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TECHNICAL NOTE

Effects of carboxy-terminal modifications of proteinase 3 (PR3) on the recognition by PR3-ANCA

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Effects of carboxy-terminal modifications of proteinase 3 (PR3) on the recognition by PR3-ANCA.

Background. Autoantibodies directed against neutrophil proteinase 3 (PR3-ANCA) from patients with Wegener's granulomatosis and microscopic polyangiitis recognize conformational epitopes of PR3. During maturation of neutrophils, PR3 undergoes amino-terminal and carboxy-terminal processing. In contrast to amino-terminal processing, the effects of carboxy-terminal processing on recognition of PR3 by PR3-ANCA remain unknown. Carboxy-terminally modified or tagged recombinant PR3 (rPR3) molecules may be useful for the refinement of diagnostic assays and for the study of biological processes.

Methods. This study was designed to determine whether 293 cells can be used to express specifically designed carboxy-terminal variants of rPR3, and to evaluate the effects of different carboxy-terminal modifications on the recognition by PR3-ANCA in the capture ELISA.

Results. The rPR3-variants secreted into the media supernatants of transfected 293 cells escaped proteolytic processing. Furthermore, in contrast to the effects of amino-terminal propeptide deletion on PR3-ANCA binding, carboxy-terminal modifications (deletion and additions) did not significantly affect recognition by PR3-ANCA.

Conclusions. This expression system is ideally suited for the expression of custom-designed carboxy-terminal rPR3 variants, and major conformational effects of carboxy-terminal modifications seem unlikely.

Anti-neutrophil cytoplasmic antibodies (ANCA) causing cytoplasmic immunofluorescence (C-ANCA) on ethanol-fixed neutrophils are highly specific for the small vessel vasculitides Wegener's granulomatosis (WG), microscopic polyangiitis and, organ limited variants like pauci-immune necrotizing glomerulonephritis [1, 2]. Neutrophil proteinase 3 (PR3) is the principle target antigen for C-ANCA [3].

The neutrophil serine proteases including PR3 are subject to post-translational processing prior to being stored in granules in their mature form. The product of PR3 mRNA translation is a preproenzyme that undergoes three subsequent proteolytic modifications: cleavage of the signal peptide, of the amino-terminal pro-dipeptide Ala-Glu, and of a carboxy-terminal pro-heptapeptide [4–6].

Proteinase 3 recognition by PR3-ANCA depends upon the preservation of conformational epitopes [7]. Amino-terminal processing of the PR3 molecule critically affects recognition by a subset of PR3-ANCA, which may correlate with disease activity [8, 9]. Unlike the conformational and functional effects of amino-terminal processing of PR3, the physiologic significance and effects of carboxy-terminal processing remain unknown. Furthermore, the addition of custom-designed tags to recombinant PR3 (rPR3) variants could facilitate studies of intracellular processing, purification of rPR3, and subsequent use of such rPR3 variants in solid phase assays for PR3-ANCA detection, as labeled competitors in inhibition assays, or as ligands for molecular and cell binding studies. Therefore, this study was designed to determine whether 293 cells secrete rPR3 variants with intact carboxy-terminal propeptide extensions, and to evaluate the effect of different carboxy-terminal modifications of rPR3 on the recognition by PR3-ANCA.

METHODS

Unless specified otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Purified neutrophil PR3 (PMN-PR3) was purchased from Athens Research and Technology (Athens, GA, USA). The monoclonal anti-PR3 antibody (mAb), MCPR3-2, has been characterized previously [10]. The polyclonal rabbit anti-PR3 antibody was generated by immunization with PMN-PR3. The mAb directed against the c-myc poly-

Key words: ANCA, proteinase 3, small vessel vasculitis, Wegener's granulomatosis, pauci-immune glomerulonephritis, 293 cells, recombinant PR3.

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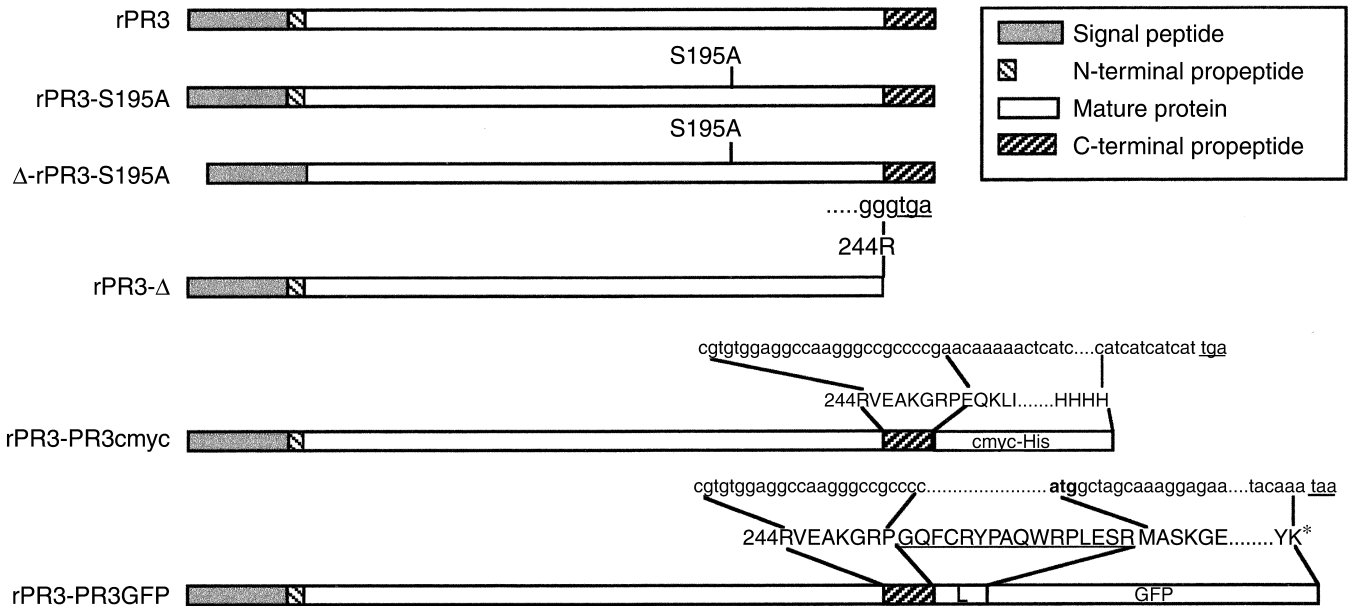

Fig. 1. Schematic diagram of cDNA constructs. The nucleotide sequence (small letters) is listed above the amino acid sequence. Stop codons are underlined. The single letter code (capital letters) and the bovine chymotrypsinogen numbering system for serine proteases of the trypsin family are used to identify amino acid residues and their positions. S195A indicates the substitution of the active site serine at position 195 by an alanine residue. 244R indicates the arginine residue after which the carboxy-terminal proheptapeptide of proteinase 3 (PR3) is cleaved. Abbreviations are: L, linker peptide; GFP, green fluorescence protein.

peptide (cmyc) and rabbit polyclonal anti-green fluorescent protein (GFP) antibody were purchased from Invitrogen (San Diego, CA, USA).

C-ANCA positive sera (titer range 1:4 to 1:256) were obtained from 58 patients with biopsy-proven WG evaluated at Mayo Clinic (34% had active disease at the time of sampling). All 58 samples were PR3-ANCA positive by capture enzyme-linked immunosorbent assay (ELISA) using PMN-PR3 as the substrate. Their use for this study was approved by the Institutional Review Board at Mayo Clinic.

Figure 1 shows the rPR3 cDNA constructs used. Amino acid numbers are based on tertiary structure similarities with bovine chymotrypsinogen A apparent from the crystal structure of human PR3 facilitating the sequence alignment of similar serine proteases [11, 12]. The original cDNA insert coding for wild-type rPR3 (rPR3) and those coding for the active site mutant, rPR3-S195A, and the amino-terminal propeptide deletion mutant, Δ-rPR3-S195A, as well as their expression in 293 cells have been described elsewhere [5, 8]. For the generation of the construct rPR3-cmyc, HindIII/XbaI restricted wild-type rPR3 was used as the polymerase chain reaction (PCR) template with the primers US124 5′-AAGCTTCCCAC CATGGCT-3′ (sense) and US126 5′-GGGCCCGGGG CGGC-3′ (antisense), generating an ApaI restriction site overhang adjacent to the codon for residue Pro-251. This PCR product was subcloned into HindIII/ApaI restricted expression vector pcDNA3/myc-His (Invitrogen). The construct rPR3-cmyc codes for an rPR3 variant carrying a carboxy-terminal 21 residue extension following Pro-251 of PR3, which consists of the c-myc peptide followed by six His-residues. This extension is recognized by antibodies to c-myc and poly-His. The poly-His extension is designed to facilitate purification of proteins by binding to nickel-resins.

The construct rPR3-GFP was generated by PCR reaction using the primers US203 5′-AAGCTTCCCACC ATGGCTCACC-3′ (sense) and US204 5′-CGGGGCG GCCCTTGGCCT-3′ (antisense) and cloning the PCR product into the pcDNA3.1/CT-GFP-TOPO vector using the CT-GFP Fusion TOPO TA Expression Kit (Invitrogen). The construct codes for a fusion protein consisting of the 229 amino acid residues of PR3, 16 linker residues, and the 239 residues of GFP. The carboxy-terminal GFP extension conveys autofluorescence of tagged proteins facilitating the tracking of their expression and cellular localization.

The cDNA construct rPR3-Δ was prepared using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The PCR primers US223 5′-CCACG CTGCGCCGTTGAGAGGCCAAGGGCCGC-3′ (sense) and US224 5′-GCGGCCCTTGGCCTCTCAACGGCG CAGCGTGG-3′ (antisense) were designed to replace the codon for Val-245 with a translation stop codon after residue Arg-244, the presumed cleavage site of the carboxy-terminal PR3 propeptide [4].

Adherent 293 cells (American Type Tissue Culture,

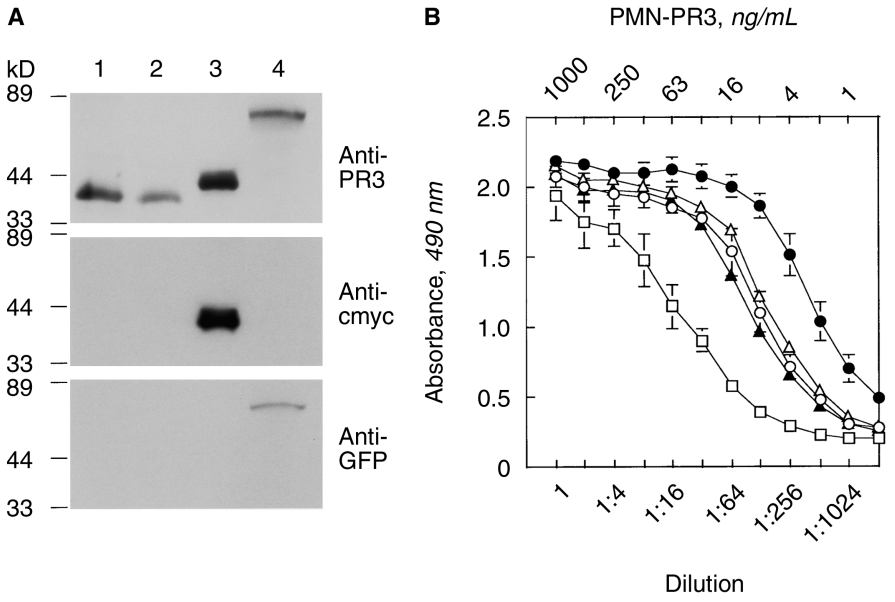

Fig. 2. (*A*) Western blots of conditioned media of 293 cells expressing wild-type rPR3 (lane 1), rPR3-Δ (lane 2), rPR3-cmyc (lane 3) and rPR3-GFP (lane 4). Proteins were separated by SDS-PAGE (12% gel) under non-reducing condition and probed with the monoclonal antibodies MCPR3-2 (anti-PR3), anti-myc (anti-cmyc), and the polyclonal rabbit anti-GFP (anti-GF) antibody. The rPR3 variants carry the expected carboxy-terminal extensions indicating the 293 cells secrete carboxy-terminally unprocessed rPR3 into the media supernatant. (*B*) The capture ELISA, using MCPR3-2 as the capturing antibody and rabbit polyclonal anti-PR3 as the detection antibody, was used to quantify the rPR3 variants in the conditioned media. Shown are the means ± SEM of three independent experiments for serial dilutions of conditioned media containing wild-type rPR3 (▲), rPR3-Δ (△), rPR3-cmyc (○), and rPR3-GFP (□), in comparison to known concentrations of PMN-PR3 (●).

Rockville, MD, USA) grown to confluence were transfected using the calcium phosphate precipitation method (Stratagene) [8]. Stable transfected 293 cell clones expressing rPR3-Δ, rPR3-cmyc and rPR3-GFP were selected in the presence of genticin (Gibco, Grand Island, NY, USA). Clones were screened for expression of rPR3 by immunoblot with MCPR3-2.

For immunoblotting, proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, transferred to nitrocellulose membranes, and probed with MCPR3-2, anti-cmyc, anti-GFP. Bound antibodies were detected using goat anti-mouse and goat anti-rabbit (Bio-Rad, Richmond, CA, USA) HRP-conjugated secondary antibodies and the ECL chemiluminescence system (Amersham, Arlington Heights, IL, USA).

For the quantification of rPR3 variants in 293 cell media supernatants a capture ELISA with MCPR3-2 as capturing antibody and polyclonal rabbit anti-PR3 for detection of bound rPR3 was used [10]. PR3-ANCA reactivity with the different rPR3 variants was measured also by capture ELISA [10]. A net absorbance of <0.100 was considered negative in this assay [8–10].

RESULTS

Expression of the carboxy-terminal rPR3 variants by stable 293 cells clones was evaluated by immunoblot using MCPR3-2 for rPR3 detection (Fig. 2A). As expected from a seven amino acid difference, the rPR3-Δ has an apparent molecular mass that is about 1 kD lower than that of rPR3. Probing the blotted proteins with anti-cmyc and anti-GFP confirmed that rPR3-cmyc and rPR3-GFP secreted into the media supernatants indeed carry the expected extensions.

To determine the affinity of the rPR3 variants for the capturing mAb MCPR3-2, to quantify levels of rPR3 secretion from different clones, and to identify optimal dilutions of media supernatants to be used in the capture ELISA for PR3-ANCA detection, serial dilutions of media supernatants were tested in the capture ELISA and compared to known quantities of PMN-PR3 (Fig. 2B). Only the dilution curve of rPR3-GFP had a slightly different slope from those of PMN-PR3 and the other three rPR3 variants, suggesting some effect of this large molecular extension on the binding to MCPR3-2. The 293 cell clones expressed 0.25 μg rPR3, rPR3-Δ, rPR3-cmyc, and 0.04 μg of rPR3-GFP per mL of serum-free media supernatants per 24 hours.

Of the 58 serum samples one was negative in the assay when the mature conformation Δ-rPR3-S195A lacking the amino-terminal propeptide was used as target antigen. This sample had a low C-ANCA titer of 1:4 and a borderline positive result in the capture ELISA with PMN-PR3 (O.D. 0.104). As observed in two previous series [8, 9], most of the PR3-ANCA sera included in this series displayed lower reactivity with amino-terminally unprocessed rPR3-S195A compared with Δ-rPR3-S195A (Fig. 3A). In contrast, the effect of carboxy-terminal propetide deletion on PR3-ANCA binding was less pronounced (Fig. 3B). It resulted in a slight gain of binding of most PR3-ANCA, and a significant correlation ($R^2 = 0.83$, $P = 0.0001$) was preserved. The best correlation was detected between rPR3 and rPR3-cmyc ($R^2 = 0.97$, $P = 0.0001$; Fig. 3C), indicating that a small carboxy-terminal extension does not affect PR3-ANCA binding.

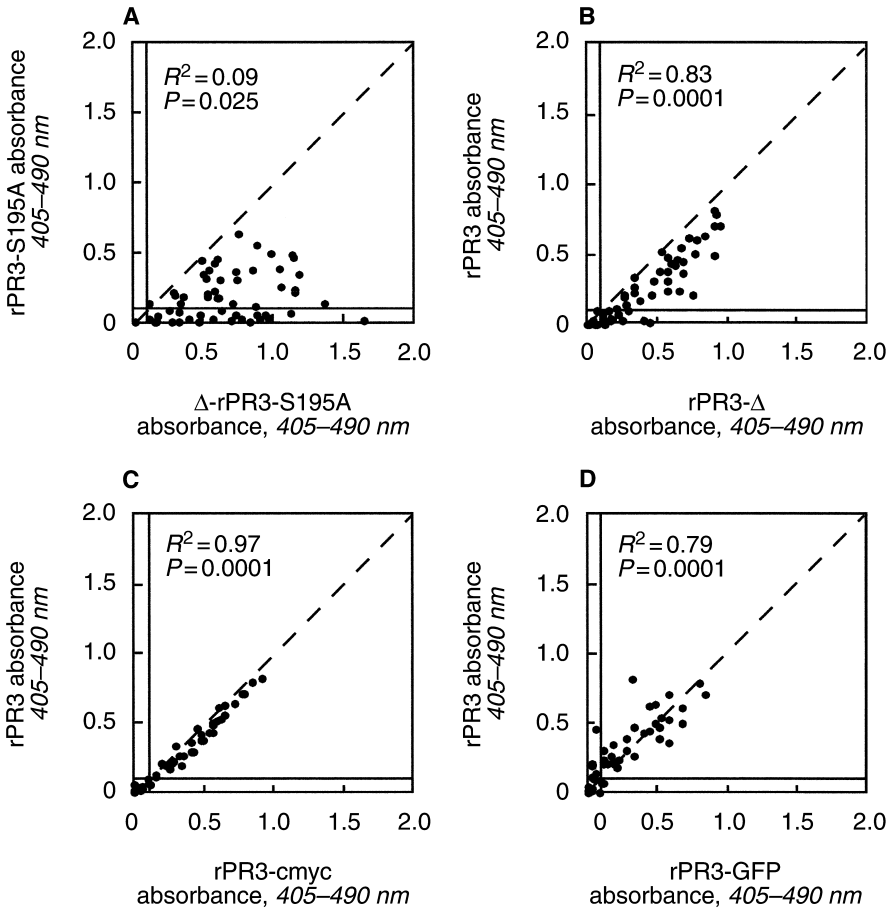

Fig. 3. Scatterplots of absorbance values obtained by capture ELISA with different rPR3 variants. (*A*) Most serum samples showed a lower reactivity with amino-terminally unprocessed rPR3-S195A than with the amino-terminally processed Δ-rPR3-S195A. The PR3-ANCA reactivities with rPR3-Δ, rPR3-cmyc, and rPR3-GFP in comparison to that with wild-type rPR3 are shown in panels *B*, *C*, and *D*, respectively. A net absorbance of <0.1 is considered negative in this assay and is indicated by solid lines. The dashed diagonal lines indicate equal reactivities with both target antigens. The significance of correlations was analyzed using the *F* test for linear regression. Two-sided *P* values of ≤0.05 were considered significant.

The carboxy-terminal addition of GFP had some effect on PR3-ANCA binding (Fig. 3D), but again, a significant correlation with the binding to wild-type rPR3 was preserved ($R^2 = 0.79$, $P = 0.0001$). In contrast to the effect of the carboxy-terminal deletion the observed differences in binding were not unidirectional. Only seven samples displayed >50% reduced binding compared to wild-type rPR3, suggesting that the binding of most PR3-ANCA is not sterically hindered by this large addition.

DISCUSSION

Our results indicate that the renal epithelial cell line 293 secretes carboxy-terminally unprocessed rPR3 into the media supernatants. Consequently, this expression system is suitable for the generation of rPR3 variants with custom-designed carboxy-terminal modifications.

Unlike the amino-terminal activation dipeptide motif, the carboxy-terminal extensions of the various neutrophil serine proteases from different species are not conserved [12–17]. The cleavage of the amino-terminal propeptide allows the formation of a salt bridge between Ile-16 and Asp-194 residues of the trypsin-like neutrophil serine proteases including PR3 with profound conformational and functional effects on the molecule [8, 18, 19]. In contrast, the impact of carboxy-terminal processing of PR3 has not been studied extensively. Here we have demonstrated that custom-designed carboxy-terminal molecular tags can serve as useful tools for studies of rPR3 processing. However, conformational changes of rPR3 induced by carboxy-terminal modifications of the molecule could have profound effects on the ability to bind ligands. Consequently, in order to interpret the results of experiments obtained with carboxy-terminally modified rPR3 molecules appropriately, the effect of such modifications on conformation needs to be known. To this end, we studied the described four carboxy-terminal rPR3 variants. The highly conformation-sensitive binding of PR3-ANCA is the most sensitive surrogate marker of conformational changes induced by mutational alterations of the PR3 amino-acid sequence. Therefore, it was used as a probe to identify significant conformational changes.

The data indicate that, in contrast to the conformational effects of the cleavage of the amino-terminal prodipeptide, the deletion of the carboxy-terminal proheptapeptide of PR3 has little conformational impact, implying that its functional role is likely to be minimal.

This suggests that the carboxy-teminus is amenable to modification without risking substantial functional effects. Indeed, the addition of the small carboxy-terminal extension (cmyc-polyHis) had no detectable effect on PR3-ANCA binding. This finding is consistent with a most recent report that carboxy-terminal poly-His tag does not seem to affect the recognition of rPR3 expressed in a baculovirus system by PR3-ANCA significantly [20].

Green fluorescent protein (GFP) has emerged as a widely used marker of recombinant molecules. The addition of this large molecular extension had little effect on PR3-ANCA binding. Clearly, steric hindrance of PR3-ANCA binding seems to be minimal, perhaps because of the interspersed 16-residue linker polypeptide. Variants of rPR3 carrying this particular tag can easily be tracked by direct fluorescence microscopy or using antibodies against GFP. Cells expressing rPR3-GFP displayed abundant intracellular autofluorescence (not shown). Hence, GFP-tagged rPR3 variants should represent useful tools for studies where direct visualization of PR3 is essential.

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