Recombinant human proteinase 3, the Wegener's autoantigen, expressed in HMC-1 cells is enzymatically active and recognized by c-ANCA

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Abstract We developed a stable expression system for conformationally intact recombinant human PR3 (rPR3) using the human mast cell line HMC-1. Like in U937 cells, the rPR3 is processed from a 34 kDa precursor to the 29 kDa mature form, primarily as the result of oligosaccharide trimming. The rPR3 binds [^{3}H]DFP and hydrolyzes the substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-pNA. The enzymatic activity is inhibited by greater than 95% by α1-PI. The rPR3 and the enzymatically inactive mutant rPR3-S176A are both packaged in granules. Thus, proteolytic autoprocessing is not required for PR3's targeting to granules. This rPR3 is the first to be recognized by most c-ANCA from WG patients and all anti-PR3 ANCA that were detected by standard anti-PR3 specific ELISA. This expression system for rPR3 represents a versatile tool for the analysis of its intracellular processing, structure-function relationships and interaction with autoantibodies.

Key words: Proteinase 3; Human; Mast cell; Neutral serine protease; Anti-neutrophil cytoplasmic antibody; Expression system

1. Introduction

The neutrophil azurophil granule constituent proteinase 3 (PR3; EC 3.4.21.76) is a neutral serine protease with substantial amino acid sequence homology with elastase, cathepsin G and azurocidin [1–3]. PR3 has enzymatic activity for elastin, fibronectin and the basement membrane proteins collagen type IV and laminin [4]. Since activated neutrophils express PR3 on their surface [5], it could facilitate their migration through basement membranes. PR3 may be instrumental in host defense and regulation of proliferation and differentation of hematopoetic cells [2,6]. PR3 can also proteolytically process interleukin 8 into a chemotactically more potent form [7], and cleave the mammalian heat shock protein hsp28 [8]. These two properties clearly distinguish PR3 functionally from elastase.

PR3 has been implicated in the pathogenesis of pulmonary emphysema [4,9] and Wegener's granulomatosis (WG) [10]. WG is a specific form of vasculitis associated with circulating anti-neutrophil cytoplasmic autoantibodies directed against PR3 (c-ANCA) [3]. The interaction of c-ANCA with PR3 exposed on the surface of cytokine stimulated neutrophils is felt to be significant for the pathogenesis of the disease [10].

The expression of recombinant PR3 (rPR3) as a fusion protein in *E. coli* and in baculovirus systems failed to generate an enzymatically active recombinant product that is recognized by the majority of c-ANCA sera [11–13]. Thereby, these reports have confirmed that most c-ANCA recognize conformational epitopes of PR3. The final conformation of PR3 is determined by its disulfide bonds, appropriate glycosylation, as well as only partially understood specific intracellular processing events, of which most eukaryotic expression systems may not be capable.

Here we present a stable expression system for rPR3 using the human mast cell line (HMC-1). The expressed rPR3 is enzymatically active and recognized by c-ANCA sera, suggesting that the recombinant product is conformationally intact. In HMC-1 cells, rPR3 is processed like endogenous PR3 in U937 cells, and targeted to granules.

2. Materials and methods

2.1. Materials

Unless specified otherwise, all materials were from Sigma, St. Louis, MO. The following anti-PR3 antibodies were used: the monoclonal antibody (moAB) WGM2, kindly provided by Dr. E. Csernok [14]; the moABs 4A3, 4A5, 6A6, and the polyclonal rabbit anti-PR3 antiserum, kind gifts from Dr. J. Wieslander [15,16]. MoABs specific for human mast cell tryptase (Dako, Carpinteria, CA), and rabbit polyclonal antibodies against human mast cell chymase (Biogenesis, Bournemouth, UK), human neutrophil elastase, myeloperoxidase (both from Dako, Carpinteria, CA), and cathepsin G (Cortex Biochem., San Leandro, CA) were also used.

2.2. Cell culture, DNA constructs, and transfection

Promyelomonocytic U937 cells obtained from the American Type Culture Collection (Bethesda, MD) were cultured according to their instructions. The human mast cell line HMC-1, a kind gift from Dr. J. H. Butterfield, was cultured as described [17]. Some transfected HMC-1 cells were subsequently cultured in RPMI/10% BCS.

U937 cell mRNA served as template for the preparation of PR3 cDNA. The following primers were prepared (mutations underlined):

1, 5′ – GCCCCCCAGCCCTGCCCTGGCGTCCGTG – 3′

2, 5′ – CGGCCAGCGCTGTGGGAGGGGCGGTTCA – 3′

3, 5′ – GTCAAAGCTTCCCACCATGGCTCACCGGCCCCCC–AGCCCTG – 3′

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Abbreviations: α1-PI, α1-protease inhibitor; ANCA, anti-neutrophil cytoplasmic antibodies; c-ANCA, cytoplasmic fluorescence pattern ANCA; p-ANCA, perinuclear fluorescence pattern ANCA; BCS, bovine calf serum; DFP, diisopropylfluorophosphate; DPPI, dipeptidylpeptidase I; IIF, indirect immunofluorescence; moAB, monoclonal antibody; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pNA, *p*-nitroanilide; PR3, proteinase 3; rPR3, recombinant PR3; WG, Wegener's granulomatosis

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4, 5′ – GTACTCTAGACGGCCAGCGCTGTGGGAG – 3′

5, 5′ – ATCTGCTTCGGAGACGCCGGTGGCCCCCTGATC – 3′

and

6, 5′ – GATCAGGGGGCCACCGGCGTCTCCGAAGCAGAT – 3′.

Reverse transcription of mRNA from U937 cells was followed by amplification by polymerase chain reaction (PCR) using primers 1 and 2, resulting in a cDNA fragment spanning from nucleotide positions 9 to 790 of the published sequence [18]. *Hin*dIII and *Xba*I restriction sites were added to the 5′ and 3′ ends, respectively, by PCR using primers 3 and 4. This insert was cloned into the *Hin*dIII/*Xba*I restricted expression vector pRcCMV (InVitrogen, San Diego, CA), resulting in the plasmid pRcCMV/PR3.

The plasmid pRcCMV/PR3-S176A was prepared to express the enzymatically inactive mutant rPR3-S176A using the splicing by overlap extension method [19]. Overlapping DNA fragments generated by PCR with primers 5 and 4, and primers 6 and 3 were spliced together and cloned into the vector pRcCMV.

The plasmids were transfected into HMC-1 cells by electroporation [20]. An rPR3 expressing cell population (HMC-1/PR3) was selected using G418 (genticin, Gibco, Grand Island, NY). Cells growing in the presence of G418 (600 μg/ml) were subjected to cloning by dilution selection. Control cells (HMC-1/VEC) were mock-transfected with the plasmid pRcCMV without insert, selected and cultured under the same conditions.

2.3. Immunofluorescence, biosynthetic labeling and immunoprecipitation

Ethanol-fixed cytospin preparations of human peripheral blood neutrophils, HMC-1, HMC-1/VEC, HMC-1/PR3 and HMC-1/PR3-S176A cells were prepared and indirect immunofluorescence (IIF) was performed as described elsewhere [21].

Biosynthetic labeling and immunoprecipitation were performed using the following modification of a protocol described elsewhere [22]. 2×10^7 U937, HMC-1/VEC or HMC-1/PR3 cells were washed twice in methionine- and cysteine-free RPMI (Gibco, Gaithersburg, MD) containing 10% BCS, and incubated overnight in methionine- and cysteine-free RPMI/10% BCS to which 100 μCi/ml of [^{35}S]methionine/[^{35}S]cysteine (ICN, Costa Mesa, CA) had been added. Cells were then washed, lysed, sonicated, normalized for trichloroacetic acid-precipitable counts, precleared with *Staphylococcus aureus* (Gibco, Gaithersburg, MD), and immunoprecipitated. The immunoprecipitates were washed three times in the radio-immunoprecipitation buffer, electrophoresed on 12% SDS-PAGE gels, and visualized by autoradiography.

For pulse-chase experiments, cells were washed, starved for 30 min in methionine- and cysteine-free RPMI/5% BCS, and incubated for 30 min in the same media supplemented with 100 μCi/ml of [^{35}S]methionine/[^{35}S]cysteine (pulse labeling), washed once again, resuspended, and cultured in RPMI/10% BCS for the various time periods (chase). For each time point 10×10^6 cells were collected and analyzed.

2.4. Deglycosylation of immunoprecipitates

Immunoprecipitates of metabolically labeled cells were prepared, washed, resuspended in 10 μl of 0.5% SDS, and incubated for 2 min at 95°C. After addition of 90 μl of digestion buffer (20 mM Na_2HPO_4, 10 mM NaN_3, 50 mM EDTA, 0.5% (v/v) Nonidet P-40, pH 7.2), the sample was divided in two. One sample served as the control; 0.4 U N-glycosidase F (Boehringer, Mannheim, Germany) was added to the second sample, and both were incubated at 37°C for 15 h. The samples were then precipitated in 30% trichloroacetic acid, washed in cold acetone and analyzed by SDS-PAGE and autoradiography.

2.5. [^{3}H]Diisopropylfluorophosphate labeling

20×10^6 cells were lysed in 500 μl of 1% Triton X-100, 20 mM Tris, pH 6.8 and sonicated. Serine proteases were labeled by incubating the cell lysate with [^{3}H]diisopropylfluorophosphate (DFP) (1 μCi/μl at a specific activity of 8.4 Ci/mmol; DuPont NEN, Wilmington, DE) for 15 min at 4°C. The cell lysate was precleared with *S. aureus* prior to immunoprecipitation and analysis by SDS-PAGE and autoradiography.

2.6. Enzymatic activity assay

5×10^5 cells suspended in 20 mM Tris, 0.5 M NaCl, pH 7.5 each were placed in microtiter wells, centrifuged at $900\times g$, and fixed in 95% ethanol at 4°C. Substrates were dissolved at concentrations of 10 mM in DMSO, and diluted to 2 mM in 0.1 M Tris, pH 8.1. Plated cells were treated with 50 μl of 1% Triton X-100 in 0.1 M Tris, pH 8.1 for 30 min at 37°C. 50 μl of substrate solution were added to the wells, incubated at 37°C, and the hydrolysis of the substrate was detected colorimetrically. Data are expressed as absorbance of *p*-nitroanaline at 405 nm minus absorbance at 490 nm. Inhibitors, when used, were added during the first 30 min incubation.

3. Results and discussion

3.1. Phenotype of HMC-1 cells

The phenotype of the HMC-1 cells used was determined by IIF using antibodies against PR3, elastase, cathepsin G, myeloperoxidase, mast cell chymase and tryptase. HMC-1 cells (70% of cells) expressed only mast cell tryptase. The tryptase expression is consistent with a report by Nilsson, but in contrast to the original description of the HMC-1 cell line [17,23]. We could not confirm Nilsson's observation of 5% of HMC-1 cells expressing cathepsin G. This could be the result of different antibodies used. However, previously reported discrepancies in the phenotype of HMC-1 cells have been attributed to secondary alterations occurring during prolonged passage in vitro [17,23].

3.2. Expression of rPR3 in HMC-1 cells

Sequence verification of the expression vector pRcCMV/PR3 confirmed that the amino acid residue Arg-228 was missing from the originally published sequence [18,24]. Expression of PR3 mRNA of the expected size (0.9 kb) in HMC-1/PR3 cells was detected by Northern blot analysis (performed as described elsewhere [25]), but not in HMC-1/VEC or non-transfected HMC-1 parent cells (not shown).

To determine whether HMC-1/PR3 cells expressed rPR3 protein, IIF was performed on ethanol-fixed cytospin preparations. A strong granular cytoplasmic fluorescence signal was detected in HMC-1/PR3 cells with all anti-PR3 antibodies including the conformation sensitive WGM2 and c-ANCA (Fig. 1B,E). No signal was detected in non-transfected HMC-1 or HMC-1/VEC control cells with either of the antibodies (Fig. 1F), or when non-immune control antibodies were used on HMC-1/PR3 cells (Fig. 1D). Confocal microscopy confirmed that rPR3 is stored in granules in HMC-1/PR3 cells (Fig. 1C). The active site mutant rPR3-S176A also was recognized by all anti-PR3 antibodies and appeared to be deposited in granules (not shown).

Immunoprecipitation experiments (Fig. 2A) showed that all specific anti-PR3 antibodies, with the exception of WGM2, specifically immunoprecipitated rPR3 from extracts of HMC-1/PR3 cells, but not of HMC-1/VEC cells. Normal mouse IgG, normal rabbit and human sera served as non-immune controls. The most prominent specific immunoprecipitate from HMC-1/PR3 cells had an apparent molecular mass of 29 to 34 kDa, consistent with that of native neutrophil and U937 cell PR3 [9,26].

Two additional protein species of about 47 and 83 kDa were co-immunoprecipitated by the PR3 specific antibodies from HMC-1/PR3, but not HMC-1/VEC cells. These were also immunoprecipitated from U937 cells which endogenously produce PR3 (Fig. 2B). The existence of these previously not characterized larger proteins was originally shown by Gold-

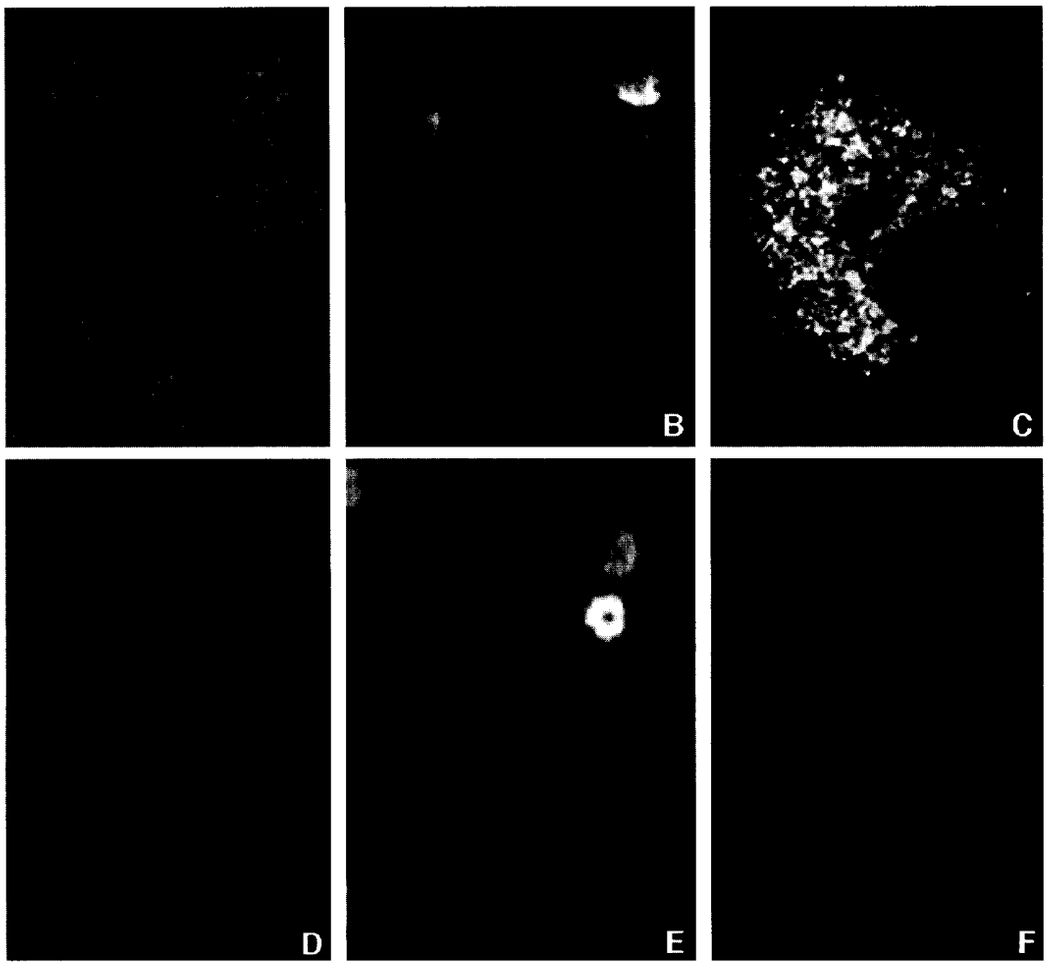

Fig. 1. Indirect immunofluorescence of stably transfected HMC-1 cells. (A) Phase contrast view of HMC-1/PR3 cells; (B) staining of rPR3 with the conformation-sensitive moAB WGM2. (C) Confocal micrograph of HMC-1/PR3 cells stained with the polyclonal rabbit antiserum shows rPR3 deposited in HMC-1 cell granules. (D) HMC-1/PR3 cells incubated with normal human control serum, (E) and c-ANCA serum. (F) c-ANCA serum incubated on HMC-1/VEC cells.

schmeding et al. [27] in immunoprecipitates obtained with c-ANCA patient sera from neutrophil granule extracts. The nature of the 47 and 83 kDa proteins remains unknown. It is unlikely that they represent multimers of PR3 since the gels were run under reducing conditions. The possibility that they represent related protein products from different genes has been excluded by the control experiments. The fact that these proteins do not bind [^{3}H]DFP could indicate that they represent inhibitor or chaperoning molecules that are bound to PR3 intracellularly.

3.3. Intracellular processing of rPR3

The sequence homologies and structural similarities between PR3, elastase and cathepsin G make it likely that these enzymes are all processed in a similar fashion [18,28]. Elastase and cathepsin G have been shown to be processed to the mature enzymatically active enzyme by cleavage of an N-terminal activation dipeptide [29]. Also, there is evidence for cleavage of a C-terminal propeptide of cathepsin G [29]. A most recent report suggests that PR3 is indeed processed in a similar fashion in U937 cells [26]. To investigate the suitability of our recombinant system for studies of intracellular processing of PR3, we evaluated the fate of pulse labeled rPR3 in HMC-1/PR3 cells, and compared it to that of endogenous PR3 in U937 cells. The data indicate that PR3 is processed from a 34 kDa precursor to the 29 kDa form in both HMC-1/PR3 and U937 cells (Fig. 3A). The process appears to be completed after 6 h in HMC-1/PR3 cells, and after 3 h in U937 cells. Some unprocessed 34 kDa precursor is secreted into the media. The observed drop in molecular size is too large to be explained by the proteolytic processing of the protein core, which would account for a molecular mass reduction of about 1 kDa. The observed reduction in molecular mass is more likely a consequence of sugar side chain trimming, as has been reported for U937 cells [26]

The cDNA sequence of PR3 indicates two potential N-linked glycosylation sites [18,24]. Native PR3 immunoprecipitated from neutrophil azurophil granule extracts showed an increased electrophoretic mobility in SDS after digestion with N-glycosidase F [27], suggesting that native neutrophil PR3 is indeed glycosylated. The observed drop in molecular mass after N-glycosidase F treatment (Fig. 3B) indicates that the processing of rPR3 from a 34 kDa to a 29 kDa form is indeed largely the result of the processing of N-linked glycans. Deglycosylation of immunoprecipitates of total HMC-1/PR3 cells lysates results in a double band on SDS-PAGE (Fig. 3B), whereas deglycosylation of single time points from the pulse-chase experiments results in a single band (not shown).

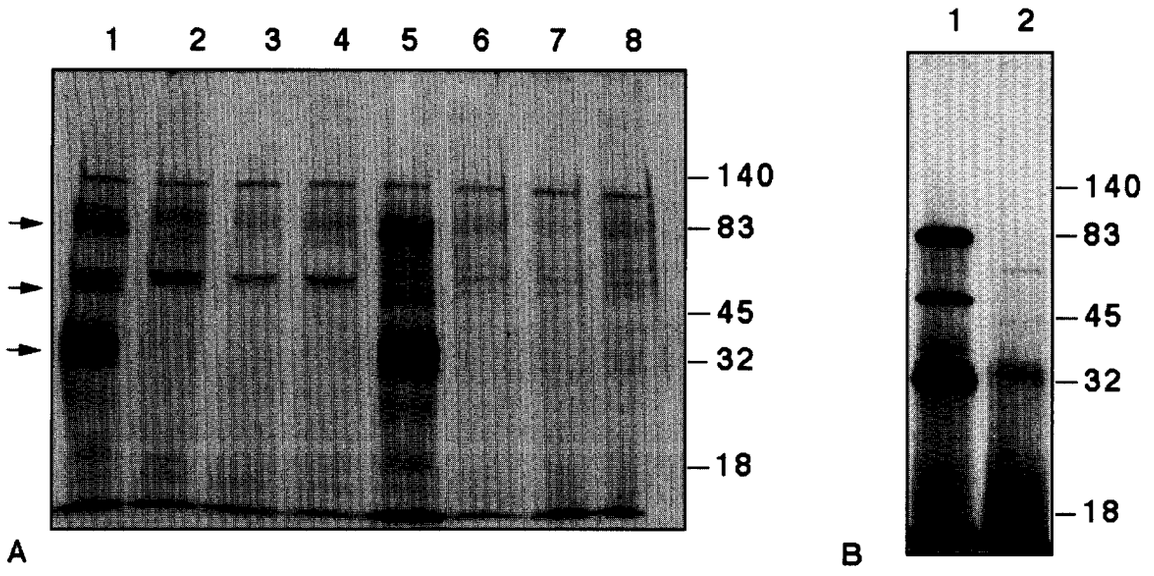

Fig. 2. Immunoprecipitation of [^{35}S]methionine/[^{35}S]cysteine-labeled cell extracts. (A) Cell lysates of HMC-1/PR3 cells (lanes 1,3,5,7) and of HMC-1/VEC cells (lanes 2,4,6,8) were immunoprecipitated with the polyclonal rabbit anti-PR3 serum (lanes 1,2), non-immune rabbit control serum (lanes 3,4), the moAB 4A3 (lanes 5,6) and non-immune mouse IgG (lanes 7,8). The 29–34, 47 and 83 kDa proteins specifically immunoprecipitated from HMC-1/PR3 cells are indicated by arrows. (B) Immunoprecipitation of endogenous PR3 from U937 cells with the moAB 4A3 (lane 1) and non-immune control mouse IgG (lane 2) confirms the presence of the 47 and 83 kDa protein species in U937 cells. Autoradiography exposure times were 8 h (A) and 21 days (B).

This indicates that proteolytic cleavage of a 1 kDa propeptide of the peptide core of rPR3 occurs which is consistent with appropriate proteolytic processing of the C-terminus.

The enzymatically inactive mutant rPR3-S176A is processed and targeted to granules like the wild-type rPR3, indicating that proteolytic autoprocessing is not required, and that the processing of rPR3 is determined by the presence of the processing machinery in the host cell. The HMC-1/PR3 cell system is particularly useful for the study of intracellular processing events, because it allows the application of in vitro mutagenesis, and because of the high rPR3 expression resulting in immunoprecipitation signals being detectable after 24–72 h, compared to 21–28 days for U937 cells.

3.4. Enzymatic activity of rPR3

One of the enzymatic characteristics of serine proteases is their ability to bind the serine protease inhibitor DFP. [^{3}H]DFP bound to rPR3 of 29–34 kDa size (Fig. 4A), but not to the 47 and 83 kDa PR3-related proteins, confirming that only the 29–34 kDa rPR3 contains an active site serine. As expected, the rPR3-S176A mutant was unable to bind [^{3}H]DFP (Fig. 4A).

PR3 preferentially cleaves substrates with small aliphatic amino acids at the P_1 site, and *N*-methoxysuccinyl-Ala-Ala-Pro-Val-pNA represents its most specific synthetic substrate [4]. This was hydrolyzed by lysates of HMC-1/PR3, but not HMC-1/VEC or HMC-1/PR3-S176A cells (Fig. 4B).

The activity of rPR3 against this substrate was inhibited by greater than 95% in the presence of 1 mM DFP or 1.85 μM α1-PI, its major natural inhibitor (Fig. 4B). No inhibition was observed in the presence of 0.1 TIU/ml aprotinin, 1 mM *N*-ethylmaleimide or 35 μM Dansyl-Glu-Gly-Arg-chloromethyl ketone which inhibits enzymes with specificity for basic amino acids at the P_1 site, such as tryptase (not shown). 20% inhibition was observed in the presence of 1.2 μM recombinant eglin C (not shown). No hydrolysis of the substrate succinyl-Ala-Ala-Ala-pNA was observed by HMC-1/PR3, HMC-1/VEC or HMC-1/PR3-S176 A cells under these assay conditions (not shown). Together, these findings indicate that rPR3 recognizes high and low molecular weight substrates and that the substrate and inhibitor spectrum is consistent with what has been reported for native PR3 purified from neutrophils [4].

To estimate the amount of rPR3 expressed by HMC-1/PR3 cells, we compared the HMC-1/PR3 cell activity against *N*-methoxysuccinyl-Ala-Ala-Pro-Val-pNA with that of neutrophils in this assay. Since neutrophils contain enzymes other than PR3 that have activity against this substrate, such as elastase, and since PR3 is not inhibited by aprotinin, neutrophil lysates were assayed in the presence of 0.1 TIU/ml apro-

Table 1
Diagnoses and anti-PR 3 assay results of 40 c-ANCA positive patients

	n = positive on		
	PMN cytospins	HMC-1/PR3 cytospins	Anti-PR3 ELISA
Wegener's granulomatosis	29	18	12
Microscopic polyangiitis	8	6	6
Ulcerative colitis	1	1	1
Sclerosing cholangitis	1	0	0
Somatoforme pain syndrome	1	0	0
Total	40	25	19

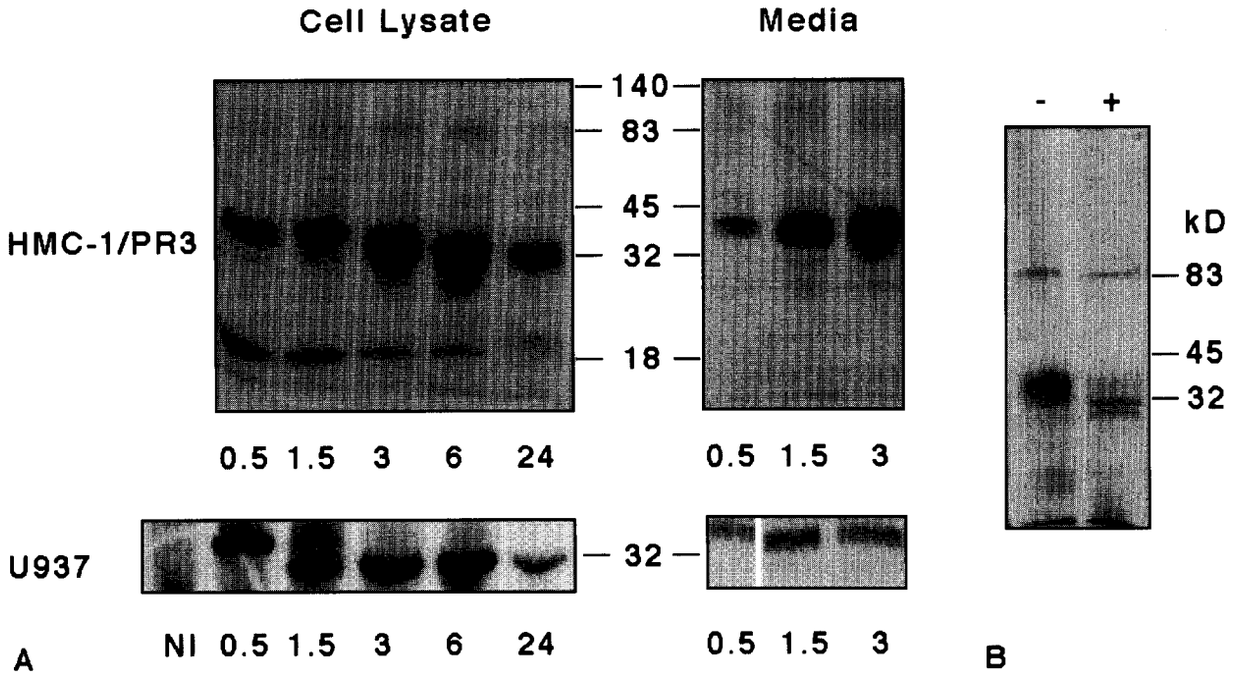

Fig. 3. (A) Pulse-chase experiments indicate intracellular processing from a 34 kDa rPR3 precursor to a 29 kDa rPR3 form in HMC-1/PR3 and U937 cells. The unprocessed 34 kDa form of rPR3 appears to be secreted into the cell media. The time intervals (h) of the chase are indicated at the bottom of the panels. 10×10^6 cells per time point were lysed and immunoprecipitated using the rabbit polyclonal anti-PR3 antibody. Autoradiography exposure times were 40 h for HMC-1/PR3 cell lysates and media, 21 days for U937 cell lysates and 5 days for U937 cell media. (B) Digestion of rPR3 with N-glycosidase F. 20×10^6 HMC-1/PR3 cells were metabolically labeled overnight and immunoprecipitated. The immunoprecipitate was divided into two equal aliquots, which were incubated with (+) or without (−) N-glycosidase F overnight under identical conditions, followed by SDS-PAGE (12% gel). Autoradiography exposure time was 40 h.

tinin. The aprotinin-resistant activity of one neutrophil against this substrate is equivalent to that of two HMC-1/PR3 cells.

The enzymatic activity data also confirm that HMC-1 cells possess the intracellular armentarium required to appropriately process neutrophil serine proteases. As demonstrated for elastase, cathepsin G and lymphocyte granzymes, the cleavage of a two-amino-acid residue propeptide is required for PR3 to assume its conformation dependent enzymatic function [29,30]. Its cleavage is performed by dipeptidylpeptidase I (DPPI) [31,32], which appears to be missing in non-hematopoetic eukaryotic cells [30,33]. In U937 cells the activation dipeptide of PR3 appears to be cleaved by a cysteine protease distinct from DPPI, while elastase is activated by DPPI [26]. The fact that rPR3 is activated to a fully active enzyme in HMC-1 cells suggests that this unidentified processing enzyme is also present in HMC-1 cells, or, alternatively, that rPR3 can also be activated by DPPI.

3.5. Recognition of rPR3 by c-ANCA sera

The most recent report of rPR3 expression in a baculovirus system has confirmed that that the majority of c-ANCA recognize conformational epitopes on PR3, and that insect cells are not able to process rPR3 into a conformationally intact form [13]. To determine whether rPR3 expressed by HMC-1/PR3 cells is recognized by c-ANCA, 40 consecutive serum samples that had previously been determined to be c-ANCA positive by standard IIF on neutrophil cytospin preparations [21] and control sera (5 normal, 5 anti-myeloperoxidase ANCA positive, 10 containing various patterns of ANA and 5 containing anti-mitochondrial antibodies) were obtained from the clinical laboratory. The samples were tested independently by three investigators by IIF on cytospin preparations of HMC-1/PR3 and HMC-1/VEC cells, and in parallel for PR3-reactivity by commercial ELISA [34]. After completion of the data collection the clinical records of the c-ANCA positive patients were reviewed to correlate their clinical diagnoses with the test results (Table 1).

No cytoplasmic signal was detected when these 40 antisera were used on cytospin preparations of HMC-1/VEC control cells (Fig. 1F). Also, no specific signal was detectable when normal (Fig. 1D) or any of the other control sera were used.

The data confirm that routine c-ANCA testing by IIF using neutrophil substrates may detect cytoplasmic fluorescence even in patients without any ANCA associated disease [10,15]. The two sera for which this was the case did not contain antibodies against PR3 as indicated by the test results using HMC-1/PR3 cells as substrate. The parallel use of mock-transfected control cells excludes false-positive results that could be caused by circulating antibodies directed against cytoplasmic antigens other than PR3 when neutrophils are used as test substrate [15].

A substantially higher number of anti-PR3 ANCA containing sera were recognized by the HMC-1/PR3 cell assay than by the anti-PR3 ELISA. That not all true positive samples reported by the clinical laboratory were recognized on HMC-1/PR3 cells can be explained by the fact the standard IIF on neutrophils was performed on fresh serum samples, whereas the testing on HMC-1/PR3 cells and by ELISA was preformed later, after the samples had been frozen and thawed several times. However, it cannot be excluded that some patients with WG may have ANCA directed against

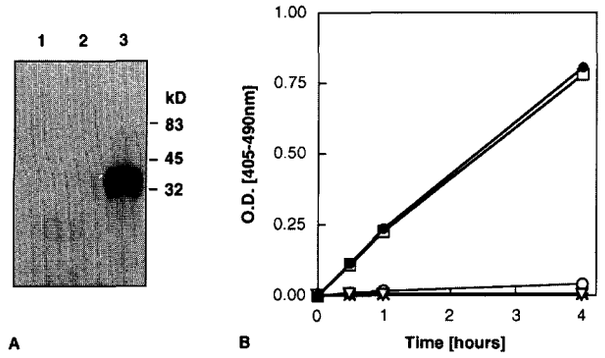

Fig. 4. Enzymatic activity of rPR3. (A) [^{3}H]DFP labeling of rPR3. Only the rPR3 expressing HMC-1/PR3 cells (lane 3), but not the mock-transfected HMC-1/VEC (lane 1) or HMC-1/PR3-S176A cells (lane 2), contain DFP-binding PR3. Autoradiography exposure time was 4 days. (B) Hydrolysis of the substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-pNA by HMC-1/PR3 (full circles), HMC-1/VEC (open triangles), and HMC-1/PR3-S176A cell (stars). Hydrolysis of the substrate is inhibited by greater than 95% by 1.85 μM α1-PI (open circles), but not by 0.1 TIU/ml aprotinin (open squares). Each data point represents the mean of duplicates. Shown is a representative examples of 5 different experiments.

target antigens other than PR3. These data indicate that our stable expression system for conformationally intact rPR3 represents a major step towards the development of new PR3-specific c-ANCA test methods that are more disease specific than the current standard IIF method, and more sensitive than currently commercially available ELISA methods.

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References

[1] Baggiolini, M., Bretz, U., Dewald, B. and Feigenson, M.E. (1978) Agents Actions 8, 3–10.
[2] Gabay, J.E., Scott, R.W., Campanelli, D., Griffith, J., Wilde, C., Marra, M.N., Seeger, M. and Nathan, C.F. (1989) Proc. Natl. Acad. Sci. USA 86, 5610–5614.
[3] Jenne, D.E., Tschopp, J., Lüdemann, J., Utecht, B. and Gross, W.L. (1990) Nature 346, 520.
[4] Rao, N.V., Wehner, N.G., Marshall, B.C., Gray, W.R., Gray, B.H. and Hoidal, J.R. (1991) J. Biol. Chem. 266, 9540–9548.
[5] Csernok, E., Ernst, M., Schmitt, W., Bainton, D.F. and Gross, W.L. (1994) Clin. Exp. Immunol. 95, 244–250.
[6] Bories, D., Raynal, M.-C., Solomon, D.H., Darzynkiewicz, Z. and Cayre, Y.E. (1989) Cell 59, 959–968.
[7] Padrines, M., Wolf, M., Walz, A. and Baggiolini, M. (1994) FEBS Lett. 352, 231–235.
[8] Spector, N.L., Hardy, L., Ryan, C., Miller, W.H., Humes, J.L., Nadler, L.M. and Luedke, E. (1995) J. Biol. Chem. 270, 1003–1006.
[9] Kao, R.C., Wehner, N.G., Skubitz, K.M., Gray, B.H. and Hoidal, J.R. (1988) J. Clin. Invest. 82, 1963–1973.
[10] Gross, W.L. and Csernok, E. (1995) Curr. Opin. Rheum. 7, 11–19.
[11] Bini, P., Gabay, J.E., Teitel, A., Melchior, M., Zhou, J.-L. and Elkon, K.B. (1992) J. Immunol. 149, 1409–1415.
[12] Szymkowiak, C.H., Johnston, T.W., Csernok, E. and Gross, W.L. (1996) Biochem. Biophys. Res. Commun. 219, 283–289.
[13] Witko-Sarsat, V., Halbwachs-Mecarelli, L., Almeida, R.P., Nusbaum, P., Melchior, M., Jamaleddine, G., Lesavre, P., Deschamps-Latscha, B. and Gabay, J.E. (1996) FEBS Lett. 382, 130–136.
[14] Csernok, E., Lüdemann, J., Gross, W.L. and Bainton, D.F. (1990) Am. J. Pathol. 137, 1113–1120.
[15] Baslund, B., Segelmark, M., Wiik, A., Szpirt, W., Petersen, J. and Wieslander, J. (1995) Clin. Exp. Immunol. 99, 486–492.
[16] Sommarin, Y., Rasmussen, N. and Wieslander, J. (1995) Exp Nephrol. 3, 249–256.
[17] Butterfield, J.H., Weiler, D., Dewald, G. and Gleich, G.J. (1988) Leukemia Res. 12, 345–355.
[18] Campanelli, D., Melchior, M., Fu, Y., Nakata, M., Shuman, H., Nathan, C. and Gabay, J.E. (1990) J. Exp. Med. 172, 1709–1715.
[19] Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 61–68.
[20] Chu, G., Hayakawa, H. and Berg, P. (1987) Nucleic Acids Res. 15, 1311–1326.
[21] Specks, U., Wheatley, C.L., McDonald, T.J., Rohrbach, M.S. and DeRemee, R.A. (1989) Mayo Clin. Proc. 64, 28–36.
[22] Eblen, S.T., Fautsch, M.P., Burnette, R.J., Snyder, M. and Leof, E.B. (1995) Cancer Res. 55, 1994–2000.
[23] Nilsson, G., Blom, T., Kusche-Gullberg, M., Kjellén, L., Butterfield, J.H., Sundstršm, C., Nilsson, K. and Hellman, L. (1994) Scand. J. Immunol. 39, 489–498.
[24] Zimmer, M., Medcalf, R.L., Fink, T.M., Mattmann, C., Lichter, P. and Jenne, D.E. (1992) Proc. Natl. Acad. Sci. USA 89, 8215–8219.
[25] Specks, U., Mayer, U., Nischt, R., Spissinger, T., Mann, K., Timpl, R., Engel, J. and Chu, M.-L. (1992) EMBO J. 11, 4281–4290.
[26] Rao, N.V., Rao, G.V., Marshall, B.C. and Hoidal, J.R. (1996) J. Biol. Chem. 271, 2972–2978.
[27] Goldschmeding, R., Van der Schoot, C.E., Ten Bokkel Huinink, D., Hack, C.E., Van den Ende, M.E., Kallenberg, C.G.M. and Von dem Borne, A.E.G.K. (1989) J. Clin. Invest. 84, 1577–1587.
[28] Jenne, D.E. (1994) Am. J. Respir. Crit. Care Med. 150, S147–S154.
[29] Salvesen, G. and Enghild, J.J. (1990) Biochemistry 29, 5304–5308.
[30] Caputo, A., Garner, R.S., Winkler, U., Hudig, D. and Bleackley, R.C. (1993) J. Biol. Chem. 268, 17672–17675.
[31] McGuire, M.J., Lipsky, P.E. and Thiele, D.L. (1993) J. Biol. Chem. 268, 2458–2467.
[32] Dikov, M.M., Springman, E.B., Yeola, S. and Serafin, W.E. (1994) J. Biol. Chem. 269, 25897–25904.
[33] Smyth, M.J., McGuire, M.J. and Thia, K.Y.T. (1995) J. Immunol. 154, 6299–6305.
[34] Lüdemann, J., Utecht, B. and Gross, W.L. (1988) J. Immunol. Methods 114, 167–174.