

Review

What you should know about PR3-ANCA

Conformational requirements of proteinase 3 (PR3) for enzymatic activity and recognition by PR3-ANCA

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Abstract

The neutrophil azurophil granule constituent proteinase 3 (PR3) is the principal antigen for anti-neutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis. The conformation of the mature PR3 enzyme results from intracellular post-translational processing. The nascent molecule undergoes proteolytic cleavage of the amino-terminal signal peptide and activation dipeptide and of a carboxy-terminal peptide extension. The conformation of PR3 is stabilized by four disulfide bonds and, to a lesser extent, by asparagine-linked glycosylation. Most anti-neutrophil cytoplasmic antibodies directed against proteinase 3 (PR3-ANCA) recognize conformational epitopes. The expression of recombinant PR3 has provided a better understanding of the significance of the various intracellular processing steps for enzymatic activity and recognition by PR3-ANCA.

Keywords: anti-neutrophil cytoplasmic antibodies, proteinase 3, recombinant proteins

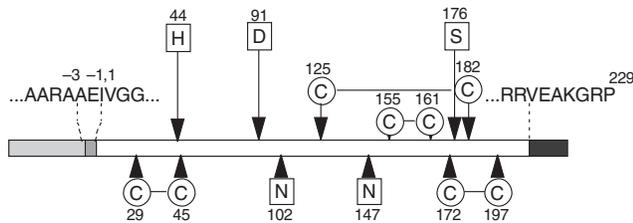
PR3-ANCA have been implicated in the pathogenesis of vasculitis [1,2], and direct functional interactions of PR3-ANCA with the PR3 molecule have been described [3–5]. Some PR3-ANCA can interfere with the complexation of PR3 with its natural inhibitor, α 1-proteinase inhibitor (α 1-PI), while others can directly inhibit the enzymatic activity of PR3. These observations suggest that different PR3-ANCA subsets bind to different epitopes on the PR3 molecule with potentially opposing functional implications. Competition studies of PR3-ANCA with anti-PR3 monoclonal antibodies further indicate that PR3-ANCA from patients are directed against a restricted number of differ-

ent epitopes on PR3 [6]. Consequently, to better understand the pathogenic potential of PR3-ANCA we must delineate the heterogeneous interactions of PR3-ANCA with its target antigen by identifying the specific epitopes reactive with PR3-ANCA and the functional impact of binding of PR3-ANCA to these specific epitopes.

Most of the biological functions ascribed to PR3 are dependent on its proteolytic activity. The expression of PR3 on the surface of activated neutrophils [7] and its enzymatic substrates, which include basement membrane proteins [8], suggests that PR3 may facilitate the migration

ANCA = anti-neutrophil cytoplasmic antibodies; α 1-PI = α 1-proteinase inhibitor; C1 = complement factor 1; ELISA = enzyme-linked immunosorbent assay; HMC = human mast cell; HMC-1/PR3-S176A cells = HMC-1 cells expressing rPR3-S176A; IL-1 β , interleukin 1- β ; IL-8 = interleukin 8; NADPH = nicotinamide adenine dinucleotide phosphate; PR3 = proteinase 3; PR3-ANCA = anti-neutrophil cytoplasmic antibodies directed against proteinase 3; rPR3 = recombinant PR3; rPR3-S176A = rPR3 with the substitution of the serine 176 residue with alanine; Δ -rPR3-S176A = amino-terminally processed rPR3-S176A expressed in 293 cells; TGF- β = transforming growth factor- β ; TNF- α = tumor necrosis factor- α .

Figure 1



Schematic diagram of the PR3 molecule. The light and dark gray bars represent the amino-terminal signal peptide and activation dipeptide, respectively. The mature enzyme is represented by the white bar, and the carboxy-terminal peptide extension is represented by the black bar. The cysteine residues forming the four disulfide bonds (horizontal black lines) are shown as circled residues. The asparagine residues at positions 102 and 147 represent two potential glycosylation sites. The histidine-44, aspartic acid-91, and serine-176 residues make up the active site. The single-letter code for amino acid residues is used. Amino acids are numbered consecutively starting with the first residue of the mature enzyme [20]. The diagram is not drawn to scale.

of neutrophils to tissue sites of inflammation. The proteolytic activity of PR3 has also been implicated in modulating the activity of platelets and endothelial cells [9] and of inflammatory mediators such as complement factor 1 (C1) inhibitor [10], IL-8 [11], tumor necrosis factor (TNF)- α [12,13], IL-1 β [13], or transforming growth factor (TGF)- β [14]. On the other hand, some potential biological activities of PR3, such as its bactericidal activity [15], the induction of IL-8 secretion from endothelial cells [16], and the blockade of neutrophil NADPH oxidase activation [17], appear to be independent of its proteolytic activity, suggesting that they are mediated by other structural domains of the molecule.

The physiological and pathogenic functions of PR3 are clearly determined by its ability to bind to other molecules, such as specific substrates for proteolytic cleavage, specific enzyme inhibitors, or PR3-ANCA. These molecular interactions depend on the intact conformation of various structural domains of PR3. The final conformation of mature PR3 is stabilized by four disulfide bonds and appropriate asparagine-linked glycosylation, and it requires the cleavage of the amino-terminal activation dipeptide [18,19] (Fig. 1). The significance of carboxy-terminal processing of PR3 is less well defined [20]. Like lymphocyte granzymes and the other neutrophil serine proteases elastase and cathepsin G, PR3 undergoes several proteolytic processing steps [18,19,20]. PR3 is synthesized as a proenzyme. After cleavage of the signal peptide and leaving the endoplasmic reticulum, the nascent PR3 molecule still carries an amino-terminal activation dipeptide, which maintains the proenzyme in its inactive conformation [20]. Subsequent cleavage of this amino-terminal propeptide allows insertion of the freed amino-terminus into the

interior of the molecule, where it interacts with the aspartic acid residue adjacent to the serine of the active site. This renders the active-site pocket of the enzyme accessible to substrates [21]. In the azurophil granules of neutrophils, PR3 is stored in its amino-terminally processed, enzymatically active form [22].

Research in the expression of recombinant PR3 (rPR3) has been driven by several quests: that to obtain abundant amounts of purified target antigen for sensitive and specific solid-phase assay systems allowing reliable detection and quantification of PR3-ANCA in sera of patients; the quest to allow modifications of the molecule for the study of intracellular processing and structure–function relationships of PR3; and the quest to allow the identification of epitopes recognized by PR3-ANCA. To these ends, rPR3 has been expressed using bacterial (*Escherichia coli*) [23,24], yeast (*Pichia pastoris*) [24], baculovirus (Sf9 cells) [21,25,26], and mammalian (HMC-1, RBL-1, 32D, and 293 cells) expression systems [27–29]. These investigations have provided valuable insights about the intracellular processing of PR3 and its effects on the structure of PR3 and about the conformational nature of PR3-ANCA epitopes.

Bini and coworkers first demonstrated that most human PR3-ANCA recognize conformational epitopes [23]. More than half of PR3-ANCA that have been boiled in sodium dodecyl sulfate no longer recognize PR3 in Western blots. Reduction of PR3 completely abolished the recognition by more than 90% of PR3-ANCA [23]. Exposure of PR3 to conditions that only partially denature the protein, such as low pH, is also sufficient to destroy PR3 antigenicity [23]. Furthermore, a recombinant PR3 (rPR3) fusion protein expressed in *E. coli* that did not contain the amino-terminal PR3 signal peptide or the activation dipeptide sequence (thus coding for mature PR3) was not recognized by PR3-ANCA, and neither was an *in vitro* translation product of rPR3 that contained the reducing agent dithiothreitol [23]. Thus, intact disulfide bonds are crucial for PR3's antigenicity. Other post-translational modifications of native PR3 not occurring in *E. coli* may also be prerequisites for proper recognition by PR3-ANCA. Another rPR3 product expressed in *E. coli* was also not recognized by PR3-ANCA [24]: this particular variant did contain the amino-terminal propeptide extension. When the same construct was expressed in the yeast *P. pastoris*, the expressed rPR3 product was recognized by 7 of 10 PR3-ANCA-positive sera when the rPR3 was used as target antigen in a capture enzyme-linked immunosorbent assay (ELISA), but only by 1 of 10 when it was used in a direct ELISA [24]. This suggests that not all conformational epitopes recognized by PR3-ANCA are displayed on rPR3 expressed in *P. pastoris*. Furthermore, the conformation of PR3-ANCA epitopes appears more vulnerable to modifications caused by the purification procedure or the binding to plastic than that of epitopes of native neutrophil PR3.

Using the baculovirus system, rPR3 has also been expressed in insect cells [21*,25,26]. rPR3 expressed in Sf9 cells assumes the conformation of the active enzyme [21*] and is recognized by the majority of PR3-ANCA [30], provided the amino-terminal activation dipeptide is cleaved *in vitro* after purification of the proenzyme. Disulfide bonds are formed appropriately in Sf9 cells. Other post-translational modifications of rPR3 are either lacking or inappropriate for the generation of the active enzyme recognized by all PR3-ANCA [25,26]. The amino-terminal propeptide is not cleaved intracellularly [21*], and asparagine-linked glycosylation of rPR3 in Sf9 cells is different from that of PR3 purified from neutrophils [26]. The majority of asparagine-linked glycosylation isoforms of rPR3 from Sf9 cells have a molecular mass of 34 kDa, whereas the majority of the native PR3 glycosylation isoforms purified from azurophil granules of neutrophils have a mass of about 29 kDa [26]. In addition, in the reported crystal structure of PR3, which is based on rPR3 expressed in Sf9 cells, only one of the two potential asparagine-linked glycosylation sites appeared occupied [21*], whereas both sites are used in rPR3 expressed in hematopoietic cells and in native PR3 from neutrophils [31].

When rPR3 is expressed in hematopoietic cells, it is processed to an active enzyme and stored in granules [27,28]. All PR3-ANCA recognize rPR3 expressed in the human mast cell line HMC-1 [32]. As indicated by immunofluorescence and capture ELISA data, the affinity of PR3-ANCA to HMC-1 cell rPR3 is similar to that of neutrophil PR3 [32,33]. This similarity suggests that the conformational epitopes recognized by PR3-ANCA are fully accessible on rPR3 expressed in HMC-1 cells. Furthermore, the recognition of rPR3 by PR3-ANCA is not affected by the substitution of the active site serine by an alanine residue (S176A), indicating that the conformational epitopes recognized by PR3-ANCA are not affected by this mutation even though it alters the active site sufficiently to render the molecule enzymatically inactive [27,33]. This mutation subsequently allowed us to express rPR3 variants in the epithelial cell line 293 [29]. The overwhelming majority of rPR3 expressed in 293 cells is secreted into the media supernatant in an unprocessed form [29]. Two rPR3 variants representing the amino-terminally unprocessed pro-form of PR3 (rPR3-S176A) and the amino-terminally processed mature form of PR3 (Δ -rPR3-S176A) were expressed in 293 cells [29]. In the capture ELISA, PR3-ANCA recognize mature rPR3 contained in HMC-1/PR3-S176A cells just as well as they recognize that contained in media supernatants of Δ -rPR3-S176A expressing 293 cells [29,33]. In contrast, the proform variant of rPR3 is not recognized by all PR3-ANCA [29]. While some PR3-ANCA sera show the same reactivity with both mature and pro-rPR3, most of them bind less well to pro-PR3 than to mature rPR3 [29]. This indicates that some PR3-ANCA epitopes are equally

accessible on mature and pro-PR3, whereas other epitopes are less or not at all accessible on pro-PR3.

The clinical relevance of the differential recognition of these rPR3 variants with different conformations is currently under investigation. Preliminary evidence obtained using our capture ELISA suggests that the correlation of clinical disease activity with PR3-ANCA reacting with pro-PR3 is more readily apparent than that of the reactivity with mature PR3 [34].

While preservation of proper disulfide bonds in the PR3 molecule is crucial for recognition of PR3-ANCA, and more PR3-ANCA appear to recognize the mature form of the enzyme than the amino-terminally unprocessed proform, the carboxy-terminal processing and asparagine-linked glycosylation appear less relevant. The fact that rPR3 secreted into the 293 cell media supernatant is carboxy-terminally unprocessed (my unpublished data) implies that carboxy-terminal processing of PR3 has little effect on the antigenicity of PR3 [29]. This interpretation is supported by the observation that an rPR3 construct carrying a C-terminal poly-histidine extension is recognized just as well by PR3-ANCA as is rPR3 without this extension [35].

Several lines of evidence suggest that asparagine-linked glycosylation of PR3 does not significantly affect recognition of PR3-ANCA. Deglycosylated neutrophil PR3 is recognized by PR3-ANCA serum and by the conformation-sensitive monoclonal antibody WGM2 with the same affinity as native PR3 purified from neutrophils [26]. When expressed in hematopoietic cell lines or in 293 cells, rPR3 with large oligosaccharides is secreted into the media. In contrast, the intracellularly retained rPR3 is not as heavily glycosylated [27–29]. PR3-ANCA recognize the heavily glycosylated rPR3 in 293 cell media supernatants as well as the rPR3 stored in granules of HMC-1/PR3 cells and as purified neutrophil PR3 [29,33]. Conversely, asparagine-linked glycosylation mutants of rPR3 expressed in HMC-1 cells are recognized by PR3-ANCA [31]. Consequently, even though the asparagine-linked glycosylation status of PR3 affects the enzymatic activity and thermostability of the molecule, it has little effect on the accessibility of conformational epitopes by PR3-ANCA [31].

Conclusion

The biological functions of PR3 and its recognition by PR3-ANCA are defined by the molecule's conformation. Its exact conformation is the result of several intracellular post-translational processing steps that appear to be largely restricted to cells of hematopoietic lineage. Mammalian expression systems for conformationally intact rPR3 represent very promising tools for the identification of specific PR3-ANCA epitopes and will help to clarify existing controversies about the nature of these epitopes [36,37].

References

Articles of particular interest have been highlighted as:

- of special interest
- of outstanding interest

1. Jennette JC, Falk RJ: **Pathogenesis of the vascular and glomerular damage in ANCA-positive vasculitis.** *Nephrol Dial Transplant* 1998, **13**:16–20.
2. Kallenberg CGM, Heeringa P: **Pathogenesis of vasculitis.** *Lupus* 1998, **7**:280–284.
3. van de Wiele BA, Dolman KM, van der Meer-Gerritsen CH, Nack CE, von dem Borne AEGK, Goldschmeding R: **Interference of Wegener's granulomatosis autoantibodies with neutrophil proteinase 3 activity.** *Clin Exp Immunol* 1992, **90**:409–414.
4. Dolman KM, Stegeman CA, van de Wiele BA, et al: **Relevance of classic anti-neutrophil cytoplasmic autoantibody (C-ANCA)-mediated inhibition of proteinase 3- α 1-antitrypsin complexation to disease activity in Wegener's granulomatosis.** *Clin Exp Immunol* 1993, **93**:405–410.
5. Daouk GH, Palsson R, Arnaout MA: **Inhibition of proteinase 3 by ANCA and its correlation with disease activity in Wegener's granulomatosis.** *Kidney Int* 1995, **47**:1528–1536.
6. Sommarin Y, Rasmussen N, Wieslander J: **Characterization of monoclonal antibodies to proteinase 3 and application in the study of epitopes for classical anti-neutrophil cytoplasm antibodies.** *Exp Nephrol* 1995, **3**:249–256.
7. Csernok E, Ernst M, Schmitt W, Bainton DF, Gross WL: **Activated neutrophils express proteinase 3 on their plasma membrane *in vitro* and *in vivo*.** *Clin Exp Immunol* 1994, **95**:244–250.
8. Rao NV, Wehner NG, Marshall BC, Gray WR, Gray BH, Hoidal JR: **Characterization of proteinase-3 (PR-3), a neutrophil serine proteinase.** *J Biol Chem* 1991, **266**:9540–9548.
9. Renesto P, Si-Tahar M, Moniatte M, et al: **Specific inhibition of thrombin-induced cell activation by the neutrophil proteinases elastase, cathepsin G, and proteinase 3: evidence for distinct cleavage sites within the aminoterminal domain of the thrombin receptor.** *Blood* 1997, **89**:1944–1953.
10. Leid RW, Ballieux BEPB, van der Heijden I, et al: **Cleavage and inactivation of human C1 inhibitor by the human leukocyte proteinase, proteinase 3.** *Eur J Immunol* 1993, **23**:2939–2944.
11. Padrines M, Wolf M, Walz A, Baggiolini M: **Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3.** *FEBS Lett* 1994, **352**:231–235.
12. Robache-Gallea S, Morand V, Bruneau JM, et al: ***In vitro* processing of human tumor necrosis factor- α .** *J Biol Chem* 1995, **270**:23688–23692.
13. Coeshott C, Ohnemus C, Pilyavskaya A, et al: **Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3.** *Proc Natl Acad Sci USA* 1999, **96**:6261–6266.
14. Csernok E, Szymkowiak CH, Mistry N, Daha MR, Gross WL, Kekow J: **Transforming growth factor-beta (TGF- β) expression and interaction with proteinase 3 (PR3) in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis.** *Clin Exp Immunol* 1996, **105**:104–111.
15. Gabay JE, Almeida RP: **Antibiotic peptides and serine protease homologs in human polymorphonuclear leukocytes: defensins and azurocidin.** *Curr Opin Immunol* 1993, **5**:97–102.
16. Berger SP, Seelen MAJ, Hiemstra PS, et al: **Proteinase 3, the major autoantigen of Wegener's granulomatosis, enhances IL-8 production by endothelial cells *in vitro*.** *J Am Soc Nephrol* 1996, **7**:694–701.
17. Tal T, Michaela S, Aviram I: **Cationic proteins of neutrophil azurophilic granules: protein-protein interaction and blockade of NADPH oxidase activation.** *J Leuk Biol* 1998, **63**:305–311.
18. Jenne DE: **Structure of the azurocidin, proteinase 3, and neutrophil elastase genes. Implications for inflammation and vasculitis.** *Am J Respir Crit Care Med* 1994, **150**:S147–154.
19. Gullberg U, Andersson E, Garwicz D, Lindmark A, Olsson I: **Biosynthesis, processing and sorting of neutrophil proteins: insight into neutrophil granule development.** *Eur J Haematol* 1997, **58**:137–153.
20. Rao NV, Rao GV, Marshall BC, Hoidal JR: **Biosynthesis and processing of proteinase 3 in U937 cells.** *J Biol Chem* 1996, **271**:2972–2978.
Describes the post-translational modifications including amino- and carboxy-terminal proteolytic processing of endogenously synthesized PR3 in promyelomonocytic cells.
21. Fujinaga M, Chernaia MM, Halenbeck P, Kothe K, James MNG: **The crystal structure of PR3, a neutrophil serine proteinase antigen of Wegener's granulomatosis antibodies.** *J Mol Biol* 1996, **261**:267–278.
This first structural model for PR3 based on rPR3 expressed in insect cells facilitates investigations of the role of PR3 in inflammatory diseases including Wegener's granulomatosis and neutrophil-mediated lung disease.
22. Kao RC, Wehner NG, Skubitz KM, Gray BH, Hoidal JR: **A distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters.** *J Clin Invest* 1988, **82**:1963–1973.
23. Bini P, Gabay JE, Teitel A, Melchior M, Zhou J-L, Elkon KB: **Antineutrophil cytoplasmic autoantibodies in Wegener's granulomatosis recognize conformational epitopes on proteinase 3.** *J Immunol* 1992, **149**:1409–1415.
24. Harmsen MC, Heeringa P, Van Der Geld YM, et al: **Recombinant proteinase 3 (Wegener's antigen) expressed in *Pichia pastoris* is functionally active and is recognized by patient sera.** *Clin Exp Immunol* 1997, **110**:257–264.
25. Szymkowiak CH, Johnston TW, Csernok E, Gross WL: **Expression of the human autoantigen of Wegener's granulomatosis (PR3) in a baculovirus expression system.** *Biochem Biophys Res Commun* 1996, **219**:283–289.
26. Witko-Sarsat V, Halbwegs-Mecarelli L, Almeida RP, et al: **Characterization of a recombinant proteinase 3, the autoantigen in Wegener's granulomatosis and its reactivity with anti-neutrophil cytoplasmic autoantibodies.** *FEBS Lett* 1996, **382**:130–136.
27. Specks U, Fass DN, Fautsch MP, Hummel AM, Viss MA: **Recombinant human proteinase 3, the Wegener's autoantigen, expressed in HMC-1 cells is enzymatically active and recognized by c-ANCA.** *FEBS Lett* 1996, **390**:265–270.
28. Garwicz D, Lindmark A, Hellmark T, Gladh M, Jögl J, Gullberg U: **Characterization of the processing and granular targeting of human proteinase 3 after transfection to the rat RBL or the murine 32D leukemic cell lines.** *J Leukocyte Biol* 1997, **61**:113–123.
29. Sun J, Fass DN, Viss MA, et al: **A proportion of proteinase 3-specific anti-neutrophil cytoplasmic antibodies only react with proteinase 3 after cleavage of its N-terminal activation dipeptide.** *Clin Exp Immunol* 1998, **114**:320–326.
30. Van der Geld YM, Oost-Kort W, Limburg PC, Specks U, Kallenberg CGM: **Recombinant proteinase 3 produced in different expression systems: recognition by anti-PR3 antibodies.** *J Immunol Methods* 2000, in press.
31. Specks U, Fass DN, Hummel AM, Viss MA, Tang H: **N-linked glycosylation of proteinase 3: effect on enzymatic activity and targeting to granules [abstract].** *Am J Respir Crit Care Med* 1998, **157**:A142.
32. Specks U, Wiegert EM, Homburger HA: **Human mast cells expressing recombinant proteinase 3 (PR3) as substrate for clinical testing for anti-neutrophil cytoplasmic antibodies (ANCA).** *Clin Exp Immunol* 1997, **109**:286–295.

33. Sun J, Fass DN, Hudson JA, Viss MA, Homburger HA, Specks U: **Capture-ELISA based on recombinant proteinase 3 (PR3) is sensitive for PR3-ANCA testing and allows detection of PR3 and PR3-ANCA/PR3 immunocomplexes.** *J Immunol Methods* 1998, **211**: 111–123.
34. Russell KA, Specks U: **Not all PR3-ANCA are equal: significance of subsets [abstract].** *Am J Respir Crit Care Med* 1999, **159**:A340.
35. Van der Geld YM, Smook M, Limburg PC, Kallenberg CGM: **Expression of recombinant proteinase 3, the autoantigen in Wegener's granulomatosis, in insect cells [abstract].** *Arthritis Rheum* 1999, **42**:S175.
36. Williams RC Jr, Staud R, Malone CC, Payabyab J, Byres L, Underwood D: **Epitopes on proteinase-3 recognized by antibodies from patients with Wegener's granulomatosis.** *J Immunol* 1994, **152**: 4722–4737.
37. Chang L, Binos S, Savage J: **Epitope mapping of anti-proteinase 3 and anti-myeloperoxidase antibodies.** *Clin Exp Immunol* 1995, **102**:112–119.

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