Journal of Cellular Biochemistry 99:117–125 (2006)

Membrane Proteinase 3 and its Interactions Within Microdomains of Neutrophil Membranes

Ram Fridlich, Alina David, and Irit Aviram*

The Department of Biochemistry, Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel

Abstract Proteinase 3 (PR3) is a serine protease of neutrophil granules released to the medium or into the phagocytic vesicle upon neutrophil stimulation. A fraction of the enzyme is thought to associate with the cell membrane yielding membrane PR3 (mPR3). In autoimmune disorders characterized by the presence of antineutrophil cytoplasmic antibodies (ANCA), the reaction of the latter with their target antigen mPR3 activates the cell inflicting injuries on the surrounding tissues. In a previous communication we provided evidence for the presence of mPR3 in lipid rafts obtained by lysis of neutrophils in Triton X-100 and for the mediation of PR3 binding to the membrane by a glycosylphosphatidylinositol (GPI)-anchored neutrophil protein, possibly FcγRIIIb. In the current study we employed the mild detergent Brij 58 to isolate high molecular weight (HMW) protein complexes in the void volume of a Sepharose 4B gel filtration minicolumn. HMW complexes of unstimulated neutrophils comprised PR3, FcγRIIIb, the β2 integrin CD11b/CD18 as well as the membrane and cytosolic subunits of the NADPH oxidase, $p22^{phox}$ and $p47^{phox}$/$p67^{phox}$. Treatment of neutrophils with phosphatidylinositol-specific phospholipase C (PI-PLC) reduced amounts of PR3 and FcγRIIIb in HMW complexes isolated from the treated cells, supporting our previous suggestion that FcγRIIIb acts as a membrane adaptor for PR3. FcγRIIIb of HMW fractions co-immunoprecipitated with PR3, indicating their presence in the same protein complex. Since HMW fractions contained also the majority of biotinylated proteins obtained by the reaction of neutrophils with a membrane impermeable biotinylating agent Sulfo-NHS-biotin, it was concluded that HMW proteins were derived from cell membranes. Lipid rafts isolated from Brij 58-lysed neutrophils were similar in their protein composition to the HMW complexes but not identical. J. Cell. Biochem. 99: 117–125, 2006.

Key words: proteinase 3; neutrophils; rafts; membrane complexes

Proteinase 3 (PR3), a soluble serine protease of neutrophil azurophilic, specific and secretory granules [Jennette et al., 1990; van der Geld et al., 2001], is present also on plasma membranes of freshly isolated human neutrophils [Halbwachs-Mecarelli et al., 1995; Witko-Sarsat et al., 1999; Campbell et al., 2000]. In patients of the autoimmune Wegener's granulomatosis disease (WG) PR3 has been identified as the main target antigen for c-anti neutrophil cytoplasmic antibodies (ANCA) autoantibodies [Jennette et al., 1990; Witko-Sarsat et al., 1999]. The binding of c-ANCA to membrane PR3 (mPR3) induces the release of proteases and superoxide ions which inflict damage upon body tissues [Falk et al., 1990]. Several studies suggested that the membrane expression of PR3 correlates with severity of the disease [Witko-Sarsat et al., 1999; Rarok et al., 2003].

The mode of PR3 binding to the plasma membrane of neutrophils as well as the identity of neutrophil surface molecules mediating this association have not been elucidated. We have recently provided evidence for an interaction of neutrophil mPR3 with the β2 integrin adhesion molecule CD11b/CD18 and the glycosylphosphatidylinositol (GPI)-anchored FcγRIIIb [David et al., 2003, 2005].

It has been well documented that cell membranes are composed of domains that differ in their detergent solubility. A special interest has lately been focused on lipid rafts, low-density microdomains resistant to non-ionic detergents and enriched in cholesterol and glycosphingolipids [Brown and London, 1998; Maxfield, 2002; Pike, 2004]. Rafts comprise GPI-anchored, acylated, and cholesterol-linked proteins and are believed to serve as scaffolds or platforms for

*Correspondence to: Irit Aviram, The Department of Biochemistry, Tel-Aviv University, Tel-Aviv 69978, Israel. E-mail: avirama@post.tau.ac.il

Received 26 December 2005; Accepted 20 January 2006

DOI 10.1002/jcb.20901

signaling and trafficking. The most popular method for rafts isolation consists of density gradient centrifugation of cellular extracts in non-ionic detergents.

Several studies described the presence in cell detergent extracts of large, non-covalently linked, soluble, detergent-resistant membrane macromolecular complexes [Cinek and Horejsi, 1992; Horejsi et al., 1994; Skubitz et al., 2000]. Similarly to rafts, these high molecular weight (HMW) complexes may contain signaling molecules as well as GPI-linked membrane proteins [Skubitz et al., 2000]. Different types of complexes involved in a multitude of cell functions may coexist in a given cell [Bohuslav et al., 1995].

The protein and lipid composition of rafts or of the detergent-resistant soluble macromolecular complexes may vary considerably when cells are lysed with different non-ionic detergents [Giurisato et al., 2003; Chamberlain, 2004]. Rafts are commonly prepared by solubilization of cells in Triton X-100 at 4°C. In Triton X-100-isolated neutrophil rafts we have recently shown the presence of PR3 in co-localization with the GPI-linked FcγRIIIb [David et al., 2005]. In the current study we utilized the mild detergent Brij-58 to demonstrate the presence of both PR3 and FcγRIIIb in the HMW complexes as well as in lipid rafts. Furthermore, we confirmed our previous suggestion that the GPI-anchored FcγRIIIb may serve as an adaptor for the association of PR3 with the neutrophil plasma membrane.

MATERIALS AND METHODS

Reagents

The reagents including biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (Sulpho-NHS-biotin) and Streptavidin-Peroxidase from Streptomyces avidini were purchased from Sigma Chemical Co. unless otherwise stated. Dextran and Ficol-Hypaque were obtained from Pharmacia. Protein G PLUS/Protein A Agarose was from Calbiochem. Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) was obtained from Molecular Probes.

Antibodies

Monoclonal anti-PR3 (6A6, 4A5) and rabbit anti-PR3 were purchased from Wieslab AB (Sweden); monoclonal anti-FcγRIIIb were from Biotest; Goat antisera to $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ were a generous gift of Dr. T.L. Leto (National Institutes of Health, Bethesda, MD). Goat IgG was from Sigma Chemical Co.

Preparation of Neutrophils

Human polymorphonuclear leukocytes (PMNs) were isolated from buffy coats by standard procedures of dextran sedimentation, hypotonic lysis of erythrocytes, and Ficol-Hypaque density gradient centrifugation. Isolated cells were resuspended in Krebs-Ringer phosphate (KRP)-buffered solution (131 mM NaCl, 15.7 mM NaPi, pH 7.4, 5.2 mM KCl, 2 mM glucose, 1.3 mM $MgSO_4$, 0.9 mM $CaCl_2$).

Neutrophil Fractionation

Cells (2×10^8/ml) in 10 mM potassium phosphate-buffered saline (PBS) (pH 7.0) supplemented with 1 mM EGTA, 3.5 mM phenylmethylsulfonyl fluoride (PMSF), 15 μg/ml leupeptin, were disrupted by a brief sonication [Tal et al., 1998]. Unbroken cells were removed by low-speed (250*g*) centrifugation. Granules were sedimented at 15,000 rpm for 15 min at 4°C (Eppendorf centrifuge 5403). Plasma membranes and cytosol were separated by ultracentrifugation of the granule-free supernatants at 48,000 rpm for 45 min at 4°C in a SORVAL Ti-50.

Isolation of HMW Complexes From Detergent Lysates

Cells (5×10^7) were suspended in 0.3 ml Brij-58 solubilization buffer (20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mM PMSF, 2 mM $MgCl_2$, 0.02% NaN_3, 1% Brij-58) supplemented with protease inhibitors. After 30 min of incubation on ice the lysate was cleared by centrifugation (6 min, 11,000*g*). The clear supernatant containing ca. 40% of cellular proteins was loaded on a Sepharose 4B mini-column (0.8×5 cm) equilibrated and eluted with the Brij-58 solubilization buffer in 0.3 ml fractions at 4°C. The column was calibrated with Dextran Blue, eluting in the void volume (fractions 3–4) and catalase, bovine serum albumin, ovalbumin, and carbonic anhydrase (225, 67, 45, and 30 kDa, respectively) eluting in fractions 6–10 (not shown).

Immunoprecipitation (IP) and Western Blotting (WB)

Primary antibodies were incubated with 15 μl Protein A + G for 30 min at room temperature,

centrifuged (2 min, 11,000*g*), and washed (2×) with the IP buffer (10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1% BSA) followed by two washes with the IP buffer containing 20% sucrose. HMW fraction (200 μl) was added and the mixture was incubated overnight at 4°C and pelleted. The washed pellets were resuspended in Laemmli sample buffer, boiled for 5 min, and sedimented and the supernatants were subjected to SDS–PAGE. The resolved proteins were transferred to a nitrocellulose membrane, blocked and exposed to anti-PR3 or anti-FcγRIIIb. Protein detection was carried out using the ECL protein detection system.

Isolation of Rafts

Neutrophils (5×10^7 cells) were lysed on ice in 0.5 ml of Brij-58 solubilization buffer for 30 min. The cell lysate was mixed with 0.5 ml of 80% (w/v) sucrose containing 1% Brij 58. Ultracentrifuge tubes were filled with 1 ml of 80% sucrose followed by the cell lysate in 40% sucrose, 5.5 ml 36% sucrose, and 3.5 ml of 5% sucrose. The tubes were centrifuged in the SW41 rotor of Beckman ultracentrifuge (38,000 rpm, 18 h, 4°C). Fractions of 1 ml were collected from the top.

Treatment With PI-PLC

Neutrophils (5×10^7) resuspended in PBS of pH 8 containing 5 E.U./ml of PI-PLC were incubated for 30 min at room temperature. The cells were washed twice in cold PBS, sedimented, lysed in 0.3 ml of Brij-58 solubilization buffer, and gel filtrated.

Biotinylation of Membrane Proteins With Sulfo-NHS-Biotin

Cells (5×10^7) resuspended in 1 ml PBS pH 8.0 were incubated with 0.5 mg Sulfo-NHS-biotin for 30 min at room temperature. The sample was washed twice with cold PBS pH 8.0, lysed and subjected to gel filtration.

Identification of Biotinylated Proteins on Nitrocellulose

After electrotransfer the nitrocellulose membrane was washed briefly with PBS, dried for 30 min at room temperature, and blocked with PBS containing 5% low fat milk and 0.05% Tween20. After four washes with PBS/0.05% Tween20 (5 min each wash) the membrane was incubated for 60 min with HRP-Streptavidin (1:1,000, Sigma) and washed as described above. Biotinylated proteins were detected by ECL.

RESULTS

Multiprotein Brij 58-Resistant Complexes Were Detected in the Void Volume of the Sepharose 4B Column

To characterize HMW complexes of neutrophil lysates in Brij 58, we fractionated the soluble fraction of the detergent lysate on a Sepharose CL-4B minicolumn [Cinek and Horejsi, 1992; Skubitz et al., 2000]. Calibration of the column with size markers indicated that fractions 3 and 4 containing Dextran Blue represented the void volume of the column; catalase (225 kDa) was identified in fraction 6 and smaller protein markers listed in the experimental section eluted in later fractions. In view of this, fractions 3–5 of the column were defined as HMW complexes.

Identification of PR3, FcγRIIIb, $p67^{phox}$, $p47^{phox}$, and $p22^{phox}$ in HMW Complexes

PR3 (29 kDa) was detected in most fractions of the column implying a wide range of size distribution of the enzyme in HMW as well as in low molecular weight (LMW) fractions (3–5 and 6–9, respectively) (Fig. 1A). Western blots of the HMW fractions 3–5 probed for FcγRIIIb and for the membrane and cytosolic subunits of the NADPH oxidase ($p22^{phox}$ and $p67^{phox}$/$p47^{phox}$, respectively) revealed their presence in the HMW fractions (Fig. 1B). Stimulation of neutrophils with PMA did not affect the distribution of the subunits of the NADPH oxidase and of PR3 in fractionated cells extracts (Fig. 1B). By contrast, a prominent 80 kDa protein band identified as PKCβII in HMW fractions of PMA-stimulated cells was not detected in HMW complexes isolated from resting neutrophils. PKCβII is one of the major isoforms of PKC known to phosphorylate the $p47^{phox}$ subunit of the NADPH oxidase and to translocate to the cell membrane [Korchak et al., 1998; Dekker et al., 2000]. On Western blots of the HMW complexes derived from resting cells and probed for PR3, another, hitherto unidentified 80 kDa protein was detected. The expression of this protein was drastically diminished in PMA-stimulated cells (Fig. 1B).

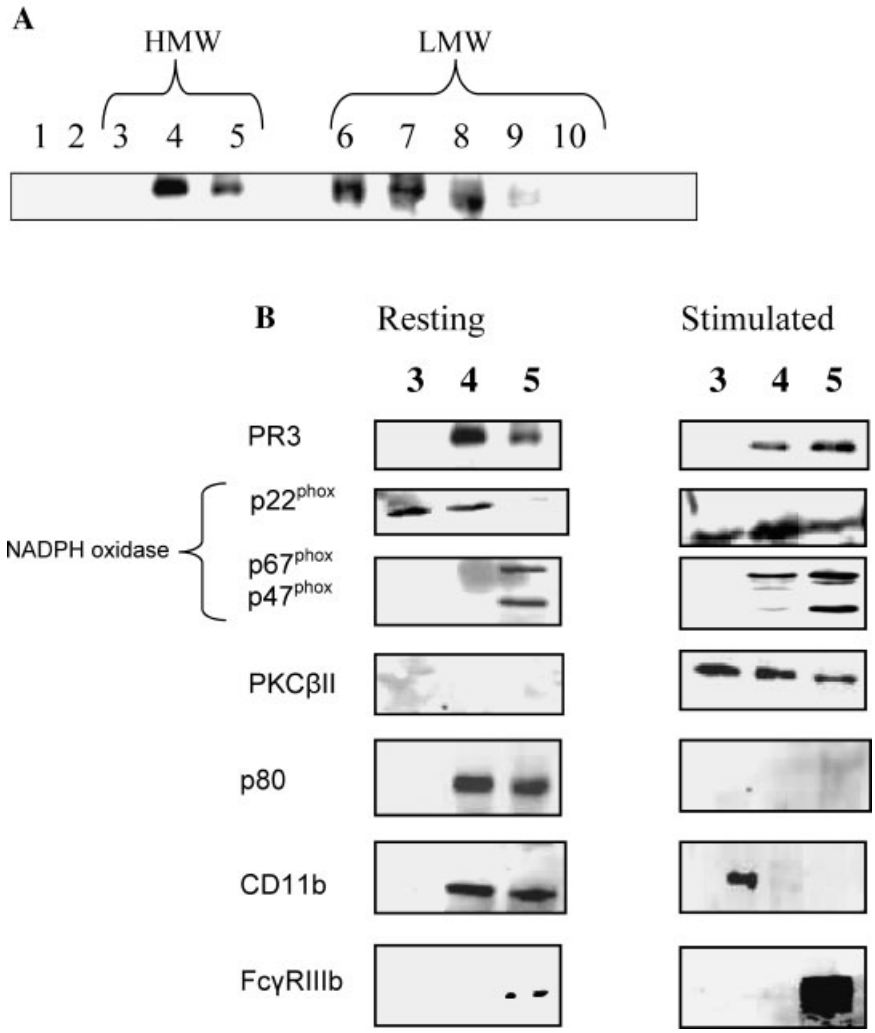

Fig. 1. The distribution of PR3 and other proteins in gel filtration neutrophil fractions. **A**: Neutrophils (5×10^7) were lysed in 0.3 ml Brij-58 solubilization buffer (30 min on ice) and clarified by centrifugation (6 min, 11,000*g*). The clear supernatant was fractionated on a Sepharose 4B minicolumn. The fractions (0.3 ml) were subjected to SDS–PAGE and the blots were exposed to anti-PR3. **B**: Western blots of the 3–5 HMW fractions of the column collected by gel filtration of resting (3–5) and PMA-stimulated cells (3′–5′) were probed with antisera listed in the experimental section.

Identification of the Source of HMW PR3

Whereas the vast majority of neutrophil PR3 is stored in intracellular granules, a small fraction of the enzyme resides on the outer face of the plasma membrane [Csernok et al., 1993; Halbwachs-Mecarelli et al., 1995; Witko-Sarsat et al., 1999]. To define whether PR3 detected in HMW fractions was derived from granules or from plasma membranes, we isolated granules and plasma membranes by differential centrifugation of neutrophil sonicates and solubilized each in the Brij 58 solubilization buffer. Chromatography of the solubilized membranes on the Sepharose 4B minicolumn (Fig. 2A) revealed the major part of mPR3 in fractions 4–5, namely in HMW fractions. An analogous fractionation of solubilized granules (Fig. 2B) yielded a different elution profile: PR3 was present mainly in fractions 7–10 of the Sepharose 4B minicolumn which contain monomeric proteins. It follows that whereas most of membrane-bound PR3 resided in macromolecular complexes, PR3 of granules was not associated into HMW complexes. This finding implies that complexes of intragranule enzymes with HMW

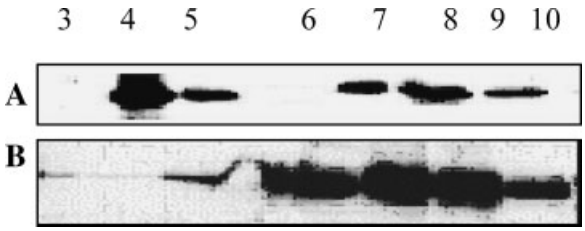

Fig. 2. Gel filtration of solubilized neutrophil plasma membranes (**A**) and granules (**B**) on Sepharose 4B minicolumns. Prior to solubilization and fractionation plasma membranes and granules of neutrophils (5×10^7 cell equivalent) were isolated by differential centrifugation. Proteins of each fraction were resolved by SDS–PAGE, transferred to nitrocellulose, and exposed to anti-PR3.

glycosaminoglycans [Avila and Convit, 1976] are not detergent resistant.

The assignment of PR3 in fractions 3–5 to membrane-associated HMW complexes was confirmed by surface biotinylation of intact neutrophils with sulfo-NHS-biotin. This biotinylating reagent does not penetrate cells and labels only surface residues. Biotinylated neutrophils were solubilized in Brij 58 and fractionated on the Sepharose 4B minicolumn. The collected fractions underwent SDS–PAGE and biotinylated proteins were visualized by streptavidin-HRP and ECL. As shown in Figure 3A, biotin residues were detected mainly in fractions 3 and 4, implicating surface proteins as the main source of the HMW complexes. In another set of experiments, fractions 3, 4 of biotinylated neutrophils were incubated with Streptavidin-agarose beads and the adsorbed proteins were resolved by SDS–PAGE. FcγRIIIb and $p22^{phox}$ were identified on the Western blots, indicating their presence in the biotinylated complexes (unpublished data).

Co-Localization of FcγRIIIb and PR3 in the Same HMW Complex

It has been implied that the GPI-linked FcγRIIIb is engaged upon binding of c-ANCA autoantibodies to their target antigen mPR3 on the surface of neutrophils [Kocher et al., 1998]. Our recent findings also suggested an association between PR3 and FcγRIIIb on the neutrophil membrane [David et al., 2005]. The concurrent presence of FcγRIIIb and PR3 in HMW fractions (Fig. 1) prompted us to confirm these findings by co-immunoprecipitation of the two proteins from the HMW fractions with monoclonal anti-FcγRIIIb or anti-PR3. The immunoprecipitates

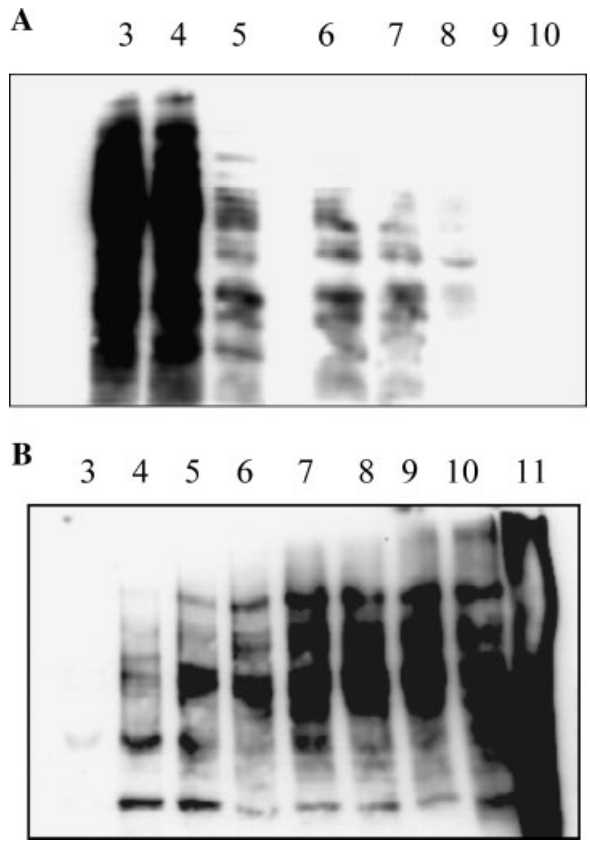

Fig. 3. Fractionation of neutrophils labeled with Sulfo-NHS-biotin. Neutrophils were labeled with the biotinylating reagent (30 min, room temp.) and lysed in Brij 58 as described. **A**: HMW complexes were fractionated by gel filtration on Sepharose 4B (fractions 3–5). **B**: Rafts were obtained by density gradient ultracentrifugation (fractions 4–7). Proteins in the fractions were resolved by SDS–PAGE, transferred to nitrocellulose, and probed for biotin by Streptavidin-HRP and ECL.

were resolved by SDS–PAGE, transferred to nitrocellulose and the blots were probed with anti-PR3 and anti-FcγRIIIb. As shown in Figure 4 PR3 co-immunoprecipitated with FcγRIIIb and

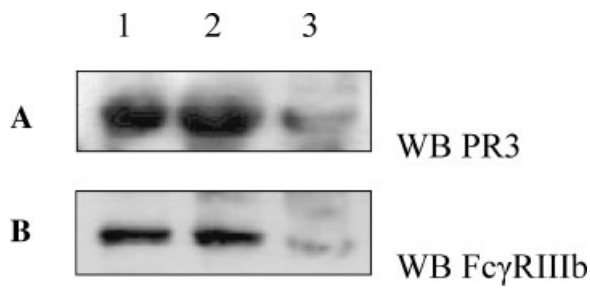

Fig. 4. Co-immunoprecipitation of PR3 and FcγRIIIb in pooled HMW fractions. HMW fractions were immunoprecipitated with (**A**, **B**) **lane 1**, monoclonal anti-PR3; **lane 2**, monoclonal anti-FcγRIIIb; **lane 3**, control IgG. Western blots of the immunoprecipitates were probed for PR3 (A) and for FcγRIIIb (B).

vice versa. This finding implied co-localization of both proteins in the same detergent-soluble HMW complex of neutrophil membranes.

Membrane FcγRIIIb and PR3 Expression Is Susceptible to PI-PLC

Neutrophil FcγRIIIb is attached to the membrane by a GPI anchor. In our recent communication we showed that cleavage of the GPI anchor by bacterial PI-PLC, results in a partial loss of neutrophil surface FcγRIIIb as well as mPR3, suggesting a possible FcγRIIIb-mediated association of PR3 with the membrane [David et al., 2005]. In view of this we tested the possibility that the interaction of PR3 with HMW complexes of the neutrophil membranes is also mediated in part by FcγRIIIb. For this purpose neutrophils were treated with PI-PLC to cleave their GPI surface linkages, lysed and size fractionated. Indeed Western blots of the gel filtration column fractions indicated a partial PLC-mediated loss of both FcγRIIIb and PR3 from the HMW fractions of PI-PLC-treated cells (Fig. 5A,B). As expected, PR3 of the LMW fractions of the column was not affected by the PLC treatment (Fig. 5C).

Isolation of Brij-58 Rafts

For the sake of comparison of protein compositions of HMW complexes and lipid rafts prepared in the same detergent, rafts were isolated by solubilization of neutrophils in Brij 58 and ultracentrifugation of the lysates in sucrose density gradients. The low-density fractions 3–5 representing lipid rafts of resting cells contained PR3, $p22^{phox}$, and $p47^{phox}$ (Fig. 6). FcγRIIIb was detected in the later raft fraction 5 and in subsequent non-raft, denser fractions of the gradient (Fig. 6). Unlike in Triton X-100-solubilized rafts [David et al., 2005], levels of PR3 in Brij 58-isolated rafts were reduced by PMA stimulation (Fig. 6), whereas subunits of the NADPH oxidase, $p22^{phox}$ and $p47^{phox}$, were not affected (data not shown). To assess the distribution of surface proteins in Brij 58-derived density gradient fractions, detergent extracts of biotinylated neutrophils were fractionated on sucrose density gradient and resolved by SDS–PAGE (Fig. 3B). Exposure of the resolved proteins to Streptavidin-HRP indicated that raft fractions 3–5 contained only a small percentage of biotin residues; most of biotinylated residues were detected in higher density fractions 7–10. Furthermore, protein determination implied that Brij 58-raft fractions comprised ca. 15% of gradient proteins (data not shown). As expected, despite the widely accepted crucial role of lipid rafts in signal transduction and trafficking, they represent a small fraction of cellular proteins.

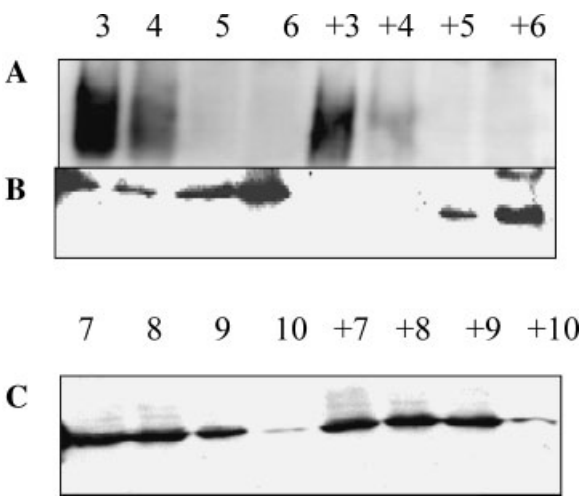

Fig. 5. The effect of PI-PLC on FcγRIIIb and PR3 in HMW fractions. Intact untreated or treated with PI-PLC neutrophils (designated +) were lysed, fractionated on Sepharose 4B, and subjected to SDS–PAGE. **A**, **B**: HMW fractions (3–5); (**C**) fractions 7–10. Proteins were electrotransferred to nitrocellulose and the membranes were probed for (A) FcγRIIIb and PR3 (B,C).

DISCUSSION

The current study was aimed at the characterization of membrane association of PR3

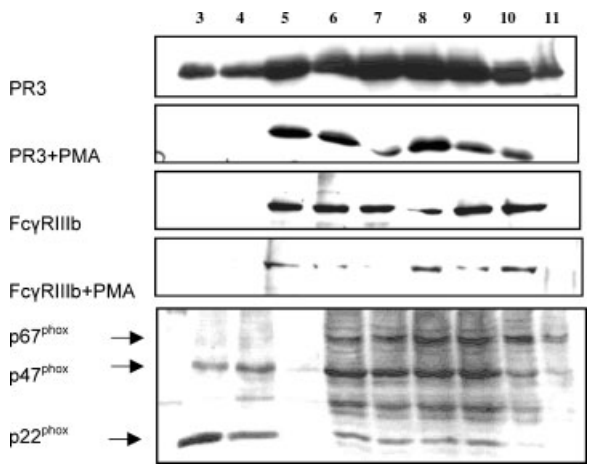

Fig. 6. Sucrose density gradient ultracentrifugation of Brij-58 neutrophil lysates. Western blots were developed with different antisera. Fractions 3–6 represent lipid ratfs.

with respect to two types of membrane complexes: the soluble HMW complexes [Cinek and Horejsi, 1992; Horejsi et al., 1994; Skubitz et al., 2000] and insoluble low-density lipid rafts [Maxfield, 2002; Pike, 2004]. Both types of multicomponent complexes were resistant to dissociation by the non-ionic detergent, Brij 58. HMW complexes isolated by gel filtration of the detergent extract of resting neutrophils contained PR3, FcγRIIIb, cytochrome b558, $p47^{phox}$, $p67^{phox}$, p80, and the β2 integrin CD11b/CD18 (Fig. 1). In PMA-stimulated cells the PKCβII isoform of protein kinase C, known to participate in activation of the NADPH oxidase and to migrate from cytosol to the membrane upon cell stimulation was also detected (Fig. 1B) [Korchak et al., 1998; Dekker et al., 2000]. The assignment of HMW complexes to plasma membrane was based on three observations: first, PR3 was detected in HMW fractions derived not only from whole cells lysates, but also from Brij-58-solubilized isolated neutrophil plasma membranes (Fig. 2A,B). Second, the HMW complexes represented the main surface-biotinylated fraction of neutrophils (Fig. 3A). Third, the levels of the GPI-linked FcγRIIIb as well as of PR3, devoid of GPI-linkages, in HMW fractions, were reduced by pretreatment of the cells with PI-PLC (Fig. 5) supporting our recent suggestion that the GPI-linked FcγRIIIb may function as an adaptor for mPR3 [David et al., 2005]. This suggestion was based on confocal microscopy-indicated co-localization of PR3 and FcγRIIIb in neutrophil cell membranes and on co-immunoprecipitation of both proteins from the membranes. Previous confocal microscopy data were consistent with the co-localization of PR3 and CD11b/CD18 in the membrane [David et al., 2003]. Despite identification of the α subunit of CD11b/CD18 in the HMW complexes analyzed in the current communication (Fig. 1), we failed to detect CD11b in Brij 58 rafts. Similarly, in studies of the compartmentalization of the GPI-anchored urokinase plasminogen activator receptor (uPAR) and its membrane partners, CD11b was not detected in neutrophil rafts [Sitrin et al., 2003]. The association of uPAR with CD11b in monocyte membranes is well documented [Bohuslav et al., 1995].

Detergent extraction of cells and membranes may give rise to distinct HMW complexes [Skubitz et al., 2000]. Co-immunoprecipitation of PR3 with FcγRIIIb and vice versa indicated the presence of these two proteins in the same HMW complex (Fig. 3). In view of the previously reported co-localization of membrane $p22^{phox}$ with PR3 and FcγRIIIb [David et al., 2005], it is conceivable that HMW complexes or a part of them may contain all three proteins.

The two subunits of the NADPH oxidase $p47^{phox}$ and $p67^{phox}$ are believed to form a ternary complex with $p40^{phox}$ in the cytosol of resting neutrophils [Park et al., 1992]. For their recruitment to the cell membrane and assembly into the active superoxide-generating complex they require neutrophil stimulation [Clark et al., 1990; Rotrosen and Leto, 1990; Park and Babior, 1992; Groemping and Rittinger, 2005]. The detection of $p47^{phox}$ and $p67^{phox}$ in the HMW fractions of resting neutrophils (Fig. 1B) was therefore as unexpected as our previous identification of these subunits of the NADPH oxidase in Triton X-100 rafts isolated from resting and activated neutrophils [David et al., 2005]. It is of note that in Brij 58 rafts isolated in the current study, only the $p47^{phox}$ subunit of the NADPH oxidase was detected (Fig. 6). The presence of cytosolic NADPH oxidase component/s in HMW and rafts, suggests that these macromolecular complexes containing also membrane subunits of cytochrome b558, are distinct from the active NADPH oxidase and reflect association of cytosolic subunits with membrane and cytoskeletal elements of resting neutrophils [Woodman et al., 1991; El Benna et al., 1999]. Differences between rafts isolated in Triton X-100 [David et al., 2005] and Brij 58 (the current study) may be ascribed to differences in the properties of these non-ionic detergents employed for fractionation [Giurisato et al., 2003; Schuck et al., 2003; Chamberlain, 2004].

Despite the use of gel filtration chromatography and of flotation on density gradients for the isolation of HMW complexes and rafts, respectively, the protein content of both appears similar though not identical. However, whereas the HMW fractions eluted in Brij 58 from the gel filtration column contained most of detergent-soluble surface biotinylated proteins (Fig. 3A), fractionation of biotinylated lysates on the sucrose gradient revealed their presence mainly in the high-density fractions where soluble proteins as well as "heavy rafts" with a higher protein/lipid ratio are expected to migrate (Fig. 3B) [Horejsi, 2005]. It should be kept in mind that in addition to the different techniques

employed for the isolation of HMW complexes and rafts, the bulk concentration of Brij 58 present during each fractionation was different: isolation of the HMW complexes was carried out in 1% Brij 58, whereas isolation of rafts from cells lysed in 1% Brij 58 was performed by centrifugation-flotation in a detergent-free sucrose density gradient. Thus unlike rafts representing membrane vesicles-associated proteins, the HMW fractions contain probably protein complexes in mixed detergent micelles [Braccia et al., 2003]. Additional studies, including comparison of the phospholipids content, will be required to characterize the basis of the differences between HMW complexes and rafts and their relation to the functional integrity of neutrophils.

REFERENCES

Avila JL, Convit J. 1976. Physicochemical characteristics of the glycosaminoglycan-lysosomal enzyme interaction in vitro. A model of control of leucocytic lysosomal activity. Biochem J 160:129–136.

Bohuslav J, Horejsi V, Hansmann C, Stockl J, Weidle U, Majdic O, Bartke I, Knapp W, Stockinger H. 1995. Urokinase plasminogen activator receptor, beta 2-integrins, and Src-kinases within a single receptor complex of human monocytes. J Exp Med 181:1381–1390.

Braccia A, Villani M, Immerdal L, Niels-Christiansen LL, Nystrom BT, Hansen GH, Danielsen EM. 2003. Microvillar membrane microdomains exist at physiological temperature. Role of galectin-4 as lipid raft stabilizer revealed by "superrafts". J Biol Chem 278:15679–5684.

Brown DA, London E. 1998. Structure and origin of ordered lipid domains in biological membranes. J Membr Biol 164:103–114.

Campbell EJ, Campbell MA, Owen CA. 2000. Bioactive proteinase 3 on the cell surface of human neutrophils: Quantification, catalytic activity, and susceptibility to inhibition. J Immunol 165:3366–3374.

Chamberlain LH. 2004. Detergents as tools for the purification and classification of lipid rafts. FEBS Lett 559:1–5.

Cinek T, Horejsi V. 1992. The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. J Immunol 149:2262–2270.

Clark RA, Volpp BD, Leidal KG, Nauseef WM. 1990. Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. J Clin Invest 85:714–721.

Csernok E, Schmitt WH, Ernst M, Bainton DF, Gross WL. 1993. Membrane surface proteinase 3 expression and intracytoplasmic immunoglobulin on neutrophils from patients with ANCA-associated vasculitides. Adv Exp Med Biol 336:45–50.

David A, Kacher Y, Specks U, Aviram I. 2003. Interaction of proteinase 3 with CD11b/CD18 ({beta}2integrin) on the cell membrane of human neutrophils. J Leukoc Biol 74: 551–557.

David A, Fridlich R, Aviram I. 2005. The presence of membrane Proteinase 3 in neutrophil lipid rafts and its colocalization with FcgammaRIIIb and cytochrome b558. Exp Cell Res 308:156–165.

Dekker LV, Leitges M, Altschuler G, Mistry N, McDermott A, Roes J, Segal AW. 2000. Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils. Biochem J 347 Pt 1:285–289.

El Benna J, Dang PM, Andrieu V, Vergnaud S, Dewas C, Cachia O, Fay M, Morel F, Chollet-Martin S, Hakim J, Gougerot-Pocidalo MA. 1999. P40phox associates with the neutrophil Triton X-100-insoluble cytoskeletal fraction and PMA-activated membrane skeleton: A comparative study with P67phox and P47phox. J Leukoc Biol 66:1014–1020.

Falk RJ, Terrell RS, Charles LA, Jennette JC. 1990. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. Proc Natl Acad Sci USA 87:4115–4119.

Giurisato E, McIntosh DP, Tassi M, Gamberucci A, Benedetti A. 2003. T cell receptor can be recruited to a subset of plasma membrane rafts, independently of cell signaling and attendantly to raft clustering. J Biol Chem 278:6771–6778.

Groemping Y, Rittinger K. 2005. Activation and assembly of the NADPH oxidase: A structural perspective. Biochem J 386:401–416.

Halbwachs-Mecarelli L, Bessou G, Lesavre P, Lopez S, Witko-Sarsat V. 1995. Bimodal distribution of proteinase 3 (PR3) surface expression reflects a constitutive heterogeneity in the polymorphonuclear neutrophil pool. FEBS Lett 374:29–33.

Horejsi V. 2005. Lipid rafts and their roles in T-cell activation. Microbes Infect 7:310–316.

Horejsi V, Cinek T, Angelisova P, Cerny J, Hilgert I. 1994. Association of GPI-anchored glycoproteins with other components of the leucocyte membrane. Braz J Med Biol Res 27:255–262.

Jennette JC, Hoidal JR, Falk RJ. 1990. Specificity of anti-neutrophil cytoplasmic autoantibodies for proteinase 3. Blood 75:2263–2264.

Kocher M, Edberg JC, Fleit HB, Kimberly RP. 1998. Antineutrophil cytoplasmic antibodies preferentially engage Fc gammaRIIIb on human neutrophils. J Immunol 161:6909–6914.

Korchak HM, Rossi MW, Kilpatrick LE. 1998. Selective role for beta-protein kinase C in signaling for O-2 generation but not degranulation or adherence in differentiated HL60 cells. J Biol Chem 273:27292–27299.

Maxfield FR. 2002. Plasma membrane microdomains. Curr Opin Cell Biol 14:483–487.

Park JW, Babior BM. 1992. The translocation of respiratory burst oxidase components from cytosol to plasma membrane is regulated by guanine nucleotides and diacylglycerol. J Biol Chem 267:19901–19906.

Park JW, Ma M, Ruedi JM, Smith RM, Babior BM. 1992. The cytosolic components of the respiratory burst oxidase exist as a M(r) approximately 240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system. J Biol Chem 267:17327–17332.

Pike LJ. 2004. Lipid rafts: Heterogeneity on the high seas. Biochem J 378:281–292.

Rarok AA, Limburg PC, Kallenberg CG. 2003. Neutrophil-activating potential of antineutrophil cytoplasm autoantibodies. J Leukoc Biol 74:3–15.

Rotrosen D, Leto TL. 1990. Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. Translocation to membrane is associated with distinct phosphorylation events. J Biol Chem 265:19910–19915.

Schuck S, Honsho M, Ekroos K, Shevchenko A, Simons K. 2003. Resistance of cell membranes to different detergents. Proc Natl Acad Sci USA 100:5795–5800.

Sitrin RG, Johnson DR, Pan PM, Harsh DM, Huang J, Petty HR, Blackwood RA. 2003. Lipid raft compartmentalization of urokinase receptor signaling in human neutrophils. Am J Respir Cell Mol Biol 30:233–241.

Skubitz KM, Campbell KD, Skubitz AP. 2000. CD63 associates with CD11/CD18 in large detergent-resistant complexes after translocation to the cell surface in human neutrophils. FEBS Lett 469:52–56.

Tal T, Sharabani M, Aviram I. 1998. Cationic proteins of neutrophil azurophilic granules: Protein-protein interaction and blockade of NADPH oxidase activation. J Leukoc Biol 63:305–311.

van der Geld YM, Limburg PC, Kallenberg CG. 2001. Proteinase 3, Wegener's autoantigen: From gene to antigen. J Leukoc Biol 69:177–190.

Witko-Sarsat V, Cramer EM, Hieblot C, Guichard J, Nusbaum P, Lopez S, Lesavre P, Halbwachs-Mecarelli L. 1999. Presence of proteinase 3 in secretory vesicles: Evidence of a novel, highly mobilizable intracellular pool distinct from azurophil granules. Blood 94:2487–2496.

Woodman RC, Ruedi JM, Jesaitis AJ, Okamura N, Quinn MT, Smith RM, Curnutte JT, Babior BM. 1991. Respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. J Clin Invest 87:1345–1351.