Cloning and functional expression of the murine homologue of proteinase 3: implications for the design of murine models of vasculitis

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Abstract Anti-neutrophil cytoplasmic autoantibodies recognizing conformational epitopes (c-ANCA) of proteinase 3 (PR3) from azurophil granules are a diagnostic hallmark in Wegener's granulomatosis (WG). Because a functional PR3 homologue has not been identified in rodents, it is difficult to assess immunopathological responses in rats or mice immunized with patients' derived c-ANCA or human PR3. Here we report the full length cDNA cloning and functional expression of murine PR3 in HMC-1 cells. Recombinant murine PR3 shows highly similar substrate specificities towards synthetic peptides and is inhibited by human α1-proteinase inhibitor like human PR3. However, neither human c-ANCA, rabbit sera nor mouse monoclonal antibodies to human PR3 recognize the murine homologue. Consequently, it is unlikely that disease observed in mice after immunization with c-ANCA or human PR3 is caused by pathogenic antibodies directed against mouse PR3. Recombinant human-mouse chimaeric variants will be a valuable new tool to localize the disease-specific immunodominant epitopes in human PR3.

Key words: Neutrophil; Proteinase 3; Anti-neutrophil cytoplasmic antibody; In vivo animal model

1. Introduction

Autoantibodies specifically directed against conformational epitopes of PR3 (c-ANCA) are a disease-specific and pathognomic feature of Wegener's granulomatosis (WG). Because disease activity and PR3-specific autoantibody titers are highly correlated, autoantibodies are regarded as an important pathogenic factor in this autoimmune disease [1–3]. However, direct evidence for the pathogenic role of c-ANCA is lacking, and pathogenic epitopes on conformationally intact human PR3 recognized by c-ANCA sera of WG patients have not been identified to date. To provide direct proof for antibody-dependent mechanisms of tissue damage and neutrophil activation, appropriate animal models are being sought by several investigators [4–6]. Recently, ANCA-like antibodies have been induced in mice by immunization with xenogeneic ANCA from WG patients [7], but the interpretation of such model systems is conjectural in the absence of any knowledge about rodent PR3 homologues [8]. Indeed, observations that rabbit antibodies raised against purified human PR3 and human PR3-specific autoantibodies did not crossreact with rodent neutrophils, were taken to indicate the absence of PR3 homologues in rodents [3].

In the present study, we have identified and sequenced the murine PR3 cDNA and demonstrate that recombinant murine PR3 expressed in a human mast cell line shows highly similar functional properties toward synthetic substrates and inhibitors as human PR3. Despite these strong similarities, autoantibodies from 40 WG patients did not bind to natively folded murine PR3 indicating that the endogenous murine homologue does not present any of those target epitopes which are required to study human ANCA-induced vasculitis in mice.

2. Materials and methods

2.1. Isolation of genomic clones for murine PR3

A murine cosmid library prepared from the nuclear DNA of embryonic stem cells (line cESI) in the vector pWE (a generous gift of S. Mudgett and R. Jaenisch) was screened using the human cDNA of PR3 previously cloned [9,10]. Partial sequences obtained for the third exon of the murine PR3 were used to design the gene specific primer DJ168 (5′-CACCCAGCACCACTGTCACAAG-3′) annealing to the coding strand between position 254 and 274 (Fig. 1). The 5′ sequence of the murine cDNA was completed by partially sequencing a genomic *Bam*HI (located at position 38 within the first exon) -*Sac*I fragment covering the promoter region and part of the first exon.

2.2. Specific amplification of cDNA ends

Messenger RNA of mouse and human bone marrow was isolated using a RNA isolation kit (Stratagene) and an oligo dT column (Pharmacia). Poly A+ RNA was converted into adaptor-tagged double stranded cDNA using the Marathon cDNA amplification kit (Clontech). The 5′ end of the cDNA of PR3 was obtained by PCR with the common primer AP1 (Clontech) and the oligonucleotide DJ168. The resulting PCR product was directly sequenced after gel purification. The 3′ end of the cDNA was amplified using the two nested primers DJ186 (5′-CTC GGC CTT ATG TGG CAT CC-3′, position 128 to 147) derived from the 5′ cDNA sequence and DJ127 (5′-TGG GTS HTG ACV GCB GCS CAY TGY-3′, position 208 to 231, a degenerate primer coding for conserved amino-acid residues around the His-57 residue, Fig. 1) in combination with the adaptor primers AP1 and AP2, respectively. The PCR products obtained were subcloned into the vector pCRII (Invitrogen) and sequenced.

2.3. Functional expression of murine and human PR3 in HMC-1 cells

The full-length murine cDNA of PR3 was amplified from bone marrow cDNA using *Pfu*I DNA polymerase (Amersham) and the primers DJ398, 5′-CCAGGTACCTGTCCATGGCTGGAAGCT (derived from position 5 to 22) and DJ399, 5′-GCCATGCATCACGTTTTGAATCCAGTC-3′ (annealing between position 735 and 752). The resulting PCR product was digested with *Kpn*I and *Nsi*I and subcloned into a modified pcDNA3.1(+)Zeo vector (Invitrogen) in a way that a nucleotide sequence coding for two different tags

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Abbreviations: α1-PI; α1-protease inhibitor (=α1-antitrypsin); ANCA, anti-neutrophil cytoplasmic antibodies; c-ANCA, cytoplasmic fluorescence pattern ANCA; HNE, human neutrophil elastase; IIF, indirect immunofluorescence; mo-AB, monoclonal antibody; NGS, normal goat serum; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PR3, proteinase 3; hPR3, human PR3; hPR3-tag, human PR3 with His-Strep-tag; mPR3, mouse PR3; mPR3-tag, mouse PR3 with His-Strep-tag; rPR3, recombinant PR3; TBS, Tris buffered saline; WG, Wegener's granulomatosis

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in tandem (MQHHHHHHHHSAWRHPQFGG, called His-Strep-tag) was attached after the codon for residue 241 (bovine chymotrypsinogen A numbering, Fig. 1).

The full-length human cDNA of PR3 was amplified by PCR from human bone marrow cDNA using the primers DJ376, 5′-CCTGGATCCCACCATGGCTCAC-3′ (nucleotide position −13 to +9 relative to the translation start site containing an artificial *Bam*HI site due to a C to T replacement at position −7) and DJ388, 5′-CCAATGCATCGTAGAACGAATCCAGTC-3′ (nucleotide position −48 to −31 relative to the stop codon followed by an artificial *Nsi*I site containing sequence). The amplified cDNA fragment was directionally cloned into a *Bam*HI and *Pst*I site of a modified His-Strep-tag containing pcDNA3.1(+)Zeo vector. The His-Strep-tag was attached to the human and murine PR3 sequence at the same position after amino-acid residue 241.

The human mast cell line HMC-1 was cultured and transfected as described [11]. To select PR3 expressing cell populations, HMC-1 cells were treated with Zeocin (Invitrogen) at a concentration of 100 μl/ml after 48 h. After selection, PR3-His-Strep-tag expressing cells were subjected to cloning by limiting dilution.

2.4. Activity assays

Proteolysis of synthetic substrates was performed as described [11]. Five times 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 for 5 min. Fixed 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. Fifty μl of substrate solution (2 mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val-pNA or succinyl-Ala-Ala-Ala-pNA in 0.1 M Tris, pH 8.1) was added to the wells, and the hydrolysis of the substrate at 37°C was detected colorimetrically. Data are expressed as absorbance of *p*-nitroaniline at 405 nm minus absorbance at 490 nm. For the inhibition experiments, detergent extracts of cells prepared as described [11] were transferred into fresh wells, and incubated with the inhibitors for 30 min at 37°C, prior to the addition of substrate.

2.5. Antibodies and immunologic procedures

The monoclonal antibodies (mABs) MCPR3-1 and MCPR3-2 were raised against human PR3 by immunization of mice with granule extracts from HMC-1/hPR3 cells (U. Specks, unpublished). MCPR3-1 and MCPR3-2 recognize PR3 from HMC-1 cells and human neutrophils in ELISAs and Western blots under nonreducing and reducing conditions. The mABs WGM2, 4A3, 5A3, 6A6, and the rabbit polyclonal anti-PR3 antibody were kindly provided by Dr. E. Csernok [12] and Dr. J. Wieslander [13]. C-ANCA positive sera with confirmed human PR3 reactivity were from patients with biopsy proven WG [11]. Indirect immunofluorescence (IIF) on ethanol fixed cytospin preparations of HMC-1/VEC, HMC-1/hPR3, HMC-1/hPR3-tag, and HMC-1/mPR3-tag cells was performed as described [11].

3. Results and discussion

3.1. The cDNA sequence and structure of murine PR3

In the absence of immunological and biochemical evidence for the existence of a rodent homologue, PR3 has been considered as an elastase-related enzyme restricted to certain mammalian species including humans, primates and the horse [3,8,14]. Our attempts to find a crosshybridizing murine gene, however, were successful after screening a murine genomic library constructed from the DNA of embryonic stem cells. The nucleotide sequence of murine PR3 is 73%, the protein sequence 69% identical to the human sequences within the coding region of the mature enzyme (amino-acid residues from +16 to 243, bovine chymotrypsinogen A numbering according to [17]) (see Fig. 1). Structural features common to catalytically active serine proteases are conserved including the His-57, Asp-102 and Ser-195 residues of the catalytic triad (square boxes in Fig. 1) and the eight cysteine residues (circled) which form intramolecular disulfide bonds. The signal peptide is two residues longer than in human PR3, the carboxy-terminal peptide tail is four residues shorter. Signal peptidase most likely cleaves between alanine at position −3 and serine at position −2 as predicted by computer programs (e.g. AnalyzeSignalase). The two residues Ser-Lys following this cleavage site represent a short propeptide in analogy to human PR3 [15,16]. The carboxy-terminal tetrapeptide Gly-Ala-Glu-Pro of murine PR3 following position 243 is probably removed during biosynthesis.

Since crystal structures have been solved for human PR3 [17] and human neutrophil elastase (HNE) [18], sequence alignments with the murine PR3 (Fig. 1) permit us to suggest that substrate specificities and biological roles of the murine enzyme have not been changed significantly during mammalian evolution. Residues that line the substrate binding pockets S1 to S3 [18] are pretty well conserved between the human and murine PR3, but differ from those of HNE [17,18]. Since Ile-190 of human and murine PR3 is replaced by a Val residue in HNE, HNE accepts larger aliphatic residues in its S1. The S2 pockets of murine and human PR3 have similar physicochemical properties, but are more polar than in HNE.

3.2. Expression of enzymatically active recombinant mouse PR3 in HMC-1 cells

HMC-1 cells were shown to have the appropriate processing machinery that allows the biosynthesis and storage of enzymatically active and conformationally intact recombinant human PR3 in cytosolic granules. To demonstrate the functional equivalence between the murine and the human gene products, we generated recombinant mouse PR3 and human PR3 with the same carboxy-terminal extension peptide (mPR3-tag and hPR3-tag) and compared their activities to human (untagged) PR3 [11]. HMC-1 cells transfected with the plasmids pcDNA3.1-Zeo/mPR3-tag and pcDNA3.1-Zeo/hPR3-tag resulted in the cell lines HMC-1/mPR3-tag and HMC-1/hPR3-tag, respectively. HMC-1/HPR3 cells expressing wild type human PR3, HMC-1/PR3-S195A expressing an inactive mutant of PR3 and sham-transfected HMC-1/VEC cells served as controls [11].

To address the question of correct processing, folding and conserved substrate specificity of murine PR3 in HMC-1 cells, the best tetra-peptide substrate known for human PR3, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-pNA (AAPV), was used to assess the catalytic activity of murine PR3. The hydrolysis of this substrate by lysates of HMC-1/mPR3-tag cells was equivalent to that of HMC-1/hPR3-tag and HMC-1/HPR3 cells (Fig. 2A). No hydrolysis of this substrate was detected in parallel control experiments using lysates of HMC-1/PR3-S195A or HMC-1/VEC cells. Like the activity of wild type hPR3 and hPR3-tag, the activity of mPR3-tag for the same substrate was inhibited by greater than 95% in the presence of 1.85 μM α1-PI (Fig. 2B). No inhibition of recombinant PR3 from either species was observed in the presence of 0.1 TIU/ml aprotinin. In contrast, the activity of 2.5 ng of purified HNE, yielding the equivalent hydrolysis of AAPV in this assay, was inhibited by greater than 95% in the presence of 0.1 TIU/ml aprotinin (Fig. 2B). Like wild type human PR3 and hPR3-tag, mPR3-tag did not hydrolyze the substrate succinyl-Ala-Ala-Ala-pNA, which was cleaved by HNE in parallel control experiments (data not shown). Taken together, these results clearly support our conclusion that the serine protease homologue identified in the murine genome is not only the closest structural, but also the closest functional equivalent for human PR3 in the mouse species. Moreover, our functional

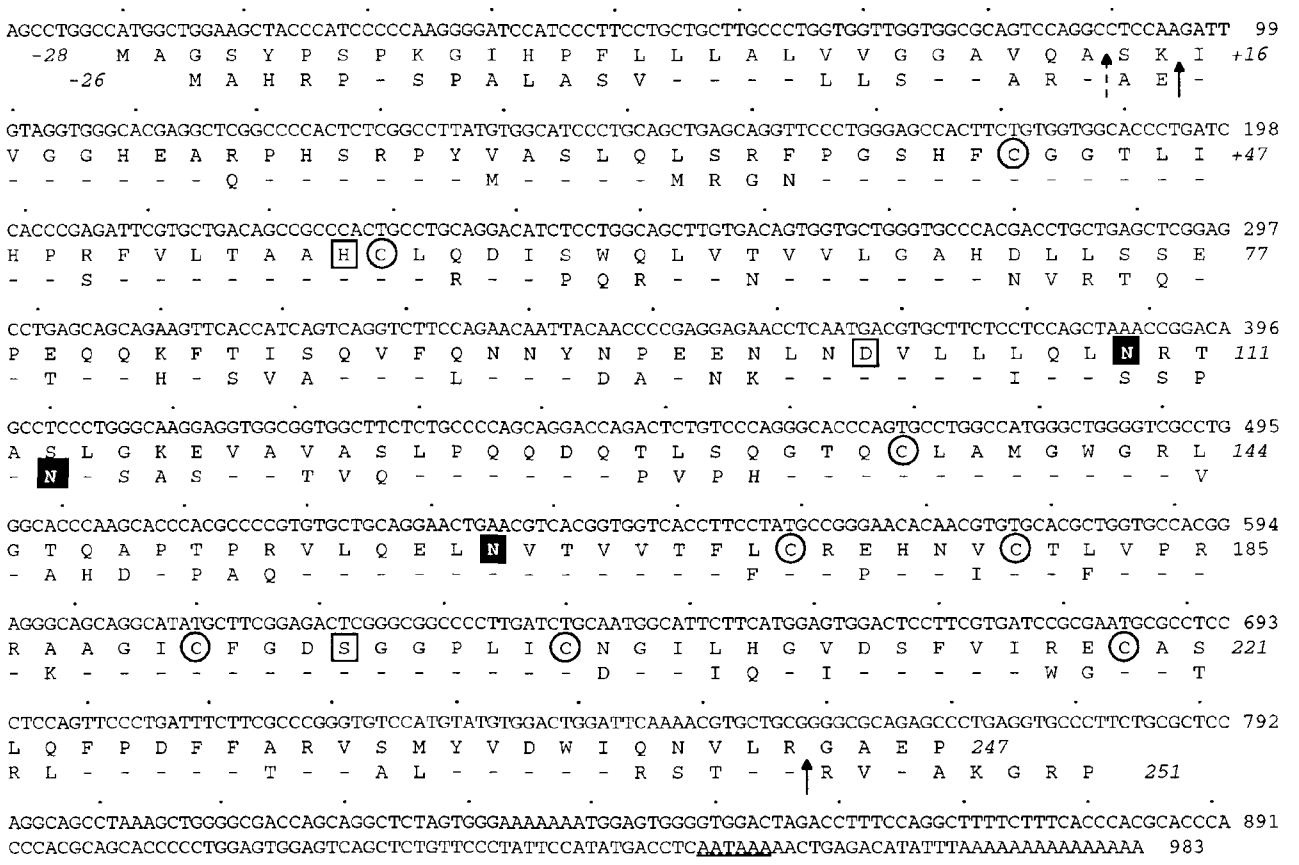

Fig. 1. Nucleotide sequence of murine PR3 and alignment of the cDNA-derived amino-acid sequence to that of human PR3. The nucleotide sequence numbering starts with the first base shown. Periods are put at every tenth position. Amino-acid residues are shown by standard one-letter symbols below the first base of the respective codon. The third row shows the amino-acid residues of human PR3 which disagree with the murine sequence, identical residues are marked by –. Protein sequences are numbered on the basis of tertiary structure similarities to bovine chymotrypsinogen A like in [17]. The residues His-57, Asp-102 and Ser-195 that form the catalytic triad, are boxed, cysteine residues are circled and *N*-glycosylation sites are highlighted by black squares. The polyadenylation signal AATAAA in the 3′ untranslated region has been underlined. The vertical solid arrows indicate the predicted sites of amino- and carboxy-terminal biosynthetic processing, the dashed vertical arrow the most likely site of cleavage by signal peptidase.

studies demonstrate that HMC-1 cells also have the capacity to correctly process the murine propeptide which requires cleavage after a lysine instead of cleavage after a glutamate residue.

3.3. Antigenic sites differ between human and murine PR3

Antigenicity of hPR3, hPR3-tag and mPR3-tag was studied by IIF using the mABs WGM2, 4A5, 5A6, 6A6, MCPR3-1 and MCPR3-2 as well as a rabbit polyclonal anti-PR3 serum that had been generated by immunization with native human PR3 [13]. None of these antibodies detected mouse PR3 in HMC-1/mPR3-tag cells. By assessing reactivity of antibodies toward mPR3-tag expressing HMC-1 cells we circumvented the problem of antigen-independent binding of immunoglobulins to mouse neutrophils. Our results clearly show that the antigenic determinants of native human PR3 that are recognized by rabbit as well as mouse monoclonal antibodies, are not preserved on mouse PR3. This probably explains why the existence of mouse PR3 has been elusive to date.

3.4. ANCA lack specificity for murine PR3

To evaluate the feasibility of vasculitis induction in mice by passive transfer of c-ANCA, active idiotypic immunization with c-ANCA [7] or immunization with human PR3, we addressed the question whether human c-ANCA have the properties to directly bind to murine PR3. Even the concept of disease induction by idiotypic immunization implies that the finally emerging anti-idiotype antibodies against c-ANCA-specific murine antibodies recognize both the human PR3 as well as the murine homologue. In previous studies the binding of murine antibodies to human PR3 from immunized mice had been substantiated [7,19], but the reactivity of murine anti-idiotype antibodies toward murine neutrophils remained speculative.

Forty c-ANCA positive unselected serum samples with high titers (median 1:256, range 1:128 to 1:1024), as determined by standard IIF using neutrophil cytospin preparations, were, therefore, evaluated in parallel on HMC-1/hPR3, HMC-1/hPR3-tag, and HMC-1/mPR3-tag cells by IIF at serum dilutions of 1:20. All 40 sera reacted with both human PR3 variants, but none reacted with mouse PR3-tag expressing cells. These data indicate that the epitopes of human PR3 recognized by human autoantibodies are not preserved on mouse PR3. Since all sera recognized the human wild type PR3 as well as the human PR3-tag-modified protein, we are able to rule out that the His-Strep-tag has changed the tertiary structure of murine PR3 to a degree that would disturb its binding to c-ANCA. Since α1-PI binding and recognition of identical

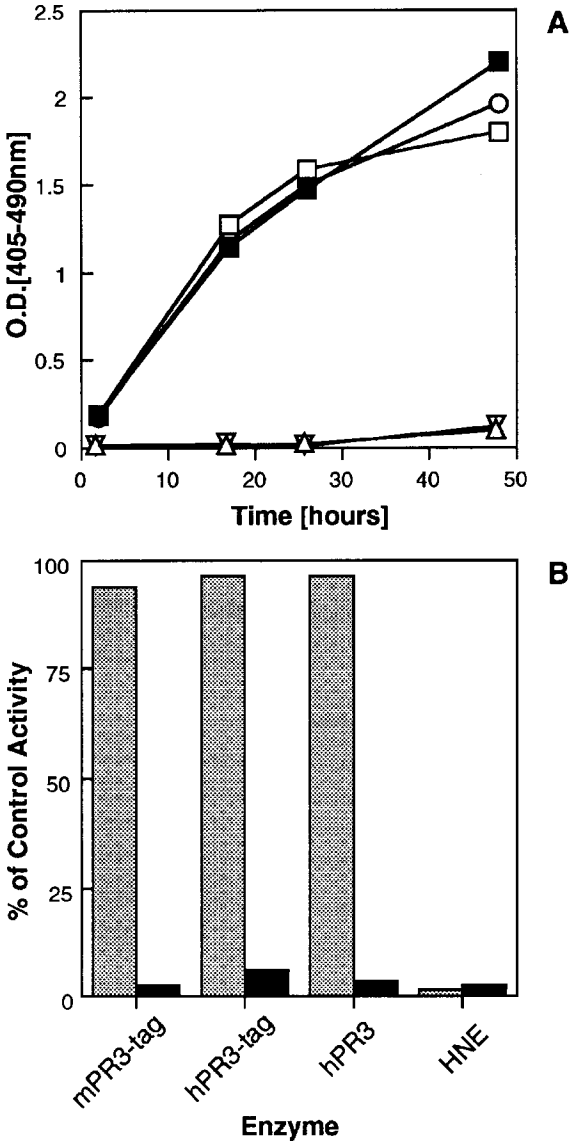

Fig. 2. Enzymatic activity of recombinant mouse PR3. (A) Hydrolysis of the substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-pNA by HMC-1/mPR3-tag (full squares), HMC-1/hPR3-tag (open squares), and HMC-1/hPR3 cells (open circles) was equivalent. No hydrolysis of this substrate was observed with lysates of HMC-1/PR3-S195A (triangles) or HMC-1/VEC cells (inverted triangles). (B) Hydrolysis of this substrate by tagged mouse and human PR3 as well as wild type human PR3 was inhibited by greater than 95% by 1.85 μM α1-PI (black bars), but not by 0.1 TIU/ml aprotinin (grey bars). HNE served as control in these experiments. Each data point represents the mean of triplicates. Shown are representative examples of five (A) and four (B) different experiments.

tetrapeptide substrates implies a certain degree of surface conservation between the murine and human PR3, the absence of cross-reactivity of c-ANCA with murine PR3 is rather surprising. These data suggest that the passive transfer of human c-ANCA and the idiotypic immunization of mice with human c-ANCA resulting in a murine vasculitis [7,19] do not provide an animal model that allows the unequivocal clarification of the pathogenicity of WG-specific autoantibodies.

The presence of a tag at the carboxy-terminus of human and murine PR3 obviously does not affect their enzymatic activities toward specific synthetic substrates, their inhibition by large molecular weight inhibitors like α1-PI, and the antigenicity of human PR3 toward c-ANCA which is conformation sensitive. Carboxy-terminally tagged PR3 molecules are sorted and stored in granules of HMC-1 cells like wild type PR3 (data not shown) and do not interfere with the subcellular routing of granule-associated serine proteases. Our observations are consistent with results from previous studies showing that deletion variants of HNE and cathepsin G lacking the carboxy-terminal propeptides have identical catalytic activities and are efficiently targeted to granules [20]. Adding a versatile peptide tag at the carboxy-terminus with affinities for protein ligands (streptavidin) and Ni-NTA matrices should facilitate the immunological characterization of c-ANCA epitopes using purified human-mouse chimaeric PR3 variants in the future.

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