Longitudinal studies of patients with ANCA vasculitis demonstrate concurrent reactivity to complementary PR3 protein segments cPR3m and cPR3C and with no reactivity to cPR3N

Peter Hewins[1,¥], **Frances Belmonte**[1], **J Charles Jennette**[1,2], **Ronald J Falk**[1,2], and **Gloria A Preston**[1,2,*]

[1] UNC Kidney Center, Division of Nephrology and Hypertension, University of North Carolina, Chapel Hill

[2] Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill

Abstract

Antibodies recognizing the complement of the middle of PR3 (cPR3m) occur in ~30% of PR3-ANCA-vasculitis patients and immunization of animals with a peptide complementary to the middle of PR3 (cPR3m) induces not only anti-complementary PR3 antibodies, but also anti-PR3 antibodies derived through an anti-idiotypic response. PR3 epitopes recognized by patient ANCA however, are not restricted to the middle of PR3. This prompted us to test for antibodies that react with proteins complementary to the terminal regions of PR3 (cPR3C and cPR3N) in PR3-ANCA patients. Anti-cPR3C reactivity was detected in 28% of patients but anti-cPR3N reactivity in only 15%. Ranked anti-cPR3C and anti-cPR3m reactivity correlated in the cohort, whereas there was no significant relationship between cPR3C and cPR3N reactivity. Serial samples from three patients' revealed that anti-cPR3C and anti-cPR3m reactivity followed a similar pattern over time. Serial samples from a fourth patient demonstrated an anti-cPR3N response without concurrent cPR3m or cPR3C reactivity. Epitope determination by mass spectrometry identified a thirteen amino acid sequence on cPR3C that contained a common binding site recognized by antibodies from three patients. This peptide sequence contains a "PHQ" motif which was reported to be the basis for cross-reactivity of anti-cPR3m antibodies with plasminogen. Why these antibodies are detected in only ~30% of the patients remains unclear. The data reveal it is not due to lack of inclusion of flanking regions of complementary PR3 during screening. Instead, quite unexpectedly, the data demonstrate that patients' antibodies react with a restricted epitope that exists in both cPR3m and cPR3C.

Keywords

Autoantigen Complementarity; ANCA vasculitis; anti-idiotypic antibodies; PR3; complementary protein pairs

---

[*]Corresponding Author: Gloria A Preston, PhD, University of North Carolina–Chapel Hill, 5008 Burnett Womack Building, CB#7155, Chapel Hill, NC 27599, Gloria_Preston@med.unc.edu, Phone 919-966-2561 ext 280.
[¥]Now at Department of Nephrology, University Hospitals Birmingham, Birmingham, UK

Introduction

The pathogenic role of Anti-Neutrophil-Cytoplasmic-Autoantibodies (ANCA) in small vessel vasculitis has been clearly established by several animal models, a host of clinical data and studies on the effects of these autoantibodies upon human neutrophils in vitro. In contrast, the initiating stimulus for autoimmunity in ANCA associated vasculitis (ASV) remains unclear and supportive evidence for classical theories, such as molecular mimicry, that would explain the development of autoantibodies directed against the ANCA antigens most closely associated with ASV, namely proteinase 3 or myeloperoxidase, has not been forthcoming. Anti-idiotypic antibodies have been recognized in ASV patients for some time but recent observations have prompted renewed interest in this area and spurred an alternative theory for the evolution of autoimmunity that may pertain not only to ASV but also to other autoimmune diseases [1,2]. Whilst searching for proteinase 3 epitopes using a bacterial expression system that incorporated blunt ended fragments of PR3 cDNA in either orientation, it was discovered that some PR3-ANCA positive patients' sera contained antibodies which reacted with peptides encoded by what is conventionally the "non-coding" (anti-sense) strand of the PR3 cDNA [3]. Proteins or peptides generated in this manner are *complementary* to their counterparts encoded by the sense strand of the gene [4]. Further investigation demonstrated that these anti-complementary PR3 antibodies bound to PR3-ANCA; in other words they exhibited idiotypic-anti-idiotypic pairing as would be anticipated. Importantly, mice immunized with complementary PR3 peptide developed antibodies against not only complementary PR3 but also PR3. This led to the theory that autoimmunity might be driven by initial exposure to a complementary antigen and the subsequent development of antibodies directed against the autoantigen (the theory of autoantigen complementarity). Evidence for the translation of anti-sense mRNA in eukaryotes is very limited but a large number of known exogenous proteins display some homology to complementary PR3 and it is possible that exposure to one of these proteins might initiate an immune response that ultimately leads to the development of PR3-ANCA vasculitis [3]. Further support for the relevance of immune responses towards complementary PR3 in ANCA vasculitis patients arises from a separate study of T cell reactivity [5]. Th1 cells specific for cPR3 were evident in ~50% of PR3-ANCA patients but not in MPO-ANCA patients. Moreover, cellular and humoral responses to cPR3 co-existed in most patients. The functional importance of anti-complementary antibodies in PR3-ANCA vasculitis patients is also revealed by the discovery of cross-reactivity of anti-cPR3 antibodies with plasminogen which is linked to retardation of clot lysis in vitro and with venous-thrombo-embolism in vivo [6]. These earlier reports focused upon immune responses directed towards a peptide complementary to the middle of PR3 (cPR3m also known as $cPR3^{105-201}$). Antibodies to cPR3m were detectable in 21% (7/34) of patients with PR3-ANCA vasculitis tested. Meanwhile, PR3-epitopes recognized by patient ANCA are diverse and located along the entire length of the molecule [7,8]. Accordingly, it is conceivable that this diversity is driven by exposure to complementary antigens or their homologues, some of which are not represented by the middle fragment (complementary-PR3-middle, cPR3m). Furthermore, co-existence of antibodies to more than one region of complementary-PR3 and changes in anti-complementry-PR3 antibody repertoire over time in individuals would be of interest, since the epitope specificity of PR3-ANCA can vary over time in individual patients and might reflect continuing exposure to diverse complementary proteins. We therefore undertook a more complete analysis of anti-complementary PR3 antibody responses in PR3-ANCA patients.

Material and Methods

Generation of complementary PR3 protein fragments

The sequence of complementary-PR3-C-terminal fragment (cPR3C) and complementary-PR3 N-terminal fragment (cPR3N) were determined from the non-coding strand of the PRTN3 gene (GenBank accession no. X55668). Complementary proteins are designated according to the position of the corresponding sense fragment within proteinase 3 (Figure 1A). Accordingly and as previously described, cPR3m represents the complement of the sense fragment extending from amino acid position 105 to 201 in proteinase 3 [3]. The nucleotide sequence encoding a complementary protein is designated by the position of the corresponding 'coding' sequence within the PRTN3 cDNA. Appropriately orientated cDNA fragments were ligated into modified pcDNA 3.1 vector containing a BM40 (Bee melittin) secretion signal and hexa-histidine tag. Recombinant proteins were produced by transiently transfected human embryonic kidney (HEK293) Freestyle cells (InVitrogen) and purified on HisTrap affinity columns and visualized by Coomassie stained gels (Figure 1B). Recombinant proteins for this study included cPR3C (amino acids 1–103), translated from the non-coding strand corresponding to PRTN 3 cDNA nucleotides 1–309; cPR3m (amino acids105–201) corresponding to sense cDNA nucleotides 310–600 and cPR3N (amino acids 203–254) corresponding to sense cDNA nucleotides 607–762 (Figure 1C). On the anti-sense strand, nucleotides 604–606 encode a stop codon.

Controls and Patients

Healthy volunteers (not sex or age matched) were used as controls (n=92). Patients (n=67) with proteinase 3 (PR3) anti-neutrophil cytoplasmic autoantibody (ANCA) positive small vessel vasculitis (ASV) were enrolled into the Glomerular Diseases Collaborative Network and Specimen Bank. An Institutional Review Board authorized entity of human information and specimens from patients who have renal diseases. Criteria for entry into the study included informed consent and biopsy proven pauci-immune crescentic glomerulonephritis. Immuno-reactivity of patient and healthy control sera (diluted 1:100) was determined by ELISA (protein coated at 5mcg/ml). Sample reactivity was calculated based on calibration against a serum or plasma sample reactive to the relevant recombinant fragment. Positivity was defined by the mean absorbance + 2 standard deviations of the healthy control sera. Anti-His antibody ELISA reactivity of all 3 protein fragments was comparable.

Epitope mapping

Total IgG was isolated from three PR3-ANCA patients whose sera reacted with cPR3C. For epitope characterization, cPR3C was allowed to bind to immobilized IgG and then subjected to proteolytic digestion. Resulting affinity-bound peptides were identified by matrix assisted laser desorption (MALDI) mass spectrometry and MS/MS. As a negative control total IgG from a 4th cPR3C negative patient (anti-GBM and MPO-ANCA positive) was subjected to the same analysis.

Production of anti-cPR3C polyclonal antibodies in rabbit

Anti-cPR3C antibody was raised in a rabbit by immunization with full length recombinant cPR3C mixed with Complete Freunds Adjuvant (Sigma). Total IgG was isolated from serum drawn on day 55.

Statistical analysis

The Wilcoxon rank sum test was used as a nonparametric alternative to the two-sample t-test for analysis of sera reactivity comparing patients and healthy controls. The Wilcoxon rank sum test was used for analysis of BVAS scores and PR3 ANCA titers comparing cPR3C

positive patients versus cPR3C negative patients. The Fisher's exact test was used for analysis of frequency of occurrence of anti-complementary protein antibodies among patients.

Results

Reactivity towards terminal complementary-PR3 fragments in PR3-ANCA vasculitis patients

To assess whether patients' sera contain antibodies reactive against complementary PR3 proteins, the production of recombinant proteins containing the appropriate amino acid sequences was required. Complementary proteins were derived by translation of the corresponding region of the conventional 'non-coding strand' of PRTN3 cDNA (Figure 1). Abbreviations include: small case 'c' – complementary; upper case 'C' – carboxy terminal region; 'm' – middle region; 'N' – amino terminal region. Fragments cPR3C, cPR3m and cPR3N comprise 103, 97 and 52 amino acids, respectively, excluding vector encoded tags and signal peptides. cPR3N protein SDS gel mobility was retarded according to the predicted molecular weight. Such erroneous mobility is attributable to the unusually high number of negatively charged amino acids (10 Asp (D) and 9 His (H) in this protein (Figure 1B).

Accordingly, cPR3C represents the complement of the N-terminal region of sense PR3, cPR3m the middle of sense PR3 and cPR3N the C-terminal region of sense PR3 (Figure 1C). We found that cPR3C antibodies were detectable in 28% (19/67) PR3-ANCA patients versus 3% (3/92) healthy controls (p<0.0001) (Figure 2A). Analysis of 20 patients with more than one sample available for testing indicated that the levels of cPR3 antibodies changed over time (Figure 2B). Ten of these 20 patients had at least one positive sample and 5 of these had persistent anti-cPR3 antibodies over time. These data were reliable and reproducible as demonstrated by testing the same serum samples three different times with different batches of recombinant protein (Figure 2C). There was no correlation between anti-cPR3 antibodies and either PR3-ANCA titers or disease activity (assessed by BVAS) (Table 1).

Reactivity towards cPR3N was considerably lower and less frequent overall. In a separate experiment, we tested 50 sera samples from 40 patients for both cPR3N and cPR3C reactivity. The results indicated that 15% (6/40) of patients and 18% (9/50) sera samples tested positive for anti-cPR3N antibodies versus 10% (4/40) healthy controls. Anti-cPR3C antibodies were detected in 27.5% (11/40) of patients and 30% (15/50) of samples, consistent with the prevalence of cPR3C positivity in the whole cohort. Reactivity of commercial anti-Histidine antibodies towards cPR3C and cPR3N was equivalent suggesting that coating of ELISA plates with each protein were comparable (data not shown). There was no significant relationship between the occurrence of antibodies against the two fragments (Fishers' exact test, p=0.10). Of the nine samples positive for anti-cPR3N antibodies, five also had anti-cPR3C reactivity, while the remaining four were negative for anti-cPR3C antibodies. We conclude that lower reactivity towards cPR3N in the majority of the patient cohort may therefore reflect a lower frequency and lower titers of anti-cPR3N antibodies in this patient cohort.

cPR3N reactivity was unusually high in one patient which prompted us to explore this patient further by testing the persistence of antibodies in samples procured over his disease course. Interestingly, at an earlier point the patient was reactive with cPR3m but ~14 months later the specificity of reactivity had changed to cPR3N (Figure 3A). This most strongly reactive sample was tested and found to be negative for cPR3C antibodies. Of potential note, the reactivity to cPR3m (month 18) coincided with Staphylococcus aureus and pseudomonas

infections. Medications at this time included doxycycline and prednisone. cPR3N reactivity (month 46) coincided with a varicella zoster infection that was treated with IV acyclovir therapy in combination with mycophenolate mofetil (MMF) for sever lung disease. Further evaluation demonstrated no consistent relationship between changes in anti-complementary-PR3 antibodies and PR3-ANCA titers over time (Figure 3A). In conformation of the specificity of anti-cPR3N-reactivity, competitive inhibition studies demonstrated inhibition of binding to solid-phase cPR3N by pre-incubation of the antibody with soluble cPR3N in a dose dependent manner (Figure 3B). Collectively, these data suggest that reactivity towards the respective terminal complementary PR3 fragments is specific and varies over time in patients with PR3-ANCA vasculitis.

Longitudinal changes in reactivity towards complementary-PR3 fragments

Whereas reactivity towards cPR3C and cPR3N represented distinct characteristics of particular PR3-ANCA sera samples, reactivity towards cPR3C and cPR3m correlated closely in the patient cohort as a whole. Three selected patients, for whom multiple longitudinal samples were available, demonstrated in further detail how reactivity towards cPR3C, cPR3N and cPR3m varied over time. Reactivity towards cPR3C and cPR3m fluctuated over time, while reactivity towards cPR3N was consistently low/negative in the three patients. Consistently low cPR3N reactivity provides an internal control compatible with the proposal that changes in cPR3C and cPR3m reactivity are specific. Similar to patient studied longitudinally in Figure 3A, there was no clear relationship between complementary antibody titers and PR3-ANCA titers in these individuals (Figure 4B). However, it is interesting that patient 3, who's complementary-PR3- reactivity remained elevated over time, also had consistently high PR3-ANCA titers (Figure 4B).

cPR3C epitope characterization

Antibody binding sites on cPR3C were sought by proteolytic digest and mass spectrometry. A single epitope, VPPHLQGGHVGPG, was detected using total IgG from 3 cPR3C ELISA positive patients with PR3-ANCA vasculitis (Figure 5). Total IgG from a fourth patient with anti-GBM antibodies and MPO-ANCA (negative for PR3-ANCA and anti-cPR3C antibodies) showed no binding of CPR3C to IgG. The similarity among epitopes identified on cPR3C, cPR3m and plasminogen (Plg) [6] are diagramed in Figure 5B. These regions overlap with amino acid sequences on native PR3 protein mapped as PR3-ANCA epitopes [9–11](Figure 5C).

Recapitulation of antibody dual reactivity in vivo

The existence of parallel reactivity towards cPR3C and cPR3m in patients' sera prompted us to consider whether these individuals harbored two uniquely reacting pools of antibodies or whether a one pool was recognizing both proteins. A rabbit was immunized with cPR3C and we examined whether antibodies produced against this antigen would react also with cPR3m. Rabbit serum reacted strongly with cPR3C protein (the immunogen) at a 1:1000 dilution (Figure 6A). Moreover, there was cross reactivity with cPR3m (at up to 1:500 dilution) in comparison to pre-immune serum. It is possible that reactivity towards cPR3m was partly attributable to anti-Histidine antibodies since the Histidine tag was present on the immunogen as well as both ELISA coat antigens. When the Histidine tag was proteolytically removed from purified recombinant cPR3m, reactivity was lost using an anti-histidine antibody. However, the rabbit anti-cPR3C antibodies were still reactive as were two patients tested earlier indicating that these antibodies react with both cPR3C and cPR3m. In further proof, competitive inhibition using soluble cPR3m diminished binding to cPR3m by ~75% whereas soluble irrelevant His-tagged recombinant protein only reduced binding by ~25%, indicating that the majority of the reactivity with cPR3m was not accounted for by anti-His antibodies (Figure 6BC). As anticipated, soluble cPR3C abrogated binding to both cPR3C

and cPR3m. Soluble cPR3m did not diminish binding to cPR3C, likely reflecting the greater avidity of polyclonal serum towards the immunogen compared to a cross reacting antigen.

Reactivity of IgG from the cPR3C immune-rabbit towards both cPR3C and cPR3m was independently demonstrated by immunoblotting (Figure 6D). Western blots performed using pre-immune IgG did not detect these proteins (data not shown). Recombinant cPR3m migrated at a higher molecular weight in non-reduced samples which we believe indicates dimerization but rabbit IgG bound to both proteins reduced and non-reduced. Furthermore, the anti-cPR3C IgG appeared to react with non-reduced plasminogen (Figure 6D). These data are compatible with our recently published evidence demonstrating that affinity-purified, patient derived anti-cPR3m antibodies also bind plasminogen [6]. Interestingly, neither unfractionated IgG from the cPR3C immune-rabbit nor affinity-purified patient anti-cPR3m IgG bound reduced plasminogen (Figure 6C and [6]), implying that the relevant epitope in plasminogen may be a conformational one. Confirmation that patient-derived anti-complementary PR3 antibodies are truly cross reactive with cPR3C, cPR3m and plasminogen would require further affinity purification experiments but the data from the rabbit-derived antibodies are compatible with this hypothesis. Of interest, sequence homology between cPR3C, cPR3m and plasminogen is limited to a 'PxHxQ' motif (Figure 5B) which may underpin antibody cross-reactivity although this requires further confirmation..

Discussion

The theory of autoantigen complementarity is still conjectural; however data are mounting that support its assertions pertaining to the development of autoimmune disease. Importantly, autoantigen complementarity provides a potential mechanism for the generation of anti- PR3 or MPO antibodies, the species of autoantibody for which existing experimental and clinical data support a pathogenic role. The studies herein reinforce and expand our earlier observations that patients with PR3-ANCA vasculitis have antibodies reactive with a complementary PR3 protein. Somewhat puzzling in our earlier studies, these antibodies were detected in only one-third of patients. One explanation was that the recombinant cPR3m protein used for screening contained only one-third of the predicted complementary PR3 molecule. Thus we hypothesized that the remaining patients would react with the missing regions that flanked cPR3m (cPR3N and cPR3C). We report that, contrary to our hypothesis, only one-third of patients tested were positive for reactive antibodies and that the sera reactive against cPR3m were also reactive with cPR3C. Although these two recombinant proteins exhibit relatively little sequence homology, they share a three residue motif which we believe may determine antibody specificity. Additionally, the identified cPR3C epitope (common to 3 separate patients) corresponds to a region of proteinase 3 that has previously been identified as an epitope for ANCA in studies by independent investigators [6]. Interestingly, anti-cPR3N reactivity was much less common and only one patient's serum exhibited strong reactivity. Two thirds of patients remain negative for reactivity against *complementary* PR3.

How and when were these antibodies produced? Robust T cell responses against complementary PR3 peptide have been documented, which were not seen in healthy controls, and were in fact greater than the responses of these same patients towards native PR3 [5]. Further investigations into the identities of proteins complementary to PR3 in patients' plasmapheresis fluid have revealed that anti-cPR3m antibodies bind to plasminogen [6]. This observation was confirmed using immunoglobulin from animals immunized with cPR3m. A functional consequence of these antibodies is that they hinder fibrinolysis and thus increase the risk of thrombo-embolic events in these patients. Whilst plasminogen seems unlikely to be the initiating stimulus for the anti-complementary PR3

immune response, these observations highlight the potential usefulness of complementary proteins in studying autoimmune disease.

Exploring autoantigen complementarity offers an alternate approach to uncover mechanisms of immunogenesis leading to autoimmune disease. Consider for the moment that anti-idiotypic antibody processes are involved in the generation of PR3-specific antibodies. It has been shown that antibodies can regulate each other by suppressing or augmenting the immune reaction in a manner that perpetuates autoimmune disease [12–14]. It is also notable that the animals used to raise anti-complementary PR3 antibodies (chicken and rabbit) developed anti-PR3 antibodies, confirming that anti-idiotypic responses can lead to antibodies that reactive with sense-PR3 as was previously observed in mice [3]. Furthermore, in animal models anti-idiotypes raised against autoantibodies induced anti-anti-idiotypes that possessed characteristics of the initial autoantibodies and caused autoimmune disease after immunization [1,15]. Mechanistically, an antibody is immunogenic by virtue of its non-germiline-encoded antigen-binding site. B cells are known to spontaneously display endogenous V region peptides on their HLA class II molecules and acivate CD4$^+$T cells [16,17].

A crucial question is the source of the actual complementary-PR3 proteins that triggered the immunological responses described here. We have previously documented that various microbial proteins exhibit homolgy to cPR3 [3]. If the source of complementary-PR3 encountered by patients who develop PR3-ANCA vasculitis is an exogenous protein, it may be that anti-cPR3 antibody positivity will associate with particular clinical manifestations. The three patients in whom we studied cPR3C and cPR3m reactivity longitudinally (Figure 4B) all had limited Wegener's granulomatosis with ENT involvement. Anti-cPR3 antibodies are clearly not exclusive to patients with ENT disease, since we have also detected them in PR3-ANCA positive patients with microscopic polyangiitis who do not have significant ENT disease by definition, but disease phenotype correlations may yet emerge through the examination of larger patient cohorts [5]. Meanwhile, we have detected antisense transcripts of PR3 in patients using an anti-sense specific primer for the reverse transcriptase reaction and PCR which could be compatible with an endogenous source of cPR3 [3]. Whether these transcripts are – or even can be – translated is unclear, although there are reports of translated anti-sense transcripts in other circumstances [18].

A recent review prosposed that "complementary proteins, which occur naturally, or result from cellular dysfunction, might be more common than recognized currently. This implies that the role of complementary proteins in autoimmunity merits increasing investigation" [2]. Understanding when and how complementary proteins initiate autoimmune disease will depend on discovering where these proteins come from. Nevertheless, there is enough evidence to warrant a closer look.

Acknowledgments

References

1. Shoenfeld Y. The idiotypic network in autoimmunity: antibodies that bind antibodies that bind antibodies. Nat Med 2004;10:17–8. [PubMed: 14702622]

2. McGuire KL, Holmes DS. Role of complementary proteins in autoimmunity: an old idea re-emerges with new twists. Trends Immunol 2005;26:367–72. [PubMed: 15927527]

3. Pendergraft WF 3rd, Preston GA, Shah RR, Tropsha A, Carter CW Jr, Jennette JC, Falk RJ. Autoimmunity is triggered by cPR-3(105–201), a protein complementary to human autoantigen proteinase-3. Nat Med 2004;10:72–9. [PubMed: 14661018]

4. Heal JR, Roberts GW, Raynes JG, Bhakoo A, Miller AD. Specific interactions between sense and complementary peptides: the basis for the proteomic code. Chembiochem 2002;3:136–51. [PubMed: 11921391]

5. Yang J, Bautz DJ, Lionaki S, Hogan SL, Chin H, Tisch RM, Schmitz JL, Pressler BM, Jennette JC, Falk RJ, Preston GA. ANCA patients have T cells responsive to complementary PR-3 antigen. Kidney Int 2008;74:1159–69. [PubMed: 18596726]

6. Bautz DJ, Preston GA, Lionaki S, Hewins P, Wolberg AS, Yang JJ, Hogan SL, Chin H, Moll S, Jennette JC, Falk RJ. Antibodies with dual reactivity to plasminogen and complementary PR3 in PR3-ANCA vasculitis. J Am Soc Nephrol 2008;19:2421–9. [PubMed: 18701607]

7. Selga D, Segelmark M, Wieslander J, Gunnarsson L, Hellmark T. Epitope mapping of anti-PR3 antibodies using chimeric human/mouse PR3 recombinant proteins. Clin Exp Immunol 2004;135:164–72. [PubMed: 14678279]

8. Rarok AA, van der Geld YM, Stegeman CA, Limburg PC, Kallenberg CG. Diversity of PR3-ANCA epitope specificity in Wegener's granulomatosis. Analysis using the biosensor technology. J Clin Immunol 2003;23:460–8. [PubMed: 15031633]

9. 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;152:4722–37. [PubMed: 8157982]

10. Van Der Geld YM, Simpelaar A, Van Der Zee R, Tervaert JW, Stegeman CA, Limburg PC, Kallenberg CG. Antineutrophil cytoplasmic antibodies to proteinase 3 in Wegener's granulomatosis: epitope analysis using synthetic peptides. Kidney Int 2001;59:147–59. [PubMed: 11135067]

11. Griffith ME, Coulthart A, Pemberton S, George AJ, Pusey CD. Anti-neutrophil cytoplasmic antibodies (ANCA) from patients with systemic vasculitis recognize restricted epitopes of proteinase 3 involving the catalytic site. Clin Exp Immunol 2001;123:170–7. [PubMed: 11168015]

12. Erlanger BF, Cleveland WL, Wassermann NH, Ku HH, Hill BL, Sarangarajan R, Rajagopalan R, Cayanis E, Edelman IS, Penn AS. Auto-anti-idiotype: a basis for autoimmunity and a strategy for anti-receptor antibodies. Immunol Rev 1986;94:23–37. [PubMed: 3492425]

13. Hill BL, Erlanger BF. Monoclonal antibodies to the thyrotropin receptor raised by an autoantiidiotypic protocol and their relationship to monoclonal autoantibodies from Graves' patients. Endocrinology 1988;122:2840–50. [PubMed: 2453350]

14. Erlanger BF. Auto-anti-idiotypy, autoimmunity and some thoughts on the structure of internal images. Int Rev Immunol 1989;5:131–7. [PubMed: 8691045]

15. Shoenfeld Y. Idiotypic induction of autoimmunity: a new aspect of the idiotypic network. Faseb J 1994;8:1296–301. [PubMed: 8001742]

16. Munthe LA, Kyte JA, Bogen B. Resting small B cells present endogenous immunoglobulin variable-region determinants to idiotope-specific CD4(+) T cells in vivo. Eur J Immunol 1999;29:4043–52. [PubMed: 10602015]

17. Munthe LA, Os A, Zangani M, Bogen B. MHC-restricted Ig V region-driven T-B lymphocyte collaboration: B cell receptor ligation facilitates switch to IgG production. J Immunol 2004;172:7476–84. [PubMed: 15187126]

18. Huelseweh B, Kohl B, Hentschel H, Kinne RK, Werner A. Translated anti–sense product of the Na/phosphate co-transporter (NaPi-II). Biochem J 1998;332 (Pt 2):483–9. [PubMed: 9601078]
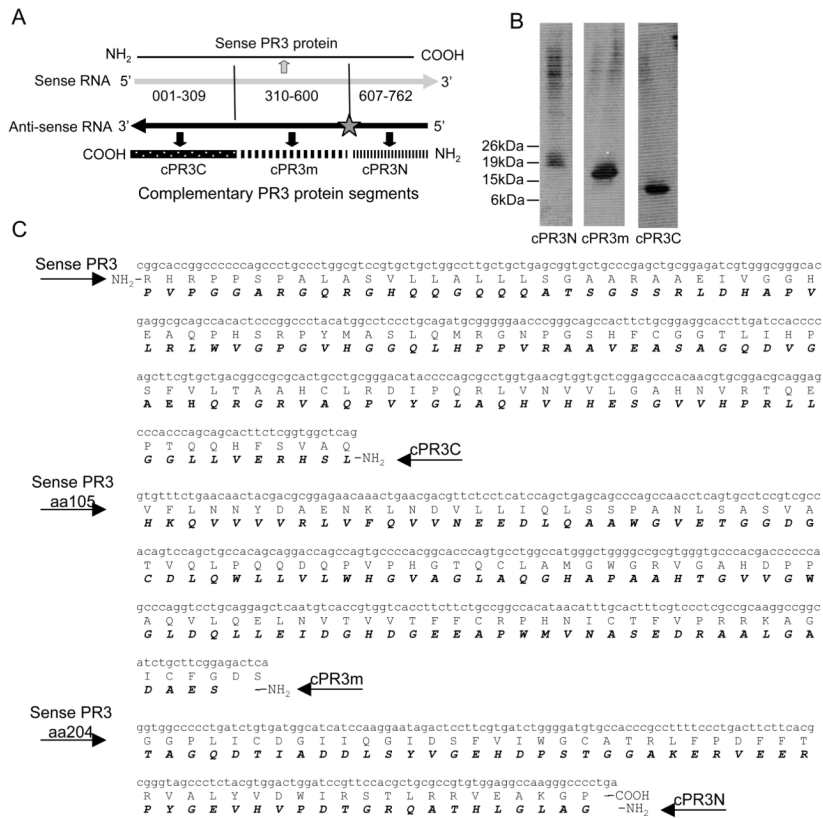
A

Sense PR3 protein
NH₂ ─────────────────── COOH
Sense RNA 5' │ 001-309 │ 310-600 │ 607-762 │ 3'
Anti-sense RNA 3' ◄─────────────────── 5'
COOH ▓▓▓▓▓▓▓▓▓ ▼ ┊┊┊┊┊┊ ▼ ‖‖‖‖‖‖‖ NH₂
cPR3C cPR3m cPR3N
Complementary PR3 protein segments

B

26kDa —
19kDa —
15kDa —
6kDa —

cPR3N cPR3m cPR3C

C

Sense PR3
NH₂-R H R P P S P A L A S V L L A L L L S G A A R A A E I V G G H
P V P G G A R G Q R G H Q Q G Q Q Q A T S G S S R L D H A P V

E A Q P H S R P Y M A S L Q M R G N P G S H F C G G T L I H P
L R L W V G P G V H G G Q L H P P V R A A V E A S A G Q D V G

S F V L T A A H C L R D I P Q R L V N V V L G A H N V R T Q E
A E H Q R G R V A Q P V Y G L A Q H V H H E S G V V H P R L L

P T Q Q H F S V A Q
G G L L V E R H S L -NH₂ ◄ cPR3C

Sense PR3 aa105
V F L N N Y D A E N K L N D V L L I Q L S S P A N L S A S V A
H K Q V V V V R L V F Q V V N E E D L Q A A W G V E T G G D G

T V Q L P Q Q D Q P V P H G T Q C L A M G W G R V G A H D P P
C D L Q W L L V L W H G V A G L A Q G H A P A A H T G V V G W

A Q V L Q E L N V T V V T F F C R P H N I C T F V P R R K A G
G L D Q L L E I D G H D G E E A P W M V N A S E D R A A L G A

I C F G D S
D A E S —NH₂ ◄ cPR3m

Sense PR3 aa204
G G P L I C D G I I Q G I D S F V I W G C A T R L F P D F F T
T A G Q D T I A D D L S Y V G E H D P S T G G A K E R V E E R

R V A L Y V D W I R S T L R R V E A K G P —COOH
P Y G E V H V P D T G R Q A T H L G L A G —NH₂ ◄ cPR3N

Figure 1.
Derivation of complementary PR3 recombinant proteins. **(A)** Nucleotide sequence was derived from the non-coding strand of human PR3 cDNA (X55668). Star denotes stop codon at 604–606 on anti-sense strand. Abbreviations: small case 'c' – complementary; upper case 'C' – carboxy terminal region; 'm' – middle region; 'N' – amino terminal region. **(B)** Coomassie-stained gel of purified recombinant proteins. cPR3C - 103 aa; cPR3m - 97aa; cPR3N - 52aa. cPR3N protein SDS gel mobility was retarded according to the predicted molecular weight. **(C)** Sequence alignment of sense PR3 with complementary amino acids. Sense sequence reads left to right. Complementary amino acids read are read in the opposite direction – right to left- shown on the bottom line in italic.
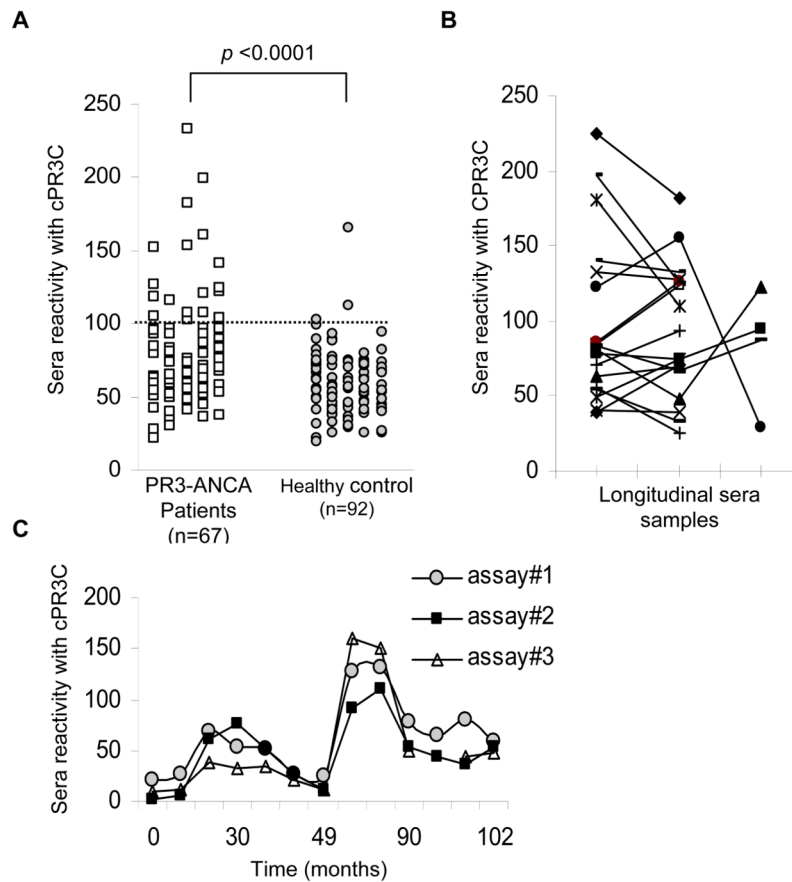
Figure 2.
Immuno-reactivity to cPR3C sera from patients with PR3-ANCA vasculitis and from healthy controls (diluted 1:100), as determined by ELISA. Reactivity values were calibrated against a known sample reactive to the relevant recombinant fragment. Positivity was defined by the mean reactivity + 2 SD (101.37) of the healthy control sera. **(A)** Of 67 patients, 19 tested positive. **(B)** For 20 of the 67 patients, ≥2 samples had been collected longitudinally. Analysis of samples from these 20 patients indicates that reactivity against cPR3C fluctuates over the course of disease. **(C)** Reproducibility of the assay. The same serum samples were tested on different days with different batches of purified recombinant cPR3C.
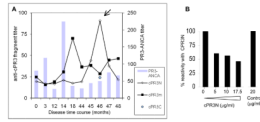
Figure 3.
Immuno-reactivity of a PR3-ANCA patient to cPR3N. **(A)** ELISA of longitudinal serum samples indicates that previously this patient's serum was reactive to cPR3m but then specificity changed to cPR3N at 45 months. At the point of highest reactivity with cPR3N (arrow), there was concurrent minimal reactivity to cPR3C and cPR3m. There is no consistent relationship between changes in anti-complementary-PR3 antibodies and PR3-ANCA titers. **(B)** Competition assay to demonstrate specificity of interaction. Results are expressed as % inhibition of serum pre-incubated without protein. Serum from the patient sample indicated by an arrow in 'A' was incubated with cPR3N protein prior to ELISA analysis. Data indicate reactivity was reduced by 54%. An irrelevant Histidine-tagged protein served as control.
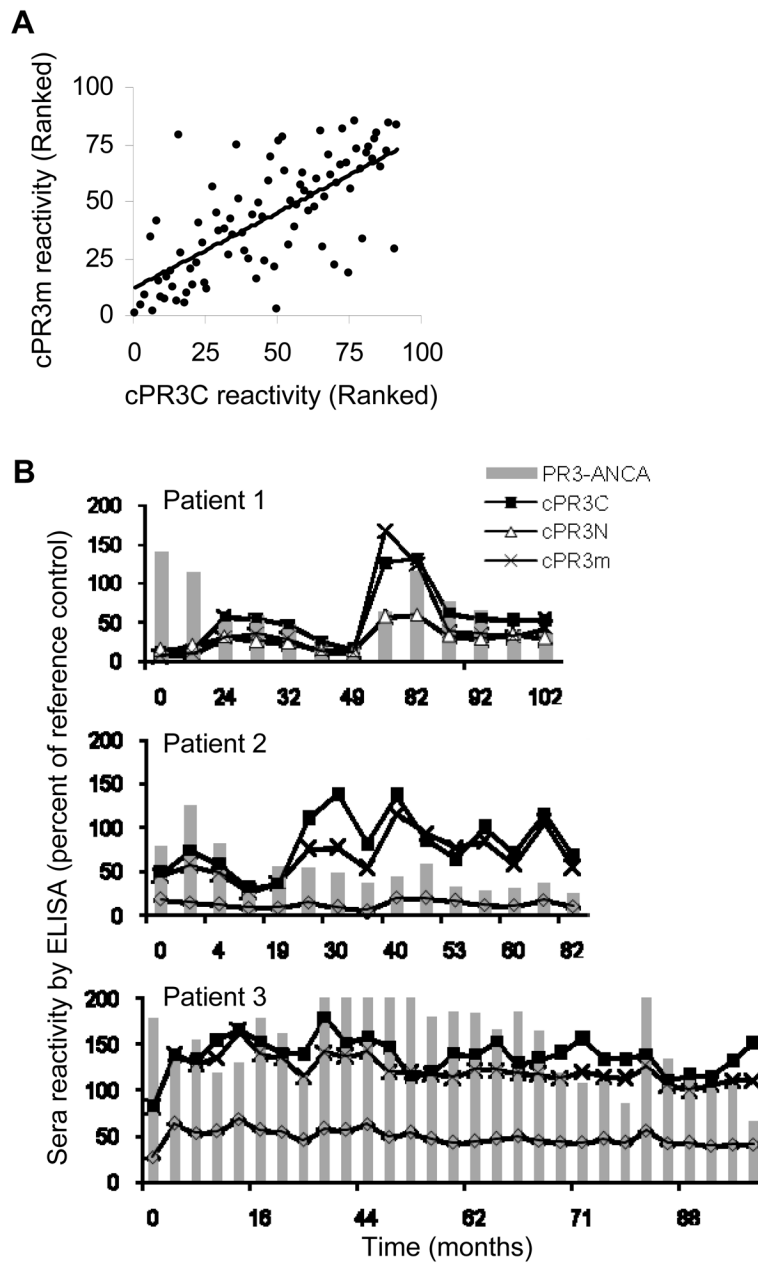
Figure 4.
Specificity of sera reactivity to recombinant complementary PR3 protein segments. **(A)** Among the cohort of 67 patients (85 separate samples) represented in Figure 2, reactivity towards cPR3C and cPR3m is closely correlated. Ranked correlation of anti- cPR3C and cPR3m reactivity per serum sample ($R^2 = 0.5056$). **(B)** Longitudinal samples from three different patients analyzed for reactivity to complementary protein fragments. Most patients demonstrated parallel anti-cPR3C and cPR3m titers with lower or absent anti-cPR3N titers. Further, there is no consistent relationship between changes in anti-cPR3 antibody and PR3-ANCA titers in patients 1 and 2(gray bar), while patient 3 who had consistently high PR3-ANCA also had consistently high reactivity to anti-cPR3C and cPR3m.
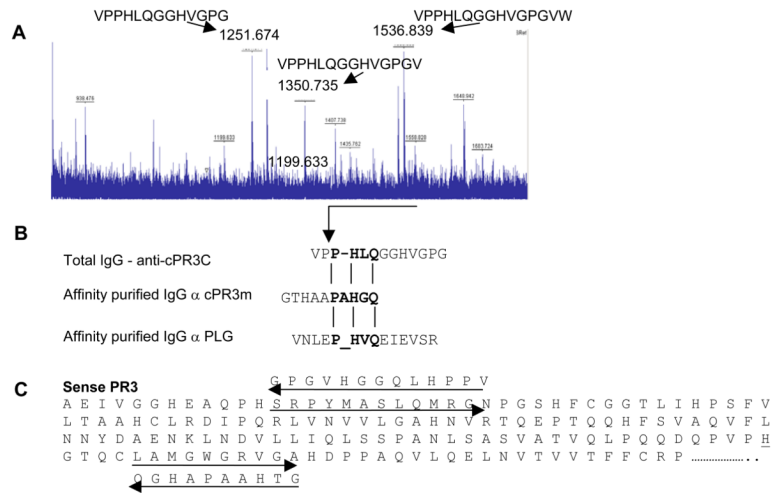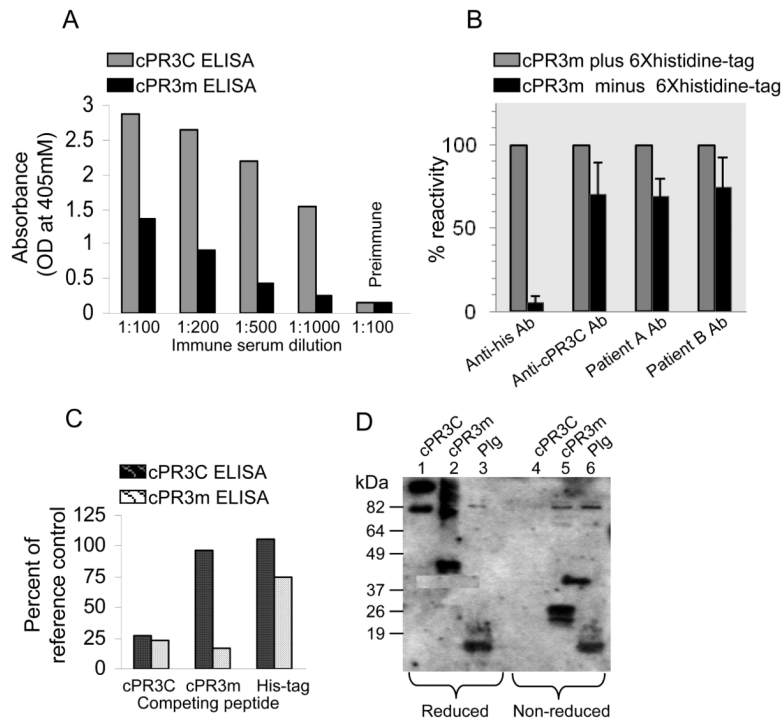
Figure 5.
Identification of regions on complementary protein fragments reactive with patients'
antibodies. **(A)** A Mass Spectrometry chromatogram showing a cPR3C epitope recognized
by a cPR3C positive patient's total IgG. **(B)** A common motif was identified in cPR3C,
cPR3m and plasminogen (see Bautz, et al [6]). **(C)** Regions on native PR3 that overlap with
cPR3C and cPR3M regions of reactivity (Adapted from Van Der Geld, et al [10]).

Figure 6.
Recapitulation of antibody dual reactivity *in vivo*. (**A**) Rabbit anti-cPR3C antibody reactivity with recombinant proteins cPR3C and cPR3m. (**B**) Anti-cPR3C- antibody reactivity with cPR3m protein is not due to cross reactivity with 6Xhistidine-tag. (**C**) Competition assay: Anti-cPR3C rabbit serum (1:200) was pre-incubated with 20mcg/ml cPR3C, cPR3m or irrelevant his tagged protein then analyzed for cPR3C or cPR3m reactivity by ELISA. Results expressed as % of absorbance obtained from serum pre-incubated without recombinant protein. (**D**) Western blot analysis of rabbit anti-cPR3C antibody specificity shows indicates dual reactivity with cPR3C and cPR3m under both reduced and non-reduced conditions. Reactivity with plasminogen (Plg) was observed under non-reduced conditions.

Table 1

Demographics of patients in the study

Parameter	Patients with ANCA Vasculitis		p value
	cPR3C negative (49/67)	cPR3C positive (19/67)	
Mean Age	51.0 (24–79)	47.2 (12–88)	
Median BVAS	3.0 (0–22)	5.0 (0–19)	*p=0.89*
Median PR3 ANCA titer	90.9 (4.8 –1430)	127.1 (1.3–174)	*p=0.36*

[*] *p* values were calculated by Wilcoxon two sample test.