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Characterization of a recombinant proteinase 3, the autoantigen in Wegener's granulomatosis and its reactivity with anti-neutrophil cytoplasmic autoantibodies**

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Abstract Using the baculovirus/insect cells system, we have expressed a recombinant proteinase 3 (PR3) — the neutrophil-derived serine protease autoantigen in Wegener's granulomatosis — as a glycosylated intracellular and membrane-associated protein. Oligosaccharides accounted for the difference in molecular weights between recombinant (34 kDa) and neutrophil-PR3 (29 kDa). Whereas rabbit-anti-PR3 IgG recognized both recombinant and neutrophil-derived PR3, autoantibodies from Wegener patient sera recognized only neutrophil-derived PR3. Although oligosaccharides were not involved in PR3 epitope recognition, autoantibodies did not recognize the amino acid primary structure of recombinant PR3. Improper disulfide bond formation and/or lack of post-translational events in insect cells, may affect the conformation of PR3, precluding its reactivity with sera from WG patients.

Key words: ANCA; Azurophil granule; Baculovirus; Proteinase 3; Wegener

1. Introduction

Proteinase 3 (PR3) belongs to the broad family of neutrophil-derived cationic glycoproteins involved in the defense against pathogens via the non-oxygen-dependent pathway [1]. Described first by Baggiolini in 1978 [2], PR3 is an elastase-like serine protease packed in azurophil granules from neutrophilic polymorphonuclear leukocytes (PMN) [3] which degrades a variety of extracellular matrix proteins [4] and can cause emphysema in hamsters [5].

A different starting point of clinical research also led to the identification of PR3 as the major, if not sole, target autoantigen of anti-neutrophil cytoplasmic antibodies (ANCA) associated with Wegener's granulomatosis (WG) [6,7]. There is mounting evidence that ANCA may play a direct role in the pathogenesis of ANCA-associated vasculitis and could be used as a marker for disease activity [8]. In vitro, ANCA can activate neutrophils to produce reactive oxygen species, degranulate and damage target cells [9]. Standardized indirect immunofluorescence tests to diagnose and monitor patients have been developed, based on the typical cytoplasmic pattern (cANCA) of alcohol-fixed neutrophils using patient's sera. Only antibodies producing this fluorescence pattern turned out to be highly specific for WG [10].

The cDNA and gene encoding for PR3 have been cloned [11–13] and the molecular genetic similarity between PR3 and WG autoantigen has been demonstrated [14]. Several studies exploring the nature of antigen recognition of cANCA have led to divergent results, some reports provided evidence that autoantibodies from WG sera recognized conformational epitopes on PR3 [15], while others described linear epitopes [16] similar to surface-exposed regions of PR3. To settle the controversy and facilitate the mapping of the epitopes of the protein, we have expressed PR3 in a baculovirus insect cell system which is supposed to carry several of the post-translational modifications found in mammalian proteins [17].

2. Materials and methods

2.1. DNA constructs

For the baculovirus construct, the full length of PR3 cDNA [11] was amplified using the Gene Amp PCR kit (Perkin Elmer Cetus, Norwalk, CT) and was cloned using standard techniques [18] into the expression plasmid pAcC4 (kindly provided by Dr. R. Stevens, Frederick, MA), between the *NcoI* and *SmaI* sites, directly behind the ATG start codon of the polyhedrin gene which is flanked by wild type virus AcMNPV DNA sequences. The complete sequence of this recombinant plasmid designated pAcC4/PR3 was shown to be correct by dideoxynucleotide sequencing (Sequenase, version 2.0, US Biochemical Corp., Cleveland, OH).

2.2. Transfection and purification of recombinant virus AcMNPV/PR3

Spodoptera frugiperda (Sf9) cells (ATCC CRL 1711, Rockville, MD) were cultured in TMN-FH medium (Biowhittaker, Walkersville, MD), 10% fetal calf serum as described [17]. Sf9 were transfected with a mixture of 3 μg recombinant plasmid vector (pAcC4/PR3), 1 μg linearized wild-type AcMNPV and 20 μl cationic liposomes (Invitrogen, San Diego, CA). The recombinant baculovirus (AcMNPV/PR3) was then cloned and purified as previously described [19].

2.3. Indirect immunofluorescence of Sf9 cells

Immunofluorescence was performed on Sf9 cells grown on coverslips and infected for 2 days with the recombinant virus at a multi-

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Abbreviations: AcMNPV, *Autographa californica* mononuclear polyhedrosis virus; AG, azurophil granules; ANCA, anti-neutrophil cytoplasmic antibody; cANCA, cytoplasmic ANCA; PR3, proteinase 3; Sf9, *Spodoptera frugiperda* clone 9 cells; WG, Wegener's granulomatosis.

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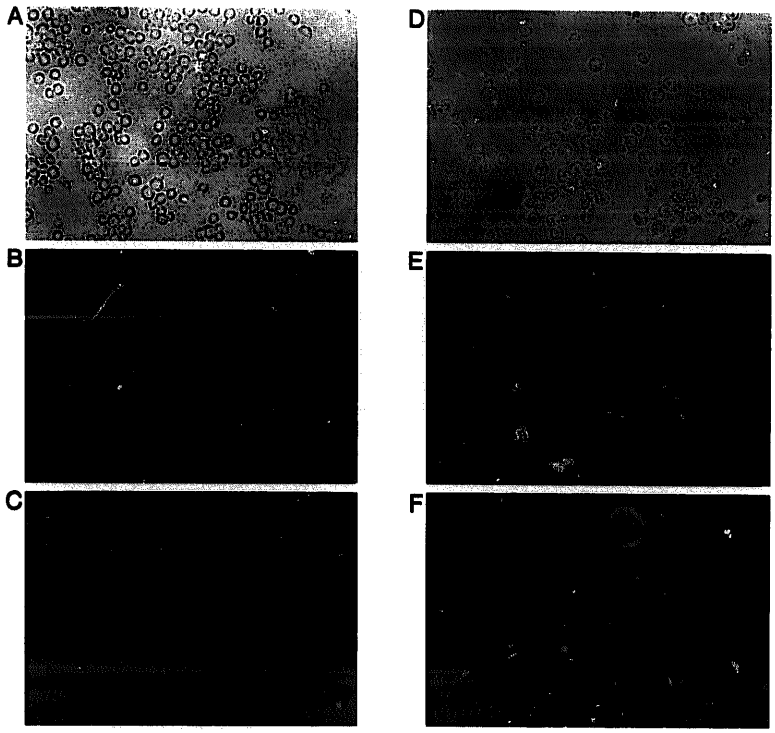

Fig. 1. Indirect immunofluorescence of Sf9 cells infected by AcMNPV/PR3. Sf9 cells infected with AcMNPV/PR3 (D, E, F) at 3 days post-infection, or non-infected Sf9 cells (A, B, C), were viewed by light microscopy (A and D) or examined by fluorescence microscopy (at magnification ×450 in B and E, and magnification ×1000 in C and F).

plicity of infections of 1 to 10 pfu/ml. Cells were fixed with methanol and incubated for 1 h with dilutions of polyclonal rabbit anti-PR3 or preimmune antibody followed by rhodamine isothiocyanate-conjugated goat IgG anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

2.4. Biochemical analysis of recombinant PR3 by Western blot

AcMNPV/PR3-infected Sf9 cells were harvested at the indicated time and washed twice in PBS. Unglycosylated PR3 was produced by adding tunicamycin (5 μg/ml, Calbiochem, La Jolla, CA) to the culture medium 1 h post-infection. Sf9 pellets were boiled in reducing buffer (1% SDS, 2% β-mercaptoethanol, 5% glycerol) and run on 12.5% SDS PAGE gel along with prestained molecular weight markers (Gibco BRL, Gaithersburg, MD). Proteins were then transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) in 20% methanol, 25 mM Tris and 192 mM glycine buffer, pH 8.3, at 100 V for 2 h. The membrane was incubated for 1 h with blocking solution (Carnation dry milk 5%+0.05% Tween 20 in PBS) and incubated successively with dilutions of polyclonal rabbit anti-PR3 [3] and secondary antibody $F(ab')_2$ of donkey IgG anti-rabbit IgG conjugated to alkaline phosphatase (The Jackson Immunoresearch Laboratories, West Grove, PA). After washing, the membrane was developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates (Sigma Chemicals, St Louis, MO).

Subcellular fractionation was performed on 100×10^6 AcMNPV/PR3-infected Sf9 cells resuspended in a relaxation buffer (100mMKCl, 3 mM NaCl, 1 mM $ATP(Na)_2$, 3.5 mM $MgCl_2$, 10 mM Pipes, pH 7.3) and disrupted by nitrogen cavitation for 20 min at 350 psi in a bomb at 4°C [20]. Nuclei and unbroken cells were pelleted by centrifugation at $500\times g$ for 10 min at 4°C. The postnuclear supernatant was centrifuged for 90 min at $100\,000\times g$.

Various solubilization protocols were carried out on Sf9 cells infected by AcMNPV/PR3 at day 3 post-infection. Sf9 cells were sonicated in a hypotonic Tris buffer (10 mM Tris, pH 7.8, 3 mM $MgCl_2$, 50 mM KCl) and centrifuged at $100\,000\times g$ for 45 min. The pellet was washed 2 times with 50 mM Tris and submitted to the different extraction protocols: acid (50mM glycine, pH 2.0, 40 min at 20°C), base (0.1 M sodium bicarbonate, pH 11.0, 40 min at 20°C), salt (2 M NaCl, 50 mM $NaPO_4$, pH 7.0, 40 min at 20°C), detergents (Triton-X100 1%, or NP-40 1%, or Cholate 1%, or CHAPS 1%, or Lubrol 1%, 20 min at 4°C), organic solvents (100% acetonitrile, 12 h at 4°C), denaturing agent (urea 8 M, pH 8, 1 h at 20°C) or strong detergents (0.1 and 1% SDS in 50 mM Tris, pH 7.8 for 30 min). The cell extract was centrifuged at $100\,000\times g$ and pellet and supernatant were analyzed for the presence of PR3. All reagents were from Sigma.

2.5. Purification of PR3 from PMN granules

Granules of human PMN were isolated [20] and PR3 was purified using an aprotinin-Sepharose column [21] followed by Matrex gel orange A chromatography [5]. The eluate was then immunoadsorbed using a PR3 monoclonal antibody (CLB, Clone 12.8, Amsterdam) [22].

2.6. Glycosylation analysis

Determination of PMN-purified PR3 glycosylation was performed using the Lectin-Link kit (Genzyme, Cambridge, MA) combined with chemiluminescent detection. Briefly, purified PMN-derived PR3 (50 μg/ml) was first subjected to Western blotting. The membrane was then incubated with a panel of biotinylated lectins, including GNA (*Galanthus nivalis* agglutinin), SNA (*Sambucus nigra* agglutinin), DSA (*Datura stramonium* agglutinin), suitable for identifying 'high mannose', or complex sialylated, or *N*-acetylglucosamine *N*-glycan chains, respectively. The nitrocellulose membrane was then incubated with horseradish peroxidase-conjugated streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL), and was revealed by ECL detection (Amersham, Buckinghamshire, UK). Deglycosylation treatment was performed as follows: 2 μg of PMN-purified PR3 in 50 mM Tris, pH 8.0 was first denatured by boiling in 0.5% SDS. The sample was diluted with 200 mM sodium phosphate buffer, pH 8.6, 10 mM 1,10-phenanthroline, and 1.25% NP-40. *N*-glycanase (Peptide-*N*-(*N*-acetyl-glucosaminyl) asparagine amidase, EC 3.5.1.52, Genzyme) was added at 10 U/ml and the reaction mixture was incubated at 37°C for 16 h. The samples were then Western blotted.

2.7. ANCA testing

High titer sera were obtained from 15 patients suffering from WG. The diagnosis was established on the basis of classic symptoms and the typical histological findings in biopsy specimens. Sera of these

patients were selected on the basis of the positivity in both indirect immunofluorescence performed on alcohol-fixed PMN and ELISA on azurophil granule extracts from neutrophils [22]. Reactivity of Sf9-derived recombinant PR3 with cANCA was successively tested: (1) by indirect immunofluorescence performed with dilutions of patient serum followed by FITC-conjugated anti-human IgG (Dako, Carpinteria, CA); (2) by ELISA on urea and SDS extracts of Sf9 cells. Briefly, ELISA plates were coated with Sf9 extracts or purified azurophil granules solubilized either in 1% Triton-X100 or in 8 M urea or in 1% SDS (50 μg protein/ml) in carbonate buffer, pH 9.6. After saturation with 1% casein, diluted serum was added and incubated for 1 h at 37°C. The second antibody was a biotinylated $F(ab')_2$ anti-human IgG (Sigma) detected with an alkaline phosphatase–streptavidin complex (Amersham, Buckinghamshire, UK) using *p*-nitrophenyl phosphate (Sigma) as substrate and OD at 405 nm was measured [22]; (3) by Western blots performed on 1% SDS Sf9 extracts diluted in sample buffer (with or without β-mercaptoethanol, as indicated). Western blotting protocol was modified since proteins were transferred in 15 mM bicarbonate buffer, pH 10.5 at 100 V for 2 h at 4°C. Dilutions of sera from WG patients were used as primary antibody and alkaline phosphatase-conjugated anti-human IgG (Sigma) was used as secondary antibody.

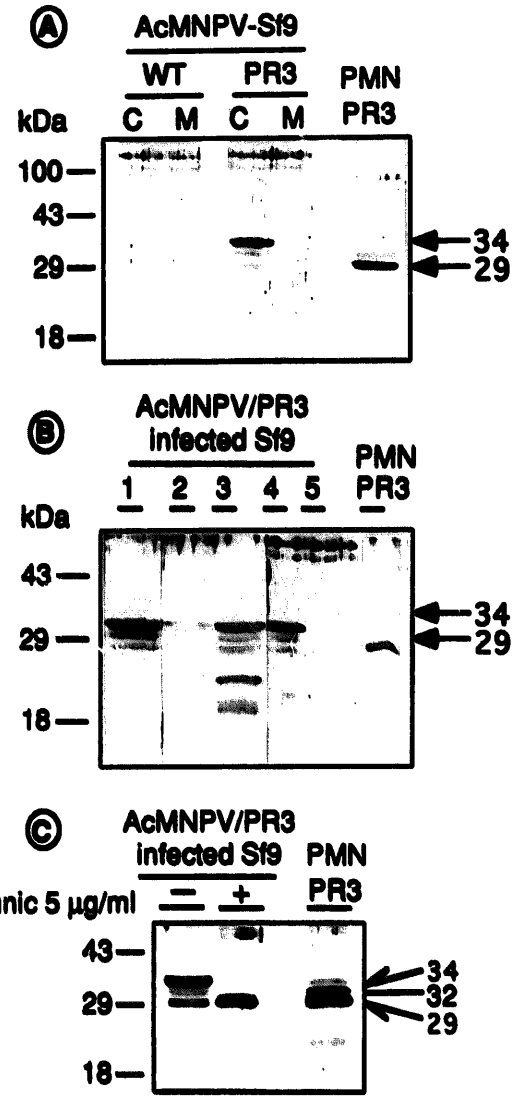

Fig. 2. Intracellular localization, glycosylation of recombinant PR3 expressed in Sf9 cells. PMN-derived azurophil granules containing native PR3 were run in the outer lane of each gel for comparison. The arrows indicate 34 and 29 kDa, the molecular weights of Sf9- and PMN-derived PR3, respectively. *Panel A*, Sf9 cells were infected with either wild type AcMNPV (WT, lanes 1–2) or the recombinant baculovirus AcMNPV/PR3 (PR3, lanes 3–4). Sf9 cells (C) or culture medium (M) were collected at 3 days post-infection (0.5×10^6 cells per lane). *Panel B*, Sf9 cells were submitted to nitrogen cavitation and an aliquot from each fraction (10^6 cells equivalent) was analyzed by Western blot: (lane 1) total cell lysate after cavitation, (lane 2) pellet after low-speed centrifugation (intact cells + nuclei), (lane 3) supernatant after low-speed centrifugation (membrane + organites + cytosol), (lane 4) pellet after high-speed centrifugation (membranes + organites), (lane 5) supernatant after high-speed centrifugation (cytosol). *Panel C*, post-sonication pellets were obtained from AcMNPV/PR3-infected Sf9 cells in the absence or presence of tunicamycin and analyzed by Western blot (20 μg/lane). PR3 produced by either untreated (lane 1) or tunicamycin-treated AcMNPV/PR3-infected Sf9 cells (lane 2) were compared with PR3 from PMN-derived azurophil granules (20 μg/lane) (lane 3). Arrows indicate the same three PR3 isoforms due to different glycosylation forms, either in PMN- or in Sf9-derived PR3.

3. Results

3.1. Recombinant PR3 is expressed in Sf9 cells infected with AcMNPV/PR3

Indirect immunofluorescence showed that no protein reacting with the rabbit polyclonal anti-PR3 IgG was detectable in non-infected Sf9 cells, whereas infection of Sf9 cells with AcMNPV/PR3 resulted in the expression of the recombinant PR3 in Sf9 cells and suggests that PR3 was membrane-associated (Fig. 1)

3.2. Recombinant PR3 produced by Sf9 cells is a 34 kDa glycosylated membrane-associated protein

Immunoblot analysis performed with the rabbit polyclonal anti-PR3 IgG showed that recombinant PR3 was produced as an intra-cellular protein, with a prominent band at 34 kDa, and 3 distinct lower bands ranging from 34 to 29 kDa, as compared with the native PMN-derived PR3 from azurophil granules at 29 kDa. Recombinant PR3 was not secreted as no PR3 was detected in the culture medium (Fig. 2A).

Subcellular fractionation of AcMNPV-infected Sf9 cells followed by immunoblotting analysis revealed that the recombinant PR3 was insoluble and membrane-associated as it was found in the pellet after high-speed centrifugation whereas no PR3 was detected in the supernatant (Fig. 2B).Unglycosylated recombinant PR3 was produced by AcMNPV/PR3-infected Sf9 cells cultured in the presence of tunicamycin, an inhibitor of the first step of *N*-glycosylation. Tunicamycin (5 μg/ml) was added 1 h post-infection and Sf9 cells were harvested 3 days post-infection. Western blot analysis showed that in the presence of tunicamycin recombinant PR3 appears as a single 29 kDa band, smaller than the PR3 produced in the absence of tunicamycin (34 kDa), and similar to the prominent band observed with PMN-derived PR3 (Fig. 2C). Oligosaccharides accounted for the difference in molecular weight observed between recombinant and PMN-derived PR3 and in glycosylation pattern.

3.3. Autoantibody recognition of PR3 in WG is glycosylation-independent

We first investigated the nature of PMN-purified PR3 glycosylation by lectin probing. Native PR3 reacted with *Sambucus nigra* agglutinin (SNA), thus indicating that PR3 bears sialic acid (Fig. 3), whereas no reactivity was found with other lectins such as GNA or DSA (data not shown). We then

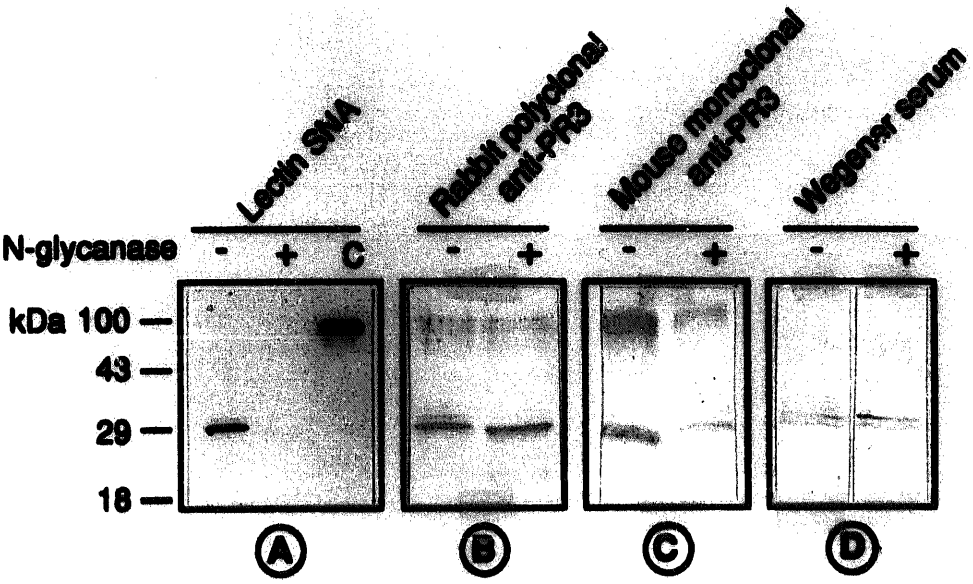

Fig. 3. PR3 glycosylation is not involved in cANCA recognition. Glycosylation analysis was performed on purified PMN-derived PR3 (2 μg/lane) treated or not with *N*-glycanase and Western blotted. On the first panel, PR3 (lane 1) and *N*-glycanase-treated PR3 (lane 2) were probed with the lectin SNA, which binds sialic acid. Transferrin (100 kDa) was used as a positive control for the lectin SNA (lane 3). On the second panel, PR3 (lane 1) and *N*-glycanase-treated PR3 (lane 2) were probed with rabbit polyclonal anti-PR3 IgG. On the third panel, PR3 (lane 1) and *N*-glycanase-treated PR3 (lane 2) were probed with mouse monoclonal anti-PR3 IgG. On the fourth panel, PR3 (lane 1) and *N*-glycanase-treated PR3 (lane 2) were probed with serum from WG patient number 1. This representative experiment was performed five times with five different WG sera and gave similar results.

treated PMN-purified PR3 with *N*-glycanase, which releases all common classes of Asn-linked oligosaccharides. As expected, the lectin SNA did not bind the *N*-glycanase-treated PR3, indicating the removal of the carbohydrate moiety. Binding of PMN-purified PR3 to the rabbit polyclonal, mouse monoclonal or autoantibodies from WG sera on Western blots has essentially the same affinity as the binding of the same antibodies to *N*-glycanase-treated-PMN-purified PR3 (Fig. 3). It should be noted that, unlike Sf9-derived recombinant PR3, PMN-purified PR3 is not heavily glycosylated.

3.4. Recombinant PR3 produced by Sf9 cells is highly insoluble

Immunoblot analysis of the pellet, as well as of the supernatant following different solubilization protocols, was performed and compared to that of non-solubilized Sf9 cell membranes. Among the 10 different solubilization protocols tested to extract PR3 from the cell membrane pellet, only 8 M urea, pH 8.0 and 1% SDS, partially solubilized PR3, whereas 0.1% SDS did not lead to detectable PR3 in the supernatant. Agents commonly used to solubilize peripheral and integral membrane proteins (acid, base, high salt concentrations, anionic, cationic or zwitterionic detergents and solvents) were unsuccessful (data not shown).

3.5. Autoantigen activity of Sf9-derived recombinant PR3

Using indirect immunofluorescence on AcMNPV/PR3-infected Sf9 cells, in the absence or presence of tunicamycin, no difference was found between sera from WG patients (n = 7) and from controls (n = 7) (data shown). Using standard ELISA techniques [22], we showed that normal control serum did not react with neutrophil azurophil granules whereas in the case of WG sera, strong reactivity was observed with azurophil granules extracted with 1% Triton-X100, as well as with 8 M urea or 1% SDS, showing that cANCA reactivity is not impaired by denaturing agents or a strong detergent (Fig. 4). In contrast, no specific reactivity was observed with Sf9-derived PR3, either in its glycosylated or unglycosylated form. We tested the reactivity of 11 WG sera on PMN-purified PR3 under non-reducing Western blotting conditions. β-mercaptoethanol (β-ME) reduction completely abolished cANCA binding for all the sera tested. Similarly to cANCA, a mouse monoclonal anti-PR3 antibody CLB clone 12.8 recognized PR3 only under non-reducing conditions (Fig. 5). Tested on SDS-extracted Sf9 cells infected with AcMMNPV/PR3 in the presence or absence of tunicamycin, the majority of the WG sera tested (9/11), as well as the mouse monoclonal anti PR3 IgG, did not show any reactivity. We did observe, however, with 2 WG sera (2/11) as well as with one control (1/8), a strong band around 30-31 kDa, in Sf9 infected with AcMNPV/PR3. In contrast to what was observed with the rabbit polyclonal anti-PR3 IgG, this band at 30–31 kDa had the same intensity in the presence or absence of tunicamycin and was β-ME-insensitive (Fig. 5).

4. Discussion

This is the first report showing the expression of a recombinant PR3, the autoantigen of WG and its biochemical characterization in a baculovirus system. Although the recombinant PR3 was not recognized by sera from WG patients, our results reveal new information on the nature of the epitope(s) recognized by cANCA.

The recombinant PR3 produced intracellularly by Sf9 cells infected with AcMNPV/PR3 can be detected on Western blots with specific rabbit polyclonal anti-PR3 IgG. It appears as a 34kDa protein, which is larger than the 29 kDa native PMN-derived PR3 and we further demonstrate that glycosylation accounts for this molecular weight difference. According to

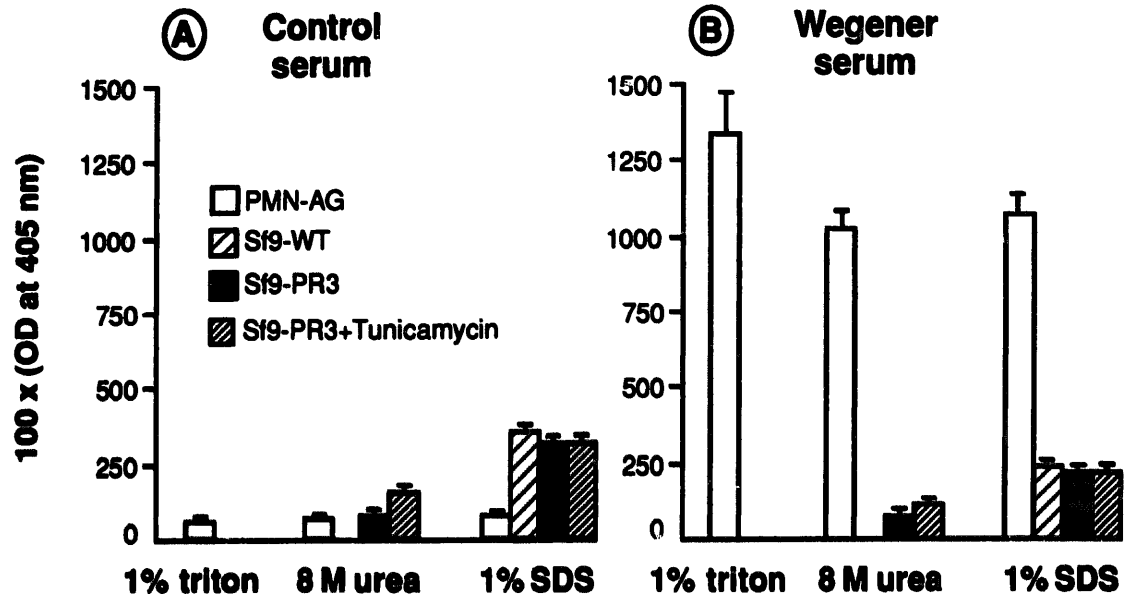

Fig. 4. ELISA comparing the binding of sera either from control or from Wegener's patients with PMN-derived PR3 or recombinant PR3 produced in AcMNPV/PR3-infected Sf9 cells. Sf9 cells infected either with wild type AcMNPV, or AcMNPV/PR3 in the presence or absence of tunicamycin were sonicated, centrifuged to obtain the membrane-associated fraction containing recombinant PR3. Sf9-derived membrane fractions or azurophil granules were extracted with different protocols including 8 M urea or 1% SDS for both azurophil granules and Sf9 cells and 1% Triton-X100 for azurophil granules. These extracts were coated in parallel on ELISA plates and sera from either a control subject (panel A) or a WG patient (panel B) were tested. This representative experiment, including a serum from a control subject and from a WG patient, was performed five times and gave similar results.

our study, native PMN-derived PR3 was weakly glycosylated, and appeared to display a complex oligosaccharide-like structure, with terminal sialic acid residues. It has been suggested that Sf9 cells lack detectable *N*-acetyl-glucosaminyl-, galactosyl-, and sialyltransferase activities, and would not allow the addition of terminal sugars normally found in the complex oligosaccharides of vertebrate cells [23]. Detailed and complete structural studies of oligosaccharides of our recombinant PR3, as well as protein sequencing, were hampered by its insolubility and failure to immunoprecipitate (data not shown). The conditions used to solubilize intracellular PR3 (1% SDS or 8 M urea) suggest that this protein could be part of an aggregate or complexed with other structures. This insolubility, which precludes simple purification protocols, renders obligatory a denaturation step with 8 M urea or guanidinium chloride followed by renaturation. Using small synthetic peptides as substrates [24], we failed to demonstrate a reproducible serine proteinase activity in urea-solubilized Sf9 membranes.

In PMN, PR3 is present in azurophil granules, which are cellular organites specific to cells from the myeloid lineage and the intracellular targeting of PR3, and of other proteins from azurophil granules, has not been elucidated but does not appear to be similar to the targeting of lysosomal enzymes via the mannose-phosphate receptor [25]. A likely explanation is that Sf9 cells are devoid of the specific enzymatic equipment as well as specific cellular organites that are required for proper folding and/or processing and/or targeting of PR3. Indeed, the similarity between the apparent molecular weight of *N*-glycanase-treated PMN-purified PR3 and unglycosylated Sf9-derived PR3 may suggest that, as described for other proteins produced in a baculovirus system, the signal sequence has, most likely, been processed. We evaluate two other constructs that code for different forms of PR3. Since the post-translational processing of PR3 involves at least three proteolytic steps to form the mature enzyme, we constructed a recombinant baculovirus coding for the mature protein, without signal sequence and propeptide. No recombinant PR3 was produced (data not shown). As it has been described that elastase or cathepsin G undergo a carboxy terminal processing, we expressed a 12 amino acid-truncated recombinant PR3. The truncated protein was still membrane associated (data not shown). It has to be noted that azurocidin, another azurophil granule-derived protein which shares 45% sequence homology with PR3, was successfully expressed as a secreted and a fully biologically active recombinant antibiotic protein in the same baculovirus system. Interestingly, the type of glycosylation — high mannose — was similar in PMN- and Sf9-derived PR3 (Almeida et al., manuscript submitted for publication). Our present results suggest that production of recombinant PR3 in a baculovirus system is more complex.

The second set of findings provides new insights into the nature of the epitopes recognized by WG autoantibodies and supports the concept of conformation-dependent binding of autoantibodies to PR3. We have shown by ELISA that PMN-derived PR3 antigenicity is conserved in 8 M urea or 1% SDS, thus demonstrating that the conformational epitopes are maintained by disulfide bonds even in denaturing conditions. Moreover, we tested WG sera using Western blot techniques after boiling the samples in 1% SDS but in the absence of β-ME and showed that WG sera did not recognize recombinant PR3, either in its glycosylated or unglycosylated form. Interestingly, the mouse monoclonal anti-PR3 had the same recognition pattern whereas the rabbit polyclonal anti-PR3 antibody recognized both PMN- and Sf9-derived PR3, regardless of its glycosylation state, in the absence or presence

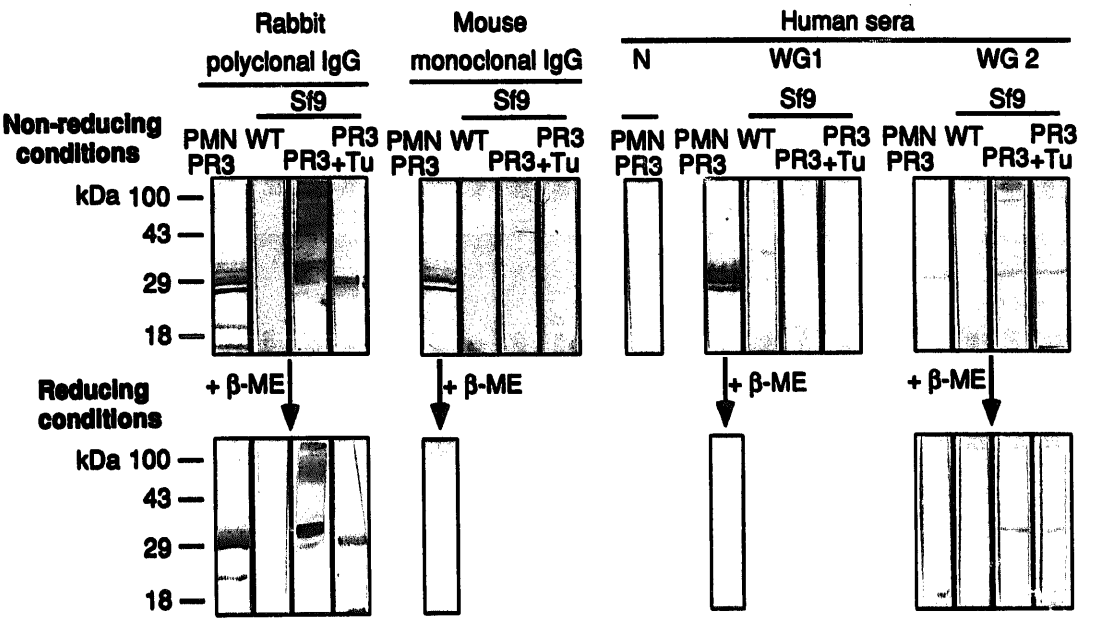

Fig. 5. Reactivity of recombinant PR3 with Wegener's sera tested by Western blot analysis. PMN-derived azurophil granules and the post-sonication pellet from AcMNPV/PR3-infected Sf9 cells were extracted with 1% SDS and the solubilized fractions were dissolved in sample buffer, under non-reducing (in the absence of β-ME, on the upper set of blots) or reducing conditions (in the presence of β-ME, on the lower set of blots). Samples were run on 12.5% SDS-PAGE gel (20 μg/lane) and analyzed by Western blot. All samples including PMN-PR3, wild type AcMNPV- or AcMNPV/PR3-infected Sf9 cells in the presence or absence of tunicamycin, were incubated either with rabbit polyclonal anti-PR3 IgG, or mouse monoclonal anti-PR3 IgG, or human sera from a normal subject (N) or WG patients: WG1 was representative of the group of 9 out of 11 high-titer WG sera which did not show any cross reactivity with AcMNPV/PR3-infected cells, and WG2 was representative of the three sera which showed a non-specific cross reactivity with a 30–31 kDa protein. Relative molecular weights of prestained markers are shown at the left. Results are representative of 5 experiments.

of reducing agent. It should be noted that this polyclonal rabbit antibody has been raised against PR3 purified by HPLC partially denatured by acid treatment [3]. The fact that mouse monoclonal anti-PR3 and the WG sera did not recognize Sf9-derived PR3 indicates that an obligate biochemical feature, present in PMN-derived PR3, is lacking in the recombinant PR3.

As shown by the PR3 cDNA sequence, 8 cysteine residues are present, allowing the possible formation of four intramolecular disulfide bonds. Recombinant PR3, produced either in an in vitro translation system or in bacteria [15], failed to be recognized by autoantibodies from Wegener's sera. The lack of reactivity of recombinant fusion protein could be attributed to an incorrect folding due to the highly reductive intracellular environment of *E.coli*. This problem should have been circumvented by expressing recombinant PR3 in a baculovirus system, as disulfide bonds are usually formed (as observed for azurocidin and other autoantigens [26]). However, the absence of serine proteinase activity and ANCA reactivity strongly suggests that recombinant PR3 has aberrant folding. Our analysis of the importance of the carbohydrate moieties in the binding of PR3 to Wegener's autoantibodies clearly shows that autoantibody recognition of PR3 epitope(s) is mainly oligosaccharide-independent in PMN-derived PR3, which already has a proper folding. Glycosylation, however, may also be involved in the folding process of PR3 as suggested by other reports showing that the N-linked oligosaccharides and their processing are critical for proper glycoprotein folding and assembly [27].

In conclusion, Sf9-derived PR3 is not recognized by cANCA, thus suggesting that its lacks an obligate biochemical feature required for ANCA recognition. Disulfide bond formation and/or post-translational events may affect the conformation of the molecule to such a great extent that the absence of such a modification, which may occur in a restricted number of cell types, would preclude the reactivity of the protein with the majority of autoantibodies found in sera from WG patients.

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