Proteinase 3 (PR3), the target antigen of antineutrophil cytoplasmic autoantibodies, which are found in patients with Wegener granulomatosis, is a neutrophil serine protease localized within cytoplasmic granules. Recently, the human neutrophil antigen NB1 was identified as a specific neutrophil cell surface receptor of PR3. We hypothesized that the unique hydrophobic cluster of PR3 that is not present on human neutrophil elastase receptor of PR3. We hypothesized that the unique hydrophobic patch on Proteinase 3, the target of autoantibodies in Wegener Granulomatosis, Mediates Membrane Binding via NB1 Receptors*§

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Proteinase 3 (PR3) is a multifunctional serine protease stored as an active enzyme within azurophilic granules of polymorphonuclear neutrophils (PMNs) together with neutrophil elastase (HNE) and cathepsin G (CG) (reviewed in Refs. 1 and 2). PR3, initially described as an elastin-degrading protease, is also known as the principal target antigen of antineutrophil cytoplasmic autoantibodies (c-ANCA) that are formed in Wegener granulomatosis patients. This latter disease is characterized by necrotizing granulomas of the respiratory tract and perivascular inflammation of small blood vessels (reviewed in Ref. 3). c-ANCA recognizing conformational epitopes on PR3 are a clinically relevant pathogenic factor and a disease-specific diagnostic marker for Wegener granulomatosis.

Resting purified PMNs from the peripheral blood of healthy individuals express variable amounts of PR3 on their membranes (4). Often two subsets of PMNs carrying low and high amounts of PR3 can be distinguished among freshly isolated PMNs of an individual. The proportion of strongly and weakly PR3-positive PMNs is stable over time and is also maintained after PMN priming and subsequent PMN activation (reviewed in Ref. 5). Upon PMN priming, additional amounts of PR3 reach the extracellular milieu either as a freely secreted or as a membrane-bound protease (reviewed in Refs. 1 and 2). This surface-exposed fraction of PR3 is directly accessible to circulating c-ANCA (6). Upon binding of c-ANCA to neutrophil membranes, PMNs are fully activated, produce reactive oxygen species, and release lysosomal enzymes from their azurophil granules, including PR3, to the pericellular environment (reviewed in Ref. 6).

An association of PR3 with membrane lipid rafts and potential interactions of PR3 with the β2-integrins CD18 and CD11b and the FcγRIIb receptor has been reported some time ago (7, 8). PR3 was also associated with a myristoylated membrane protein with a translocase activity, phospholipid scramblase 1, present in lipid rafts (9). Recently, a direct physical interaction of PR3 with NB1 neutrophil antigen, which is also known as CD177, was demonstrated (10, 11). NB1 is a 58–64-kDa glycosyl-phosphatidylinositol-anchored surface receptor, belonging to the urokinase plasminogen activator receptor superfamily, expressed exclusively by PMNs, neutrophilic metamyelocytes, and myelocytes, but not by other blood cells (reviewed in Refs. 12 and 13). Like membrane-bound PR3, neutrophil antigen NB1 shows variable expression on PMNs ranging from 0 to 100% (10). NB1, an as yet poorly characterized membrane receptor, appears to play a role in PMN transmigration as it interacts with PECAM-1 on endothelial cell membrane (14).

Interactions between the positively charged PR3 and PMN membranes cannot simply be due to a charge effect as PR3 binding was not blocked by the highly positively charged gran...
zyme B (15) or by high salt concentrations (4). Direct interactions of PR3 to reconstituted lipid bilayers and lipid vesicles were recently studied, and a dissociation constant of 4.5 μM relating to lipid vesicles with a mixed composition has been determined. A hydrophobic surface patch of PR3 consisting of Phe-166, Ile-217, Trp-218, Leu-223, and Phe-224 was suggested to insert into lipid bilayers (16, 17).

Analyzing the diversity and residue substitutions in PR3 homologs from other closely related primates, we noticed that the hydrophobic patch of human PR3 (hPR3) was not conserved on gibbon (Hylobates pileatus) PR3 (gPR3) and other primates. This finding prompted us to examine whether gPR3 is still able to interact directly with membranes and with cells that express the human NB1 receptor as a glycolipid-anchored membrane protein. Although gPR3 (Leu-166, Arg-218, His-223) did not adhere to lipid membranes and to the human NB1 receptor, a human-gibbon hybrid carrying the respective hydrophobic residues of hPR3 was capable of binding to cells that stably expressed the human NB1 receptor. In conclusion, PR3-NB1 interactions are of hydrophobic nature and retain the hPR3 molecule on cellular membranes.

EXPERIMENTAL PROCEDURES

Production and Purification of Recombinant PR3 Variants—Recombinant proPR3 of human and gibbon origin were expressed in HEK293 (human embryonic kidney) cells using the Flip-In technology (Invitrogen). The expression vector used in this study was based on pcDNA5/FRT (Invitrogen). An Igκ chain secretion signal peptide followed by an S-peptide tag was integrated into the pcDNA5/FRT vector using the oligonucleotide duplex (sequences on request) DJ2972 and DJ2973 (Metabion/Martinsried). Full-length cDNA of hPR3 was amplified with DJ3153 and DJ3156 (Metabion/Martinsried). Hereby, an enterokinase cleavage site (4 × Asp-Lys) was introduced at the N terminus of mature PR3. The resulting PCR product was cloned into the PmII/AgeI sites after digestion. This construct was named pcDNA5/FRT/hPR3-H6. Gibbon cDNA was amplified from blood granulocyte cDNA using two primer pairs, firstly DJ2924 and DJ2929 and subsequently the primers DJ3153 and DJ3156. The final gPR3 product was subcloned (pcDNA5/FRT/gPR3-H6) as described for hPR3. The two human/gibbon chimeras were obtained by replacing a 390-bp BpiI/AgeI fragment in pcDNA5/FRT/hPR3-H6 and pcDNA5/FRT/gPR3-H6 with that from the other species.

Flip-In HEK293 cells (Invitrogen) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transfection of 1.8 μg of pOG44 (encoding the Flp-In recombinase) and 0.2 μg of expression vector was done in 100 μl of serum-free OptiMEM medium containing 7 μl of FuGENE HD transfection reagent (Roche Applied Science). Two days later, the medium was replaced by Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum containing 75 μg/ml hygromycin B (Invitrogen). Hygromycin B-resistant colonies were visible after 14 days.

Recombinant human, gibbon, and hybrid proforms of PR3 were purified from culture supernatants by single step nickel-nitrilotriacetic acid chromatography. Protein concentration

FIGURE 1. Soluble, but not membrane-bound NB1 is degraded by hPR3. A, affinity-purified secreted NB1 receptor was incubated with hPR3 and analyzed by SDS-PAGE (15%) under reducing conditions. Protein bands were visualized by silver staining. The extracellular domain of the NB1 receptor (lane 1) shows the expected molecular weight (molecular weight markers in lane 3). After hPR3 incubations, the NB1 band disappeared completely, and only glycosylated hPR3 was visible (lane 2). B, confocal immunofluorescence microscopy showing plasma membrane expression of NB1 (green) on adherent CHO cells stably expressing the NB1 receptor. C, flow cytometry analysis of NB1-transfected CHO cells before (thin line) and after incubation (dotted line) with hPR3 at arbitrary units.

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![Figure 2](image)

**FIGURE 2.** CHO cells expressing the membrane form of NB1 bind both the proactive and the active form of hPR3. Suspensions of nontransfected (A and B, upper panels) and NB1-transfected (A and B, lower panels) CHO cells were incubated with active hPR3 (A) (bold blue line) or purified proform (B) (prohPR3) and stained with the anti-PR3 CLB12.8 mAb. Isotype control data are given by the gray dotted line. A.U, arbitrary units. C, fluorescent and confocal microscopy showing plasma membrane presence of hPR3 on CHO cells stably expressing NB1 receptor. DIC, differential interference contrast.

Expression of NB1 in CHO Cells—Chinese hamster ovary (CHO) cells were grown in Ham’s F12 medium supplemented with 10% fetal calf serum and 0.5% penicillin/streptomycin and were transfected with the full-length NB1 cDNA in pcDNA3.1 as described previously (14). Stably transfected cells were selected with Zeocin (200 μg/ml; Invitrogen) and subcloned. Transfectants expressing recombinant NB1 receptor were identified by flow cytometry using mAb 7D8 against NB1.

Flow Cytometry Analysis—Flow cytometry was performed on a BD Biosciences FACSsort flow cytometer. The presence of the NB1 receptor on stably transfected CHO cells before and after incubation (1 h, room temperature) with hPR3 (1–5 μg/ml) was analyzed by double labeling using a murine anti-NB1 mAb (MEM-166, Biolegend) and donkey anti-mouse (1:200, Invitrogen) for 1 h at room temperature.

NB1 receptor transfected CHO cells in suspension culture were incubated (1 h, room temperature) with 2 μg/ml purified prohPR3 and active hPR3 and stained with the anti-PR3 mAbs CLB12.8 (Pel-Clyuster), MCPR3-2 (Thermo Scientific), or 4A5 (WieslabAB). The presence of hPR3 after treatment with 2 mg/ml α1-PI (Sigma-Aldrich) and elastin (Proteo Biotech AG, Kiel, Germany) (2 h, room temperature) was analyzed using mAb 4A5. Results were obtained from three independent experiments.

Immunofluorescent Staining and Microscopy Analysis—Adherent NB1 transfected CHO cells were fixed with 3% paraformaldehyde in phosphate-buffered saline and stained using a 1:75 dilution of a murine anti-NB1 monoclonal antibody (MEM-166, Biolegend) and an Alexa Fluor 488 conjugated secondary donkey anti-mouse Ab (1:200) at room temperature for 1 h. Nonspecific binding sites were blocked with 1.5% bovine serum albumin. Confocal microscopy was performed on a Zeiss confocal microscope. Suspended NB1 receptor transfected CHO cells were incubated with 1–2 μg/ml hPR3 in phosphate-buffered saline containing 1% bovine serum albumin at room temperature for 1 h. After centrifugation and resuspension, cells were stained using a mouse anti-PR3 monoclonal antibody CLB12.8 (1:100) and Alexa Fluor 488 conjugated secondary donkey anti-mouse Ab at room temperature for 1 h.

RESULTS AND DISCUSSION

Degradation of Soluble Recombinant NB1 Receptor by hPR3—A secreted version of the His-tagged NB1 receptor was purified with mAb 7D8 affinity column and incubated with hPR3 (1 μg/ml) for 1 h. The proform of hPR3 was produced in HEK293 cells and converted to its active form by enterokinase after purification. After SDS-PAGE and silver staining, we observed a complete disappearance of the NB1 receptor band (~60 kDa).
(Fig. 1A). Similar results were obtained after incubations with commercially available HNE and CG preparations (not shown). By contrast, CHO cells expressing the entire human NB1 receptor showed positive staining after hPR3 incubations. Confocal microscopy analysis of adherent NB1 receptor-expressing CHO cells confirmed the proper sorting and surface anchorage of the receptor in CHO cells (Fig. 1B). After incubations with 1 μg/ml hPR3 at room temperature for 2 h, the NB1 receptor was still present on the cellular surface as shown by flow cytometry after anti-NB1 staining (Fig. 1C). NB1 receptors on the surface of CHO cells are evidently resistant to hPR3, as well as to HNE and CG (not shown) as opposed to its soluble form, which is rapidly degraded by all three enzymes.

**Membrane NB1 Binds Not Only the Mature hPR3 but Also Its Proform**—Proforms and mature forms of serine proteases display conformational differences and, therefore, differ in their proteolytic activities. This transition is known to affect the conformation of three surface loops, the 142–152 loop (so-called autolysis loop), the 186–194 loop, and the 217–225 loop (reviewed in Ref. 19). The 186–194 loop of hPR3 carries three positively charged residues Arg-186A, Arg-186B, and Lys-187, and the 217–225 loop carries the four hydrophobic residues Ile-217, Trp-218, Leu-223, and Phe-224 (20, 21). Using three different monoclonal anti-PR3 antibodies (CLB12.8, 4A5, MCPR3-2), we analyzed the binding of the proform and the mature form of hPR3 to NB1 on CHO cells. Both forms of hPR3 were detected on the plasma membrane of NB1 receptor-expressing CHO cells (Fig. 2, A and B). No interaction was observed with nontransfected CHO cells using recombinant proPR3 and hPR3 at concentrations of 2 μg/ml. Flow cytometry results were confirmed by fluorescent and confocal fluorescence microscopy (Fig. 2C). At high concentrations (15 μg/ml), hPR3 (but not gPR3) adhered to membranes of NB1 receptor-negative CHO cells, indicating that an additional mechanism of hydrophobic binding, direct lipid insertion (16), was operating under these conditions (supplemental Fig. 1A).

We examined whether N-linked carbohydrates were involved in membrane and receptor interactions. hPR3 from the culture supernatant of HEK293 cells is highly glycosylated in contrast to naturally occurring hPR3 from PMNs, which carries only few carbohydrate moieties (22). We confirmed these previous results by mass spectrometry analyses of natural and recombinant hPR3 before and after endoglycosidase F treatment. Using a human proPR3 variant with the natural Ala-Glu-propeptide and without N-linked glycosylation sites (22), we also found PR3 binding to NB1 receptor transfected CHO cells (not shown). As prohPR3 is proteolytically inactive and cannot degrade NB1 receptor in solution, we examined by enzyme-linked immunosorbent assay whether soluble recombinant NB1 was able to interact with immobilized prohPR3. No interaction of the two proteins was observed under these conditions, suggesting that glycolipid-anchored NB1 receptors on cell surfaces either have a different conformation or act in concert with other cell surface components.

**NB1-bound hPR3 Is Active and Inhibited by α1-PI and Elafin**—Next we examined whether NB1-bound membrane-associated hPR3 is still active toward a fluorogenic substrate Abz-VADnorVADRQ-EDDnp (21) and whether macromolecular inhibitors can access the active site region of PR3-NB1 complexes. To this end, we recorded cleavage activities in suspensions of hPR3 bound by NB1 transfected CHO cells after incubation with hPR3 was measured using the fluorescence resonance energy transfer peptide Abz-VADnVADRQ-EDDnp (29). Approximately 20% of the total hPR3 is detected in the supernatant. B, the Abz-VADnVADRQ-EDDnp peptide is cleaved by NB1 receptor transfected CHO cells loaded with hPR3, but this activity is lost after α1-PI treatment. A.U., arbitrary units. C, flow cytometry analysis of hPR3 binding by NB1 receptor transfected CHO cells before and after treatment with α1-PI, using the anti-PR3 4A5 mAb. The hPR3 signals on the surface of CHO cells (black line) are strongly reduced (dark gray dotted line) by α1-PI treatment. Isotype control staining is depicted in light gray.
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FIGURE 4. Structural differences in PR3 homologs and related neutrophil proteases. A, electrostatic surface potential of hPR3. Solvent-accessible surfaces with positive electrostatic potential are dark blue, and negative ones are in red (Deep View/Swiss-Pdb Viewer V3.7; www.expasy.ch). Hydrophobic residues that are clustered and form a solvent-exposed hydrophobic surface (light gray) are labeled with numbers according to chymotrypsinogen. B, ribbon plot of the hPR3 monomer showing ball-and-stick models for the six hydrophobic residues Phe-165, Phe-166, Ile-217, Trp-218, Leu-223, and Phe-224. C, the same as in A, but all residues on the front site of hPR3 that are substituted in gPR3 are colored in green. D, the hPR3(1FUJ), gPR3, and mouse PR3 (mPR3) and the two other neutrophil serine proteases, HNE (1PPF) and CG (1CGH), were aligned on the basis of their known three-dimensional structure (Protein Data Bank annotation) starting at position 165. Sequence numbers are taken from the Protein Data Bank entries and refer to the chymotrypsin numbering. Solvent-accessible residues contributing to a hydrophobic surface cluster in hPR3 (Phe-165, Phe-166, Ile-217, Trp-218, Leu-223, Phe-224) are labeled by an asterisk at the bottom and are highlighted by bold lettering. Substitutions of these residues by more polar or charged residues in gPR3, mPR3, HNE, and CG are shown in red letters. gPR3 residues Gly-187, Val-204, Val-208, Ala-219, and Ala-229 are shown in green letters.

Molecular Surface Differences between Human and Gibbon Proteinase 3—The primate gibbon (H. pileatus) PR3 (gPR3) was cloned from PMNs of a freshly isolated blood sample and sequenced. It differs from its human homolog by only 16 residues. Comparative structural analysis of hPR3 and gPR3 revealed the location of amino acid variations, which almost exclusively mapped to loops and modified the molecular surface. Natural substitutions on the protein surface have been selected by the evolutionary process and are, therefore, compatible with the biological function and folding pathways of PR3. The substitutions found on gPR3 are not equally distributed but are clustered on one side of the surface of two β-barrels. With one exception, all substitutions are located on the front side of the molecule in a clear distance from the active site region. Critical residues like Asp-61, Lys-99, and Arg-143 that determine substrate binding and cleavage specificity are not altered on gPR3 (21, 23). The N-terminal β-barrel carries seven substitutions (Val-35, Ser-38A, Leu-38B, Gln-60, His-63A, Gln-90, Gln-74). Nine substitutions in gPR3, two of which are highly conservative Ile-Val exchanges (Val-204, Val-208), are localized on the C-terminal β-barrel (Thr-146, Leu-166, Gly-187, Arg-218, Ala-219, His-223, Ala-229). Val-204 and Ala-229 are located in the inner portion of the molecule. The most striking difference between gPR3 and hPR3 is the replacement of three hydrophobic residues (Phe-166, Trp-218, Leu-223) by positively

solution, whereas 80% remained associated with cellular membranes (Fig. 3A). Exogenously added α1-P1 completely inhibited the activity of membrane-bound hPR3 (Fig. 3B). To investigate the fate and behavior of hPR3 after α1-P1 binding, we analyzed the cells by flow cytometry using the anti-PR3 mAb 4A5 that binds to PR3 even in a complex with NB1. Molecular surface differences (Val-35, Ser-38A, Leu-38B, Gln-60, His-63A, Gln-90, Gln-74). Nine substitutions in gPR3, two of which are highly conservative Ile-Val exchanges (Val-204, Val-208), are localized on the C-terminal β-barrel (Thr-146, Leu-166, Gly-187, Arg-218, Ala-219, His-223, Ala-229). Val-204 and Ala-229 are located in the inner portion of the molecule. The most striking difference between gPR3 and hPR3 is the replacement of three hydrophobic residues (Phe-166, Trp-218, Leu-223) by positively

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charged or more polar residues (Leu-166, Arg-218, His-223) on the surface of gPR3. In particular, the positively charged Arg-218 interrupts the surface-exposed hydrophobic patch on gPR3 (Fig. 4, A–C). The strongly hydrophobic patch of hPR3 is also not found on PR3 homologs from other species and on HNE or CG (Fig. 4D). By contrast to hPR3, the latter two enzymes bind to PMNs by charged dependent mechanisms via heparan sulfate- and chondroitin sulfate-containing proteoglycans (24, 25). We therefore hypothesized that hydrophobic surfaces of the NB1 receptor and hPR3 account for the membrane binding of hPR3. This hypothesis implied that only hPR3, but not gPR3, was able to interact with human NB1-expressing cells.

Mapping of the NB1-interacting Region on hPR3—These considerations were consistent with our initial experimental results indicating that hPR3, but not gPR3, was able to bind to NB1-expressing CHO cells (Fig. 5, A and B, and supplemental Fig. 1C). To define the NB1-interacting area at the structural level, we constructed human-gibbon hybrids with the N- and C-terminal β-barrels, which are the smallest independently folding subdomains of serine proteases. These two subdomains of chymotrypsin-type serine proteases are homologous to each other and can be reassembled into catalytically active hybrid molecules that retain the functional properties of the parental subdomains (26). We produced in HEK293 cells the g/hPR3 hybrid (N-terminal β-barrel from gPR3 and C-terminal β-barrel from hPR3) and the h/gPR3 hybrid (N-terminal β-barrel of human origin), which lacks three hydrophobic residues in the 217–225 loop. Only the g/hPR3 hybrid, but not the h/gPR3 variant, was detected on the surface of NB1-transfected CHO cells (Fig. 5, C and D). As the most radical changes affected the surface area of closely clustered hydrophobic residues (Phe-165, Phe-166, Ile-217, Trp-218, Leu-223, Phe-224) and no other area of the C-terminal subdomain, there is little doubt that Phe-166, Trp-218, and Leu-223 are located within the contact area between NB1 and hPR3.

Covalent complexation between serpins like α1-PI and serine proteases triggers a number of conformational changes in the bound protease. In particular, the surface loop 217–225 is distorted relative to the free structure, as reported for the α1-PI-pancreatic elastase complex (27). As this segment of hPR3 contains four hydrophobic residues, Ile-217, Trp-218, Leu-223, and Phe-224, and forms a γ-turn between Ile-217 and Gly-219 with Trp-218 in the center (20), distortion of the 217–225 loop in the complex with α1-PI most likely disrupts the binding site for NB1. As a consequence, hPR3 dissociates from the NB1 receptor during its interaction with α1-PI. Our findings explain why cell-bound PR3 autoantibodies were not detectable on PMN membranes in whole blood from Wegener granulomatosis patients as membrane-bound hPR3 is rapidly removed from the NB1 receptor at the usually high levels of α1-PI in human blood (28).

The approach chosen by us is original and insightful and clarifies the hydrophobic nature of PR3 interactions with cellular membranes at the structural level. The binding of hPR3 to cellular surfaces has attracted a great deal of attention for various reasons in the past. Different mechanisms for its hydrophobic interactions with membranes have been inferred, but the molecular basis for this unique property of hPR3 has so far not been clarified. Hence it is important to map the structural determinants that mediate this unusual property of hPR3. Membrane-bound human PR3 of PMNs is the genuine target of c-ANCA that can activate PMNs. Surface-bound autoantibodies trigger perivascular inflammation in Wegener granulomatosis. Surface-bound PR3 may also contribute to pericellular proteolysis and tissue damage around cytokine-primed PMNs in general. The surface-accessible epitopes of PR3/NB1 complexes are presumably the prime target of pathogenic anti-PR3 autoantibodies.

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