by PR3 and Determination of N-Terminal Sequence of the Fragments. Recombinant IL-32α (10 μg) was incubated with PR3 (5 μg) for 30 min in 20 mM phosphate buffer, pH 8, at 4°C. Loading buffer containing 2-mercaptoethanol was added, and the sample was boiled for 10 min. The sample was applied to a 10% acrylamide gel and, after electrophoresis, transferred to a polyvinylidene difluoride membrane. The membrane was stained, and the bands corresponding to the cleaved-IL-32α fragments were excised for N-terminal microsequence analysis (National Jewish Research Center, Denver, CO).

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