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The presence of membrane Proteinase 3 in neutrophil lipid rafts and its colocalization with FcγRIIIb and cytochrome b558

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Abstract

Proteinase 3 (PR3), the target autoantigen of antineutrophil cytoplasmic antibodies in the autoimmune vasculitis, Wegener's granulomatosis, is a serine proteinase stored in granules of human neutrophils. PR3 is expressed also on the plasma membrane of unactivated neutrophils, and this expression increases in primed or stimulated cells. In the current study, we demonstrate the presence of PR3, FcγRIIIb, and cytochrome b558 of the NADPH oxidase in neutrophil lipid rafts. Activation of neutrophils with PMA, fmet-leu-phe, or TNFα known to increase the membrane expression of PR3 did not affect the amount of PR3 in rafts. Unexpectedly, the cytosolic subunits of the NADPH oxidase, $p67^{phox}$ and $p47^{phox}$, the recruitment of which to the membrane requires cell stimulation, were detected in the rafts of unstimulated neutrophils. Treatment of neutrophils with the cholesterol-sequestering agent methyl-β-cyclodextrin (MβCD) reduced raft $p22^{phox}$ and PR3. MβCD diminished membrane FcγRIIIb upregulating membrane PR3 (mPR3) and CD11b/CD18. In addition, MβCD significantly reduced PMA-induced activity of the NADPH oxidase without altering fmet-leu-phe-elicited activity. Antibody-mediated cross-linking of membrane PR3 caused activation of ERK and JNK kinases and their translocation to rafts. Confocal analysis revealed colocalization of mPR3, FcγRIIIb, and $p22^{phox}$ in the membrane, confirmed by their coimmunoprecipitation. Cleavage of neutrophil GPI-anchors by PI-PLC reduced mPR3 and FcγRIIIb, implicating a GPI-protein, possibly FcγRIIIb, in the attachment of PR3 to the membrane.

Keywords: Proteinase 3; FcγRIIIb; Neutrophil; Rafts; NADPH oxidase

Introduction

Proteinase 3 (PR3), a serine protease of neutrophil azurophilic, specific, and secretory granules [1], was identified as the main autoantigen in the autoimmune Wegener granulomatosis disease [2,3] characterized by the presence in the blood of the patients of antineutrophil cytoplasmic antibodies (cANCA). PR3 is a multifunctional protein [4], which may participate not only in killing of invading microorganisms, but also in myeloid differentiation [5], regulation of NADPH oxidase activity [6], and apoptosis [7].

PR3 has been detected on the plasma membrane of freshly isolated human neutrophils [8–11]. Binding of cANCA to the membrane-bound PR3 induces degranulation and activation of the superoxide-generating NADPH oxidase, the products of which inflict damage to vascular tissues characteristic of Wegener granulomatosis [12].

The mode of binding of PR3 to the plasma membrane of neutrophils as well as the identity of surface molecules involved in this association have not been elucidated. The distribution of PR3 on the surface of PMN is bimodal [8], possibly due to a genetic variation [13]. We have recently suggested a direct or indirect interaction of membrane-bound PR3 with the β2-integrin adhesion molecule CD11b/CD18 (Mac-1) [14].

Experimental evidence obtained during the last decade has pointed out to the existence of distinct microdomains in cell membranes [15–19]. A special interest focused on lipid rafts, non-ionic detergent-resistant microdomains enriched in cholesterol and glycosphingolipids. Rafts isolated by flotation-centrifugation of cellular detergent lysates in density gradients comprise glycosylphosphatidylinositol

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(GPI)-anchored proteins, acylated proteins, and cholesterol-linked proteins [19]. In response to intra- or extracellular stimuli that induce formation or breakage of specific protein–protein interactions, rafts undergo changes in size and composition. They are believed to serve as scaffolds or platforms for signaling and trafficking.

In the present study, we investigated the distribution of PR3 in neutrophil cell membrane microdomains. Our data demonstrate the presence of a substantial fraction of membrane PR3 in neutrophil lipid rafts and its reduction upon extraction of cholesterol with methyl-β-cyclodextrin (MβCD). We show also that membrane-associated PR3 decreased by treatment of neutrophils with bacterial PI-phospholipase C which cleaves the GPI-anchors, suggesting an association of PR3 with a GPI-anchored protein. In view of this, we investigated the possible relationship between PR3 and the GPI-linked FcγRIIIb (CD16), the most abundant Fc receptor in neutrophils [20–23]. Our data, presented below, indicate colocalization of PR3 with FcγRIIIb and with the $p22^{phox}$ subunit of cytochrome b558 of the NADPH oxidase in the membrane.

Materials and methods

Reagents

Tumor necrosis factor α (TNF-α) was obtained from Pepro-Tech Inc. (Rocky Hill, NJ); Dextran 70 and Ficoll–Hypaque were from Amersham Biosciences. All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Antibodies

Monoclonal anti-PR3 (6A6, 4A5) and rabbit anti-PR3 were purchased from Wieslab AB; monoclonal anti-FcγRIIIb and anti-CD11b were from Biotest; anti-phospho antibodies to ERK and JNK were from Santa Cruz Biotechnology, Inc., rabbit anti-phospho p38 was from BioSource International and monoclonal anti-flotillin-1 was purchased from BD Transduction Laboratories. 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). All fluorescent antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Goat IgG was from Sigma Chemical Co.

Preparation of neutrophils

Human polymorphonuclear leukocytes (PMNs) were isolated from fresh buffy coats by standard procedures of dextran sedimentation, hypotonic lysis of erythrocytes, and Ficoll–Hypaque density gradient centrifugation [6,24]. 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 (pH 7.0) supplemented with 1 mM EGTA, 3.5 mM phenylmethylsulfonyl fluoride (PMSF), 15 μg/ml leupeptin, were disrupted by a brief sonication [6]. Unbroken cells were removed by low-speed ($250 \times g$) 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 at 48,000 rpm for 45 min at 4°C in a SORVAL Ti-50.

Raft isolation [24]

Neutrophils (10^8 cells) were lysed on ice with 0.5 ml lysis buffer (0.5% Triton X-100, 25 mM Tris–HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 40 mM Na_3VO_4 plus protease inhibitors) for 30 min. The cell lysate was mixed with an equal volume of 80% (w/v) sucrose containing 0.5% Triton X-100. Ultracentrifuge tubes were filled with 1 ml of 80% sucrose followed by 1 ml of the cell lysate in 40% sucrose, 5.5 ml 36% sucrose, and 3.5 ml of 5% sucrose. The gradients were centrifuged in a Beckman ultracentrifuge (rotor SW41) at 4°C for 18 h at 38,000 rpm. Fractions (1 ml) were collected from the top.

Treatment with MβCD

Neutrophils at 10^7/ml in KRP were incubated for 15 or 30 min at 37°C with 10 mM MβCD. The reagent was washed out by centrifugation.

Precipitation of proteins with TCA and Western blot analysis

Proteins in 1-ml fractions were precipitated with 20% TCA (1 ml) for 20 min in cold and pelleted; the pellets were washed (3×) with 1 ml acetone, dissolved in Laemmli sample buffer [25], boiled for 5 min and subjected to SDS-PAGE. Resolved proteins were transferred to nitrocellulose and Western blots were developed by incubation with appropriate antisera followed by HRP-conjugated secondary antibodies. Labeled proteins were detected using the luminol-*p*-coumarine enhanced chemiluminescence technique (ECL).

Flow cytometry [14]

Resting (unactivated) neutrophils (10^7/ml in RPMI 1640/5% fetal calf serum/0.01% azide) or prestimulated cells were incubated with a primary antibody for 45 min on ice, washed, and further incubated in the dark (45 min) with fluorescent secondary antibodies. Washed cells were fixed with 1% *p*-formaldehyde at room temperature and analyzed