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In Vitro Processing of Human Tumor Necrosis Factor-α*

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Sylvie Robache-Gallea‡, Valérie Morand‡, J. Michel Bruneau‡, Bernard Schoot§, Eric Tagat‡, Evelyne Réalo‡, Salem Chouaib¶, and Sergio Roman-Roman‡‖

From the ‡Domaine Thérapeutique Immunologie and §Recherche Centrale, Roussel Uclaf, 102 route de Noisy, 93230 Romainville, France and ¶INSERM CJF 9411 Institut Gustave-Roussy, 39 rue Camille Desmoulins, 94805 Villejuif, France

Tumor necrosis factor (TNF)-α is initially synthesized as a membrane-bound, cell-associated 26-kDa protein that is further cleaved to yield the soluble 17-kDa form. By using a radiolabeled *in vitro* translated TNF-α precursor we detected a serine proteinase processing activity present in crude membrane preparations of monocytic cells able to generate a 17-kDa active protein. A similar processing pattern was obtained using purified neutral serine proteinase proteinase-3 (PR-3). Moreover, while a secretory leukocyte proteinase inhibitor (a natural serine anti-proteinase) did not affect the *in vitro* TNF-α processing, IgG preparations containing high titers of anti-PR-3 autoantibodies completely blocked this activity. The NH_2-terminal sequencing of the reaction products obtained with either membrane preparations or PR-3 showed that cleavage occurs in both cases between Val^{77} and Arg^{78}. These results together with cellular expression and localization of PR-3 suggest a potential role for this enzyme as an accessory TNF-α processing enzyme.

Initially described for its anti-tumor activity (1), tumor necrosis factor (TNF)[1]-α is actually a pleiotropic cytokine that plays a key role as mediator of inflammation and cellular immune response (2). This cytokine has been shown to be involved in the pathology of diseases such as septic shock, cancer, AIDS, rheumatoid arthritis, or malaria (3, 4). The gene for TNF-α encodes for a surface transmembrane biologically active 26-kDa precursor, that is subsequently cleaved to release the 17-kDa soluble protein (5). It has been suggested that the membrane-bound form of TNF-α can be implicated in the paracrine activities of TNF-α in tissues while systemic activities of TNF-α may be associated with the secreted form (5).

Several studies in human and murine models have suggested that TNF-α release may be dependent on the activity of one or more serine proteases. For example, N^{α}-*p*-tosyl-L-arginine methyl ester, a specific serine proteinase inhibitor, has been reported to suppress the secretion of TNF-α without affecting the level of TNF-α mRNA or the expression of its cell surface form (6). Serine proteinase inhibitors were also shown to suppress the secretion of TNF-α from murine activated macrophages (7). Moreover, mice pretreated with the serine proteinase inhibitor α_1-antitrypsin (α_1-AT) were not able to secrete TNF-α in response to D-galactosamine/lipopolysaccharide thus becoming fully protected against D-galactosamine/lipopolysaccharide-induced hepatitis (8). Recent reports suggest, however, the implication of a metalloprotease in the processing of TNF-α (9). Indeed, a metalloproteinase activity capable of generating the 17-kDa moiety from recombinant TNF-α precursor was partially purified from the monocytic cell line THP-1 membranes. A series of hydroxamate inhibitors of matrix metalloproteases have been shown to inhibit the release of TNF-α without reducing the cell-associated activity and to protect mice challenged with lethal doses of endotoxin (9–11).

In this report, we describe the processing of *in vitro* translated 26-kDa TNF-α using cellular fractions derived from human monocytes or monocytic cell lines. Such processing generated active 17-kDa TNF-α and could be blocked with serine proteinase inhibitors. Experimental evidences suggest that proteinase-3 (PR-3) is the enzyme responsible for this *in vitro* observed activity. The potential physiological relevance of these findings are discussed.

EXPERIMENTAL PROCEDURES

Reagents—Human leukocyte neutrophil elastase and cathepsin G are from Calbiochem Biochemicals. PR-3 and sera containing anti-neutrophil cytoplasmic autoantibodies (ANCA) were obtained from Wieslab AB (Lund, Sweden).

α_1-AT, 3,4-dichloroisocoumarin (DCIC), E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), leupeptin, and pepstatin were purchased from Sigma. Methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone (MeOSuc-Ala-Ala-Pro-Val-CMK) was from Bachem, Inc. (Torrance, CA). Human secretory protease inhibitor (hSLPI) was from R&D systems (Abingdon, UK).

Synthetic substrates MeOSuc-Ala-Ala-Pro-Val-pNA and MeOSuc-Ala-Ala-Pro-Met-pNA were from Sigma.

Cells—HL-60, U937, Raji, and Jurkat human cell lines were obtained from American Type Culture Collection, Rockville, MD. Cells were grown in suspension (at 37 °C, 5% CO_2) in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 10 mM Hepes, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin and streptomycin.

Human monocytes were obtained from healthy donors' leukophoresis bags. Briefly, peripheral blood mononuclear cells were separated by standard Ficoll-Hypaque density gradient centrifugation. The enriched population of monocytes and lymphocytes were plated into dishes containing RPMI supplemented with fetal calf serum and incubated for 30 min at 37 °C. The dishes were extensively washed with RPMI, leaving only adherent monocytes.

Preparation of Membrane/Particulate Fractions—Cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (PBS). The cells were collected by centrifugation at 1200 rpm, resuspended at a density of 10^8/ml in lysis buffer (Tris, 10 mM, pH 7.5, EDTA, 1 mM), and homogenized at 200 rpm with a motor-driven Potter-Elvehjem (Teflon/glass) homogenizer. The homogenate was centrifuged at 400 × *g* for 5 min. Pellets were discarded, and the supernatant was ultracentrifuged at 160,000 × *g* for 2 h. The cytosol fractions (supernatants) were stored at −80 °C, and pellets containing membrane/particulate fraction were

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‖ To whom correspondence should be addressed. Tel.: 33-1-49914422; Fax: 33-1-49915257.

[1] The abbreviations used are: TNF, tumor necrosis factor; α_1-AT, α_1-antitrypsin; ANCA, anti-neutrophil cytoplasmic autoantibodies; CMK, chloromethylketone; DCIC, 3,4-dichloroisocoumarin; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; MeO, methoxy; Suc, succinyl; pNA, *p*-nitroanilide; PBS, phosphate-bufffered saline; PR-3, proteinase-3; SLPI, secretory leukoproteinase inhibitor; WG, Wegener's granulomatosis; PAGE, polyacrylamide gel electrophoresis.

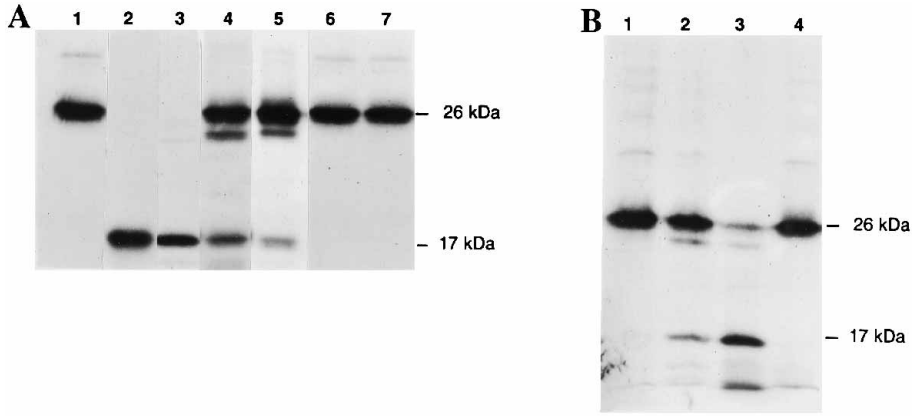

FIG. 1. ***In vitro* processing of human TNF-α precursor by crude membrane fractions prepared from different cell sources.** *A*, the 26-kDa *in vitro* translated TNF-α precursor (*lane 1*) was incubated for 1 h at 30 °C with 100 μg of membrane/particulate fraction proteins prepared from HL-60 (*lane 3*), U937 (*lane 4*), human monocytes (*lane 5*), Jurkat (*lane 6*), or Raji (*lane 7*). The reaction products were visualized by autoradiography after immunoprecipitation and SDS-PAGE. ^{125}I-Labeled 17-kDa TNF-α is shown in *lane 2*. *B*, the 26-kDa *in vitro* translated TNF-α precursor (*lane 4*) was incubated with 1, 10, and 100 μg (*lanes 1*, *2*, an *3*, respectively) of HL-60 membrane fraction proteins, and reaction products were analyzed as described above.

resuspended at a density of 7×10^8 cells/ml in membrane buffer (10 mM Tris, 250 mM sucrose), homogenized with 15 passes of the Teflon/glass homogenizer at 200 rpm, and stored at −80 °C. The protein concentrations of the fractions were determined by a colorimetric BCA assay (Pierce).

Preparation of Radiolabeled Precursor TNF-α—A 0.8-kilobase cDNA, obtained from HL-60 RNA and containing the entire coding sequence of TNF-α precursor, was inserted into *Kpn*I/*Sac*I-digested pBS-SK+ plasmid DNA and propagated in *Escherichia coli*. Purified plasmid was transcribed *in vitro* by using a T7 RNA polymerase and translated *in vitro* in a rabbit reticulocyte lysate system (TNT™ T7-coupled reticulocyte lysate system, Promega Biotech Inc.) in the presence of 40 μCi of [^{35}S]Cys (1 mCi = 37 MBq; Amersham Corp.) for 1 h at 30 °C to produce [^{35}S]Cys-labeled 26-kDa TNF-α.

Assay for in Vitro TNF-α Precursor Cleavage—Cleavage of TNF-α precursor was performed by incubating 200 μl of *in vitro* translated TNF-α precursor with different amounts of crude membrane cellular extract in a final volume of 1.5 ml in 20 mM Hepes, pH 7.5, 2 mM dithiothreitol, 10% (v/v) glycerol. Each reaction was incubated for 1 h at 30 °C and immunoprecipitated with 10 μl of rabbit anti-human TNF-α polyclonal antisera (PS30, Monosan) and 60 μl of protein A-Sepharose (Pharmacia). Sepharose pellets were washed four times, resuspended, and boiled for 3 min in 80 μl of 0.25 M Tris-Cl, pH 6.8, 10% SDS, 0.5% bromphenol blue, 0.5 M dithiothreitol, and 50% glycerol. Samples were migrated on a 13.5% SDS-polyacrylamide gel and autoradiographied.

Preparation of Human TNF-α Precursor Mutants—Mutants were generated by polymerase chain reaction with oligonucleotides encoding an Ala/Val site where the valine residue at position +1 is either deleted or substituted by glycine or alanine.

Two overlapping fragments were generated in a initial reaction using as template a wild-type TNF-α precursor cDNA obtained from HL-60 RNA and a complementary set of oligonucleotides, both of which include the point mutation. In a subsequent reaction the two fragments were joined using flanking oligonucleotides as primers.

TNF-delVal1 mutant, where the amino acid valine at position +1 is deleted, was obtained with a 33-mer oligonucleotide (upstream) 5′-TCGAGAAGATGATCTTGCCTGGGCCAGAGGGCT-3′ and a 28-mer oligonucleotide (downstream) 5′-GGCCCAGGCAAGATCATCTTCTC-GAACC-3′.

TNF-Gly1 mutant, where the amino acid valine at position +1 is substituted by a glycine, was obtained with a 24-mer oligonucleotide (upstream) 5′- TCGAGAAGATGATCTGCCTGCCTG-3′ and a 24-mer oligonucleotide (downstream) 5′-CAGGCAGGCAGATCATCTT-CTCGA-3′.

TNF-Ala1 mutant, where the amino acid valine at position +1 is substituted by an alanine, was obtained with a 24-mer oligonucleotide (upstream) 5′-TCGAGAAGATGATCTGGCTGCCTG-3′ and a 24-mer oligonucleotide (downstream) 5′-CAGGCAGCCAGATCATCTT-CTCGA-3′.

The cDNAs encoding for the mutated TNF-α proteins were cloned into the *Kpn*I/*Sac*I-digested pBS-SK+ plasmid DNA. The mutations were verified by sequence analysis.

Sequencing of Cleavage Product—The TNF-α precursor was translated *in vitro* as described above except that 80 μCi of L-[3,4-^{3}H]valine (1 mCi = 37 MBq; Amersham) was used as radiolabeled amino acid. Cleavage reactions were performed using 100 μg of HL-60 membrane proteins or 1 μg of purified PR-3. Radiolabeled TNF-α product was purified from the processing reaction by migration on SDS-PAGE gel followed by transfer to poly(vinylidene difluoride) membrane. NH_2-terminal sequencing of radiolabeled cleavage product was performed by automated Edman degradation on a 470 A gas phase microsequencer (Applied Biosystems, Inc.). Each degradation cycle product was counted in a liquid scintillation counter (Beckman Instruments).

Determination of Enzymatic Activity—Proteolysis of the synthetic substrates for elastase and PR-3 (MeO-Suc-Ala-Ala-Pro-Val-pNA) or cathepsin G (MeO-Suc-Ala-Ala-Pro-Met-pNA) was assayed in 200 μl, total volume, consisting of 2 mM of the corresponding substrate in PBS with 0.1% (v/v) Tween-80, 1.25%(v/v) dimethyl sulfoxide, and 25 nM enzyme. pNA release was followed by continuously measuring the change of absorbance at 405 nm at 30 °C during a 1-h period using a Microplate reader ThermoMax (Molecular Devices). Specific activities determined under these conditions for elastase, PR-3, and cathepsin G were 20, 0.2, and 6 units/mg, respectively. One unit corresponds to 1 μmol/min released pNA.

RESULTS

In Vitro TNF-α Processing by Crude Cell Membrane Preparations—Crude membrane and cytosol fractions were prepared from the myelomonocytic cell lines HL-60 and U937 as well as from human monocytes and tested for their ability to cleave *in vitro* translated and [^{35}S]Cys radiolabeled 26-kDa TNF-α. Reaction products were analyzed by SDS-PAGE and autoradiography after immunoprecipitation with an anti-TNF-α polyclonal antibody. While cytosol fractions did not exhibit any processing activity, incubation of TNF-α precursor with crude membrane fractions prepared from these cells generated a 17-kDa band that comigrated with a recombinant ^{125}I-labeled TNF-α (Fig. 1*A*). A 24-kDa band could be systematically detected under these conditions. In the *in vitro* assay, the crude membrane extract derived from HL-60 was significantly more active than those from monocytes and U937. No processing could be observed when TNF-α precursor was incubated with crude membranes obtained from Jurkat (human T cell line) or Raji (human B cell line). Titration of HL-60 membrane fractions showed that the processing activity was dose-dependent (Fig. 1*B*). Thus 100 μg of total protein derived from the HL-60 membrane fraction almost completely processed the 26-kDa precursor in 60 min. Interestingly, when 100 μg of crude membrane extracts were used in the assay, the 24-kDa band disappeared, suggesting that this band could result of the use of an intermediate cleavage site located in the 14-kDa precursor portion of TNF-α. When assayed using the L929 assay, the cleavage product was as active as the precursor form, indicating that the 17-kDa protein resulting from the *in vitro* processing was biologically active (data not shown).

Effect of Protease Inhibitors on the in Vitro Processing of TNF-α and Activity of Purified Serine Proteinases—In order to characterize the enzymatic nature of the proteolytic activity found in the crude membrane extracts of HL-60, we tested the effect of a series of protease inhibitors in the TNF-α cleavage assay. The results shown in Fig. 2 indicate that the serine proteinase inhibitors DCIC, α_1-AT, and MeO-Suc-Ala-Ala-Pro-

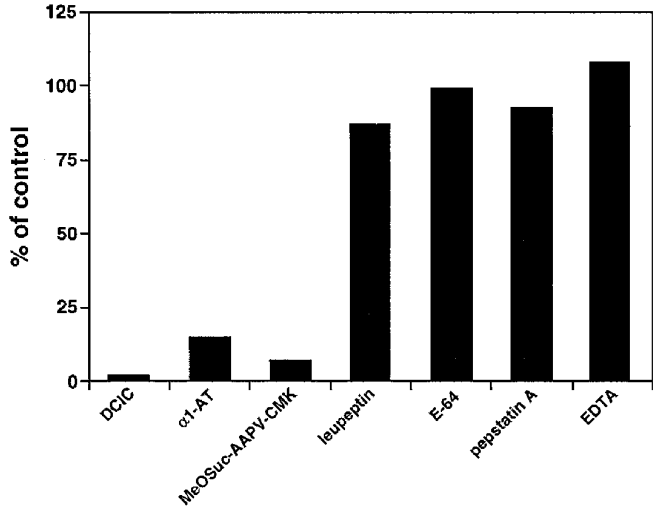

FIG. 2. **Effects of protease inhibitors in the *in vitro* TNF-α processing activity.** The 26-kDa *in vitro* translated TNF-α precursor was incubated with 10 μg of HL-60 crude membrane fraction proteins in the absence or presence of 250 μM DCIC, 1 mg/ml α_1-AT, 250 μM MeO-Suc-Ala-Ala-Pro-Val-CMK, 5 mM EDTA, 200 μM E-64, 500 μM leupeptin, or 50 μM pepstatin A, before immunoprecipitation, SDS-PAGE, and autoradiography. Results were analyzed by scanning and are expressed as percentage of the activity found in controls performed in the presence of the solvents used for each inhibitor.

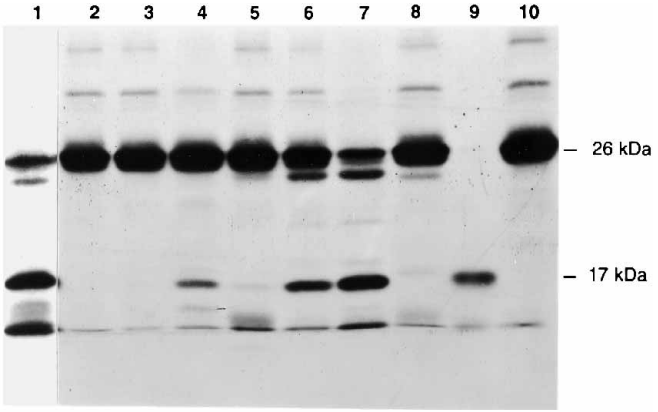

FIG. 3. ***In vitro* processing activity of human TNF-α precursor by purified serine proteinases.** The 26-kDa *in vitro* translated TNF-α precursor (*lane 10*) was incubated for 1 h at 30 °C in the presence of 8, 80, or 800 ng of neutrophil elastase (*lanes 2*, *3*, and *4*) or PR-3 (*lanes 5*, *6*, and *7*) or 1 μg of cathepsin G (*lane 8*), and reaction products were analyzed by SDS-PAGE and autoradiography after immunoprecipitation. In *lane 1* is shown the *in vitro* processing obtained with 100 μg of HL-60 crude membrane fraction proteins. ^{125}I-labeled 17-kDa TNF-α is shown in *lane 9*.

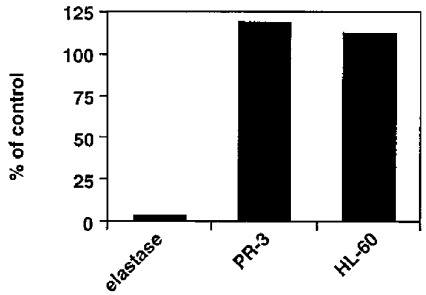

FIG. 4. **Effects of SLPI on TNF-α *in vitro* processing.** Aliquots of 800 ng of elastase, 80 ng of PR-3, or 100 μg of HL-60 crude membrane preparation proteins were incubated with 1 μg of SLPI for 10 min at room temperature before cleavage assay on the *in vitro* translated TNF-α precursor, immunoprecipitation, SDS-PAGE, and autoradiography. Results were analyzed by scanning and are expressed as percentage of the activity found in controls performed in the absence of SLPI.

Val-CMK efficiently inhibited the generation of 17-kDa TNF-α by HL-60 membrane proteins. In contrast, E-64, pepstatin, EDTA, and leupeptin, which are specific inhibitors of cysteine, aspartate, metallo, and serine/cysteine proteinases, respectively, failed to inhibit the *in vitro* processing of TNF-α. These results strongly suggest that the *in vitro* TNF-α processing activity detected in HL-60 membrane proteins is dependent on one or several serine proteinases.

In order to further confirm previous results, we studied the *in vitro* processing activity of three purified serine proteinases: human leukocyte elastase, cathepsin G, and PR-3. As shown in Fig. 3, whereas cathepsin G did not efficiently process the TNF-α precursor, elastase and PR-3 generated a 17-kDa protein in a dose-dependent manner. It should be noted that PR3 was more efficient than elastase to generate the 17-kDa TNF-α. Moreover PR-3 reproduced the same pattern of proteolysis (17- and 24-kDa bands) previously found with HL-60 membrane fractions. Among the natural serine proteinase inhibitors, the secretory leukoproteinase inhibitor (SLPI) has been shown to inhibit both elastase and cathepsin G but not PR-3 (12, 13). The proteolytic activity of elastase and PR-3 on the synthetic substrate MeO-Suc-Ala-Ala-Pro-Val-pNA was studied in the presence of different concentrations of recombinant SLPI. This molecule completely inhibited the elastase activity at a 5:1 molar ratio without affecting the PR-3 activity (data not shown). In the *in vitro* TNF-α cleavage assay, SLPI (1 μg) inhibited the weak processing activity of elastase without affecting the PR-3 one (Fig. 4). In addition, the same concentration of SLPI did not inhibit the processing activity of HL-60 membrane fraction, suggesting that elastase was not implicated in this reaction.

Effect of Purified IgG from Wegener's Granulomatosis (WG) Patients on the in Vitro Processing of TNF-α—Classic ANCA are specific markers for active WG. These antibodies are specifically directed against PR-3 (14–16). Recently, IgG from patients with active WG were shown to significantly inhibit PR-3 proteolytic activity (17). We therefore evaluated the inhibitory capacity of IgGs purified from five WG patients' sera on the PR-3 enzymatic activity measured on the synthetic substrate MeO-Suc-Ala-Ala-Pro-Val-pNA. IgG derived from the serum IO5·PR inhibited the PR-3 activity at a 50-fold molar excess without affecting the activity of elastase. The incubation of purified PR-3 or elastase with 100 μg of IO5 IgGs inhibited the PR-3 but not the elastase-mediated TNF-α *in vitro* processing activity (data not shown). At the same concentration, IO5 IgGs completely inhibited the processing activity of the monocytes and HL-60-derived membrane fractions (Fig. 5). These results support that PR-3 is most probably the enzyme responsible for the *in vitro* cleavage of 26-kDa TNF-α by HL-60 or monocyte-derived crude membrane preparations.

Identification of the Cleavage Site of 26-kDa TNF-α by HL-60 Membrane Proteins—We have constructed three mutants in which the residue Val^{77} (the NH_2-terminal amino acid in the secreted TNF-α) has been either deleted or substituted by Gly or Ala. HL-60-derived membrane preparations were unable to generate any 17-kDa protein from either the deleted (Fig. 6) or glycine-substituted (data not shown) mutants. Interestingly, the generation of the 24-kDa band was not modified in these two mutants. In contrast, any major changes could be detected in the mutant in which Val^{77} was substituted by an Ala (data not shown). These results indicate that Val^{77} is crucial for the *in vitro* detected TNF-α processing activity. To identify the amino terminus of the 17-kDa cleavage product, [^{3}H]Val-labeled 26-kDa TNF-α was cleaved by HL-60-derived membrane fraction, immunoprecipitated, electrophoresed, transferred to

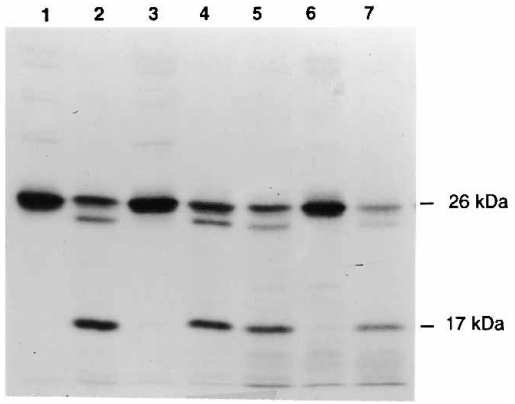

FIG. 5. **Effect of a ANCA positive serum-derived IgG on the *in vitro* TNF-α cleavage activity.** Aliquots of 10 μg of HL-60 membrane fraction proteins (*lanes 2*, *3*, and *4*) or 100 μg of human monocyte membrane fraction proteins (*lanes 5*, *6*, and *7*) were incubated for 30 min at 37 °C with buffer (*lanes 2* and *5*), 100 μg of PR·IO5-derived IgGs (*lanes 3* and *6*) or 100 μg of control human IgG (*lanes 4* and *7*) before the cleavage assay on the *in vitro* translated TNF-α precursor (*lane 1*).

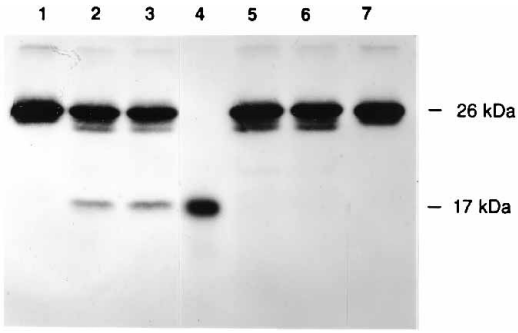

FIG. 6. ***In vitro* processing activity of human wild type or mutant TNF-α precursors by HL-60 membrane preparations.** *In vitro* translated wild type TNF-α precursor (*lane 1*) or a valine deletion mutant precursor (*lane 7*) were incubated for 1 h at 30 °C with 80 ng of PR-3 (*lanes 2* and *5*, respectively) or 10 μg of HL-60 membrane fraction proteins (*lanes 3* and *6*, respectively). The reaction products were visualized by autoradiography after immunoprecipitation and SDS-PAGE. ^{125}I-labeled 17-kDa TNF-α is shown in *lane 4*.

poly(vinylidene difluoride) membrane, and subjected to automated sequencing. Peaks of radiolabeled Val were detected at cycles 12, 15, and 16 (Fig. 7). The same radioactivity pattern was observed when purified PR-3 was used in the *in vitro* cleavage assay (data not shown). Our results show that cleavage of the TNF-α precursor by both PR-3 and HL-60 membrane preparations occurs between Val^{77} and Arg^{78}.

DISCUSSION

Using an *in vitro* TNF-α precursor cleavage assay, we have identified a serine proteinase activity in the crude membrane fractions from monocytic cells, which is capable of generating a bioactive 17-kDa TNF-α form. Experiments carried out to characterize the enzymatic nature of this activity suggest that the neutral serine proteinase PR-3 or a related enzyme is responsible for this effect. First, purified PR-3 processed the TNF-α precursor with a pattern identical to the one obtained with the crude membrane preparations. In addition to the 17-kDa protein, a 24-kDa band was observed when the TNF-α precursor was incubated with active membrane fractions or PR-3. Second, SLPI, a natural serine anti-proteinase secreted by cells of mucosal surfaces that interacts with both cathepsin G and elastase but is devoid of inhibitory activity against PR-3 (12, 13), did not affect the proteolytic activity of the membrane preparations. Third, purified IgG prepared from an ANCA-positive serum (previously shown to specifically interfere with

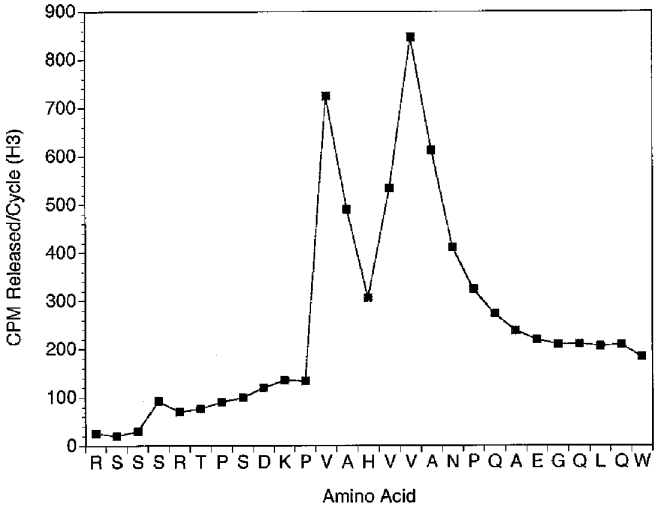

FIG. 7. **Sequence analysis of 17-kDa cleavage product.** [^{3}H]Val-labeled TNF-α precursor was cleaved with 100 μg of HL-60 crude membrane preparation proteins and the cleavage product was sequenced after SDS-PAGE and transferred to poly(vinylidene difluoride) membrane. Fractions from the sequence run were counted for associated radioactivity. Peaks of radioactivity were found in cycles 12, 15, and 16 corresponding to the Val amino acids of mature TNF-α, which are plotted on the ordinate axis. Amino acids are depicted by the single-letter code.

the PR-3 *in vitro* proteolytic activity) completely inhibited the *in vitro* processing activity of the membrane fractions. Finally, the NH_2-terminal sequence of the 17-kDa product derived from the proteolysis with both crude membrane extracts or purified PR-3 were shown to be identical.

Different biological properties of PR-3 have been reported. PR-3 degrades a variety of extracellular matrix proteins including elastin (18), fibronectin, type IV collagen, and laminin (12). In addition PR-3 has a potent antimicrobial activity against both bacteria and fungi (19, 20). It cleaves and inactivates the human C1 inhibitor leading to activation of the classical complement pathway (21), and it has been recently demonstrated that PR-3 has a potentiating effect of platelet activation (22) and may play an important role in neutrophil-mediated endothelial damage (23). Finally, PR-3 has been shown to process *in vitro* interleukin-8 (24) and the nuclear factor-κB subunit p65 (25). At the cellular level, PR-3 is not only localized in the azurophil granules of granulocytes, but is also present in small granules of monocytes (26), in human endothelial cells (27), and in mastocytes (28). Several stimuli such as TNF-α or IL-8 can even induce translocation of PR-3 from the intragranular loci to the cell surface of polymorphonuclear leukocytes (29).

The present study demonstrates that PR-3 is capable of cleaving *in vitro* synthesized TNF-α precursor in a site-specific manner between Val^{77} and Arg^{78}, thus generating a 17-kDa TNF-α with an Arg at its NH_2 terminus. The importance of this site was confirmed by using TNF-α mutants in which Val^{77} was either deleted or changed by Ala or Gly (Fig. 6). Accordingly, studies previously conducted to map the active site of PR-3 showed that the preferred P1 residue is a small aliphatic amino acid such as valine or alanine (30). As described above, an additional 24-kDa band was generated in the *in vitro* cleavage assay by PR-3, thus indicating the existence of a second proteolytic site in the TNF-α precursor. This second proteolytic site is more probably located in the 14-kDa prosequence because (i) it disappeared with high amounts of membrane preparations (Fig. 1*B*) or when longer incubation times were performed (data not shown), and (ii) membrane preparations did not cleave the recombinant soluble 17-kDa TNF-α (data not shown). A potential site theoretically susceptible to generate a

24-kDa protein is located between alanine 15 and leucine 16. This is in agreement with the studies on the primary specificity of PR-3 against the insulin-B chain showing that a major site of cleavage was an alanine/leucine bond (12).

Recently, the existence of a Zn^{2+}-containing endopeptidase capable of cleaving the 26-kDa TNF-α to a 17-kDa form beginning at Val^{77} was reported (9, 11). Val^{77} was previously shown to correspond to the NH_2 terminus of the TNF-α secreted by cultured cell lines (31, 32). This, together with the *in vivo* efficacy of metalloprotease inhibitors to block TNF-α secretion (9–11), strongly suggests that the enzyme primarily responsible for TNF-α processing is a metalloprotease. Our results suggest, however, that accessory sites and perhaps accessory enzymes could exist to generate active TNF-α. Indeed, we demonstrated that Val^{77}-Arg^{78} is a possible alternative cleavage site. Additional sites could exist since it was shown that deletion of residues between Val^{77} and Pro^{88} did not affect the generation of active TNF-α (33). Furthermore, pulse-chase studies suggest that the processing of TNF-α primarily takes place at the cell surface (34, 35), raising the possibility of an extracellular cleavage of TNF-α by serum proteinases. Interestingly, PR-3 is present in large amounts in the serum of normal subjects and its levels are significantly high in patients with connective tissue disease (36). Along with this line, the serine proteinase inhibitor $α_1$-AT was shown to block TNF-α release *in vitro* (37) and *in vivo* (8). More recently, the TNF-α concentration in synovial fluid of rheumatoid arthritis patients was shown to be inversely correlated with $α_1$-AT activity (38).

Altogether our results show that PR-3 can play a role in cleaving the TNF-α precursor to generate a bioactive form. The relevance of PR-3-mediated TNF-α processing under normal and pathological situations remains to be elucidated.

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