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Solubilization of glycosyl-phosphatidylinositol-anchored proteins in quiescent and stimulated neutrophils

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Abstract

In human neutrophils, alkaline phosphatase (AlkPase), a low-affinity receptor for IgG (FcRIIIB), and complement decay accelerating factor (DAF) are glycosyl-phosphatidylinositol (GPI)-anchored proteins. Varying greatly in biological function these three integral membrane proteins exhibit regulated cell surface expression in neutrophils. Defined by their common membrane-linkage motif, AlkPase, FcRIIIB, and DAF can be released from the lipid bilayer by the action of phosphatidylinositol-specific phospholipase C and are relatively resistant to low temperature extraction with Triton X-100 (TX-100). In this study we show that neutrophil AlkPase, FcRIII, and DAF display differential extractibility; they are relatively insensitive to TX-100 solubilization at 4° C, but are readily extracted with TX-100 at 37° C or by the detergent octyl glucoside at 4° C. The differential extractibility of these GPI-anchored proteins is the same in unstimulated cells, where these proteins exist primarily in an intracellular pool, and stimulated cells, where they are expressed principally at the cell surface. However, no differential extraction effect is observed with two neutrophil transmembrane proteins, complement receptor 1 (CD35, CR1) and MHC Class I in either stimulated or unstimulated cells.

Keywords: Neutrophil; Glycosyl-phosphatidyl-anchored protein; Cell stimulation; Exocytosis; Detergent

1. Introduction

Recently significant progress has been made in our understanding of glycosyl-phosphatidylinositol (GPI)-anchored proteins. Lacking a membrane-spanning polypeptide domain these integral membrane proteins are defined by their covalent attachment to GPI embedded in the outer leaflet of the lipid bilayer. Proteins in this growing family display great functional diversity and are rather ubiquitous in terms of species distribution having been described in organisms ranging from protozoans to mammals [1,2]. In the present study we examine three GPI-anchored proteins in human neutrophils, namely: alkaline phosphatase (AlkPase; orthophosphoric-monoester phosphohydrolase, alkaline optimum: EC 3.1.3.1); a low-affinity receptor for IgG (FcRIIIB; CD16); and complement decay accelerating factor (DAF; CD55). AlkPase has been a useful enzymatic marker in neutrophil developmental and pathological studies even though its physiological substrate(s) remains to be identified (e.g., [3,4]). FcRIIIB acts by binding complexed IgG thus facilitating clearing of antigen-antibody complexes. The low-affinity receptor exhibits specificity for IgG1 with as many as $2 \cdot 10^5$ molecules having been estimated on granulocytes [5]. DAF is a potent inhibitor of complement activation acting upon C3 convertase and curbing autologous complement-mediated cytolysis. DAF is found in a wide variety of tissues and circulating cells; stimulated neutrophils have been reported to express $\approx 2 \cdot 10^4$ molecules at the cell surface [6]. While these three proteins differ greatly in biological function, they have certain common properties worth noting. In addition to sharing the same membrane-linkage motif, they are all expressed at relatively low levels on the cell surface in

Abbreviations: AlkPase, alkaline phosphatase; DAF, complement decay accelerating factor; fMLP, formyl-methionine-leucine-phenylalanine; FcRIIIB, low-affinity receptor for IgG; GPI, glycosyl-phosphatidylinositol; MHC, major histocompatibility complex; OG, octyl glucoside; TX-100, Triton X-100.

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unstimulated neutrophils. All three can also be rapidly up-regulated to the cell surface in a stimulus-dependent fashion [7–10].

Given their unique membrane attachment, GPI-anchored proteins have been the focus of recent efforts examining protein post-translational processing. Polarized epithelial cells have been used extensively for such studies. Epithelial cell lines transfected to express GPI-anchored proteins or transmembrane-GPI protein chimeras have been used to describe the assembly and preferential apical sorting of these proteins destined for the cell surface [11,12]. Directed by a C-terminal signal peptide, the GPI-precursor protein acquires a preassembled GPI-anchor in the endoplasmic reticulum through the action of a putative transamidase complex. As the newly lipid-anchored proteins progress through the Golgi compartment, the detergent extractibility of the GPI-anchored protein changes dramatically. In the *medial-trans* Golgi and beyond the previously extractible GPI-anchored protein becomes highly resistant to Triton X-100 (TX-100) solubilization at low temperatures in the MDCK epithelial cell line [13]. Together with their susceptibility to liberation from the membrane by phosphatidylinositol-specific phospholipase C, the resistance to TX-100 extraction at low temperatures are defining characteristics of GPI-anchored proteins [14–18]. Recently an association between GPI-anchored proteins, membrane glycosphingolipids and cholesterol has been indicated, strongly suggesting an interaction between these three membrane elements [13,19,20].

In the present study we examine the solubilization behavior of three human neutrophil GPI-anchored proteins, AlkPase, FcRIIIB and DAF relative to complement receptor type 1 (CD35; CR1) and major histocompatibility complex Class I (MHC Class I), two membrane-spanning proteins. The fact that AlkPase, FcRIIIB and DAF are endogenously expressed in intracellular stores that can be up-regulated to the cell surface in a stimulus-dependent manner in these non-polarized cells allows a unique comparative examination since virtually all GPI-anchored proteins studied to date are constitutively delivered to the cell surface. We show that neutrophil GPI-anchored AlkPase, FcRIIIB and DAF display similar solubilization properties that are quite different from the properties of the transmembrane proteins CD35 and MHC Class I. Moreover, we show that the differential solubilization of these GPI-linked proteins is the same in stimulated and resting cells. A preliminary account of a portion of this work has been presented in abstract form [21].

2. Materials and methods

2.1. Reagents

The following chemicals were purchased from Sigma (St. Louis, MO): aprotinin, bovine serum albumin, 5-bromo-4-chloro-3-indolylphosphate, CHAPS, citric acid, cytochalasin B, diisopropylfluorophosphate, dextran (M_r 485 000), EDTA, Ficoll-Histopaque 1083, Hanks' balanced salt solution, Hepes, molecular weight markers (SDS-7B), *N*-formyl-methionine-leucine-phenylalanine, nitroblue tetrazolium, nocadazole, octyl glucoside, phenylmethylsulfonyl fluoride, *p*-nitrophenylphosphate, poly(L-lysine), sodium deoxycholate, sodium fluoride, sucrose, TAPS, Tricine, Tris, and Triton X-100. Sodium orthovanadate and sodium pyrophosphate were supplied by Morton Thiokol (Danvers, MA). Sodium cacodylate and MOWIOL were supplied by Polysciences (Warrington, PA). Nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH). Nonfat dry milk used in immunoblotting experiments was supplied by Carnation Co. (Los Angeles, CA). 1,4-Phenylenediamine was obtained from Aldrich (Milwaukee, WI). The BCA Protein Assay kit was supplied by Pierce (Rockford, IL). All other chemicals were at least reagent grade.

2.2. Antibodies

Mouse monoclonal anti-FcRIIIB IgG (clone: gran 1) was provided by Dr. Clark L. Anderson (Ohio State University, Columbus, OH). The mouse monoclonal antibody to human complement decay accelerating factor (DAF) was purchased from Wako Chemicals (Richmond, VA). The murine monoclonal antibodies to human CD35 (clone: yz-1), and MHC Class I (clone: w6/32) were provided by Dr. Richard M. Jack (Harvard Medical School, Boston, MA). Alkaline phosphatase-conjugated goat anti-mouse IgG was purchased from Promega (Madison, WI). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse $F(ab')_2$ was obtained from Zymed Laboratories (San Francisco, CA).

2.3. Cell preparation and stimulation

Human neutrophils were isolated from whole blood collected in citrate-dextran tubes and purified over Ficoll-Histopaque at 4° C from healthy adult males as described elsewhere [22]. Suspension cells were kept in Hanks' balanced salt solution (supplemented with 0.1% bovine serum albumin but lacking Ca^{2+} and Mg^{2+}) on ice prior to use. Neutrophils were washed three times in excess PBS (137 mM NaCl, 2.7 mM KCl, 6.45 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.35) and then stimulated in suspension with the chemotactic peptide *N*-formyl-methionine-leucine-phenylalanine (fMLP) (10^{-7} M) in PBS^+ (PBS plus 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 7.5 mM glucose) for 15 min at 37° C. Informed consent was obtained from the volunteers who provided blood after an explanation of the nature and consequences of the studies. In addition, all investigations involving human material were performed with the approval of the Human Investigations Committee, The Ohio State University, in accord with an assurance

filed with and approved by the Department of Health and Human Services.

2.4. Protein extraction

Unstimulated (untreated and stored on ice) and fMLP-stimulated cells ($20 \cdot 10^6$) were lysed in 500 μl HT buffer (Hepes (25 mM), Triton X-100 (1%), pH 7.5) at either 4° C or 37° C for 20 min with brief intermittent vortexing. In another series of experiments examining AlkPase extraction, the HT buffer was supplemented with diisopropylfluorophosphate (100 μM). Following solubilization the 37° C lysates were chilled on ice for 5 min before all samples were centrifuged at $14000 \times g$ at 4° C for 30 min. In some experiments cellular lysates were centrifuged at $100000 \times g$ in a Beckman L8-55 M ultracentrifuge for 60 min. The resultant soluble (supernatant) and insoluble (pellet) fractions were separated and processed for either the enzymatic measurement of AlkPase activity or for immunoblotting experiments. In samples prepared for immunoblotting, HT lysis buffer was supplemented with a cocktail of proteinase and phosphatase inhibitors: aprotinin (1 mg/ml), EDTA (4 mM), phenylmethylsulfonyl fluoride (200 μM), sodium fluoride (100 mM), sodium orthovanadate (2 mM) and sodium pyrophosphate (20 mM).

Cells were also 'double extracted' with Triton X-100 (TX-100). This method involved lysing cells at 4° C, separating the fractions as described above, but in this case the resultant insoluble fraction was resuspended in fresh HT and incubated at 37° C for an additional 20 min. The resulting three fractions (namely, 4° C-soluble, 37° C-soluble and 37° C-insoluble) were then assayed for AlkPase activity.

The potential role of the cytoskeleton in the solubilization behavior of AlkPase was investigated by pretreatment of living cells with the cytoskeletal disrupting drugs nocadazole (17 μM) or cytochalasin B (21 μM) at 22° C for 30 min prior to TX-100 extraction. The effect of detergent properties (i.e., nonionic, zwitterionic and ionic) on AlkPase extractibility was tested further. In this case octyl glucoside (60 mM), CHAPS (20 mM), or sodium deoxycholate (9 mM) were substituted for the nonionic TX-100 in the Hepes lysis buffer.

2.5. AlkPase enzyme assay

To quantify the partitioning of AlkPase following detergent extraction, aliquots of the soluble and insoluble fractions were assayed for AlkPase activity. This was accomplished by assaying fractions in TTS buffer (TAPS (100 mM), Tricine (50 mM), $MgSO_4$ (2 mM), sucrose (5%), pH 9.3) at 37° C employing *p*-nitrophenylphosphate (2 mM) as the substrate and monitoring spectrophotometrically the accumulation of product, *p*-nitrophenol, at 410 nm [8]. In addition, the up-regulation of AlkPase to the cell surface following stimulation with fMLP was assessed in intact cells as we have described previously [8]. The protein concentration of the fractions was determined using the BCA protein assay. The AlkPase activity values for the soluble and insoluble fractions (calculated as μmol product formed per μg protein per min) were summed for each treatment condition and designated as 100%. Values for each fraction were thus defined as a percentage of the 100% summation.

2.6. Immunoblotting

The relative partitioning of other neutrophil membrane-associated proteins from 4° C and 37° C Triton-extracted cells (stimulated and control) was determined by Western blotting methods. Fractions from 4° C octyl glucoside-solubilized cells were also examined. Briefly, protein fractions of equal concentration were resolved on 7.5 or 12% SDS-PAGE gels and electroblotted onto nitrocellulose membranes. The membranes were blocked with 1% non-fat dry milk and probed with a panel of primary antibodies. The well-characterized monoclonal antibodies used in these immunoblotting experiments were directed against FcRIIIB (clone: gran 1; ascites fluid 2.5 μg/ml), DAF (2 μg/ml), CD35 (clone: yz-1; 2.2 μg/ml), and MHC Class I (clone: w6/32; 2 μg/ml) and used at the indicated concentrations. Primary antibody recognition was detected by probing the blots with an alkaline phosphatase labeled-secondary antibody and visualized using a nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate detection system. The relative protein partitioning into soluble and insoluble fractions was quantitated following immunoblot analysis using Optimas Image Analysis software (ver. 4.1) from Bioscan (Edmond, WA). Briefly, digitized images were acquired from 35 mm negatives of all extraction blots using a CCD 72 camera (Dage-MTI; Michigan City, IN). The mean gray value was calculated from boxes of equal area drawn to circumscribe the bands of interest. Following correction for background, the mean gray value for the soluble and insoluble lanes were summed for each treatment condition and designated as 100%. Values for each fraction were then calculated as a percentage of the 100% summation.

2.7. Immunocytochemistry

The subcellular distribution of FcRIIIB was assessed in resting and fMLP-stimulated neutrophils using indirect immunofluorescence techniques as we have described previously [23]. Briefly, unstimulated or stimulated cells were fixed in suspension with 4% paraformaldehyde in sodium cacodylate (100 mM) buffer containing 5% sucrose at 22° C for 90 min. The cells were then washed three times by repeated centrifugation-resuspension in the cacodylate-sucrose buffer before being embedded by centrifugation into 10% gelatin prepared in the same buffer. The gelatin was allowed to solidify and was then cut into small pieces

which were, in turn, infiltrated with 2.3 M sucrose in cacodylate buffer (100 mM) prior to freezing in liquid N_2 that had been converted to slush under a vacuum. Semi-thin cryosections (≈ 0.5 μm in thickness) were then cut from the gelatin blocks using a Reichert-Jung Ultracut E41 cryomicrotome. Using a small wire loop the cryosections were transferred to round glass coverslips (12 mm diameter) which had been pre-coated with poly(L-lysine) (0.25%). To block non-specific protein binding sites, the immobilized sections were then incubated in 1% non-fat dry milk-PBS containing sodium azide (0.02%) at 22° C for an hour. FcRIIIB was subsequently localized following incubation with the murine monoclonal antibody to FcRIIIB (25 μg/ml). Antigen labeling was detected using a FITC-labeled goat anti-mouse $F(ab')_2$ secondary antibody. The samples were washed and mounted in MOWIOL containing 1,4-phenylenediamine (1 mg/ml) to retard photobleaching of the fluorochrome. Control samples were processed in the same manner except, in this case, incubation with the primary antibody was omitted. The samples were observed and photomicrographs taken with a Nikon Optiphot microscope.

3. Results

3.1. Differential solubilization of neutrophil alkaline phosphatase

Unstimulated cells

Membrane-associated neutrophil AlkPase was only slightly solubilized ($\approx$ 15%) by extraction of unstimulated cells with TX-100 at 4° C, while the bulk of the enzyme activity remained associated with the TX-100-insoluble cellular material (Fig. 1). Interestingly, AlkPase was readily extracted when the lysis temperature was raised to 37° C; in this case > 85% of AlkPase partitioned into the soluble fraction. This dramatic temperature-dependent shift in AlkPase extractibility could not be accounted for by a differential partitioning in total cellular protein, since nearly the same distribution of protein was found at both temperatures (Fig. 1). Under all treatment conditions the distribution of total protein following detergent extraction was constant with $\approx$ 40% of the total protein solubilized and $\approx$ 60% remaining insoluble. In addition, the differential partitioning of AlkPase is independent of endogenous neutrophil proteinase action, since neutrophils lysed in the presence of diisopropyl fluorophosphate, a potent proteinase inhibitor, revealed a similar extraction profile (data not shown).

Double extraction experiments were also conducted to assess AlkPase solubility. As shown above, TX-100 lysis at 4° C resulted in only nominal extraction of AlkPase; however, when the 4° C insoluble pellet was resuspended in fresh lysis buffer, re-extracted at 37° C for 20 min, solubilization of the GPI-anchored AlkPase activity was

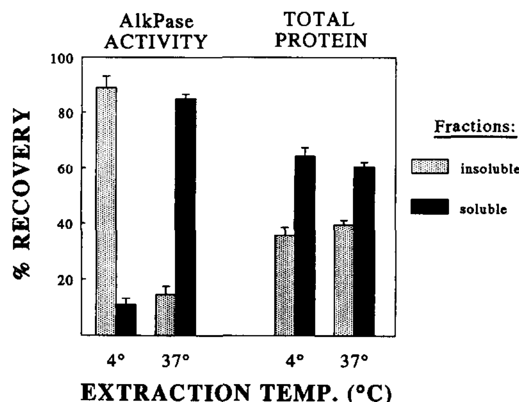

Fig. 1. Solubilization of GPI-anchored AlkPase from neutrophils with the detergent Triton X-100 is temperature-sensitive. When cells are lysed at 4° C with TX-100 for 20 min AlkPase is only slightly solubilized ($\approx$ 15%) with the bulk of this activity found in the insoluble cellular material. When the lysis temperature is raised to 37° C AlkPase is effectively extracted being found predominately in the soluble fraction ($\approx$ 85%). Note that this dramatic shift in distribution of the solubilized enzyme is not due to a differential partitioning of total cellular protein. $n = 6$.

nearly complete (> 90%) (Fig. 2). While the second extraction solubilized almost all of the AlkPase activity this fraction contained only 10% of the total cellular protein. This double extraction procedure provided a means of rapidly preparing neutrophil extracts enriched in AlkPase, and presumably, other GPI-anchored proteins.

The association of AlkPase with the 4° C Triton-insoluble cellular matrix suggested the possibility that AlkPase may interact with cytoskeletal elements enriched in the detergent resistant matrix. However, a direct interaction with the underlying cytoskeleton is not likely due to the nature of the GPI-anchor. A possible association with cytoskeletal components was tested for by treatment of cells with cytoskeletal disrupting drugs, nocodazole or

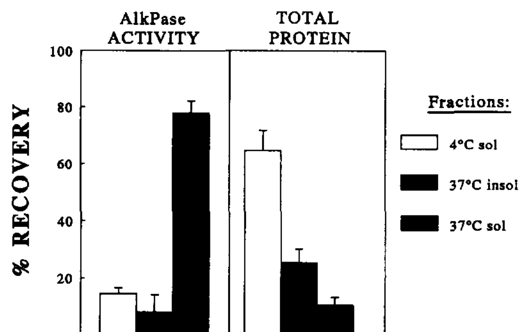

Fig. 2. The GPI-linked AlkPase that was insoluble with extraction using Triton X-100 at 4° C can be solubilized by re-extraction at 37° C. Lysis of neutrophils with TX-100 at 4° C results in only nominal extraction of AlkPase with the bulk of the enzyme found in the insoluble fraction (see Fig. 1). However, when the insoluble cellular material from 4° C extracted cells is re-extracted at 37° C for 20 min the initially insoluble AlkPase is nearly completely solubilized (> 90%). Note that 4° C extraction solubilizes the greatest percentage of total protein while the subsequent 37° C incubation extracts the least. $n = 3$.

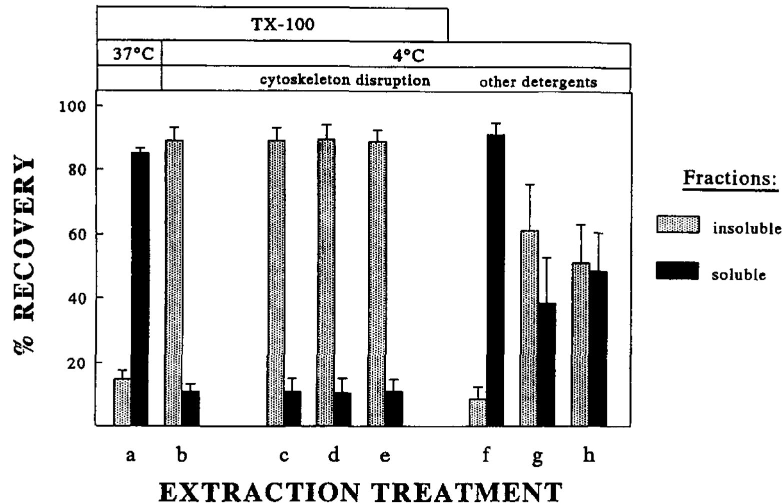

Fig. 3. Extraction of GPI-linked AlkPase is slightly altered by other detergents, but does not involve interactions with the underlying cytoskeleton. Octyl glucoside, a nonionic detergent, efficiently solubilizes AlkPase at 4° C when substituted for TX-100. All cells were lysed in Hepes-TX-100 (HT) at 4° C for 20 min (except a, f, g, h as noted below); the resultant soluble and insoluble fractions were then assayed for the enzymatic activity of AlkPase. Treatment conditions are defined as follows: (a,b) cells were lysed at either 37° C (a) or 4° C (b). (c–e) Prior to 4° C extraction, cells were pretreated with the cytoskeletal disrupting drugs cytochalasin B (21 μM) (c); nocodazole (17 μM) (d); or DMSO (0.25% solvent control) (e) for 30 min at room temperature in PBS. (f–h) Triton X-100 was replaced in the lysis buffer by the detergents octyl glucoside (60 mM) (f); CHAPS (20 mM) (g); or sodium deoxycholate (9 mM) (h). $n = 3$.

cytochalasin B, prior to the 4° C HT lysis. No difference was observed in the 4° C extractibility of AlkPase when comparing cells treated with these drugs or the control samples containing the DMSO solvent, and in each case only ≈ 15% of the AlkPase activity was extracted (Fig. 3b–e).

Additional experiments compared the effects of other detergents on AlkPase extractibility. In these studies TX-100 was replaced by CHAPS (zwitterionic), sodium deoxycholate (ionic) and octyl glucoside (nonionic) in the extraction buffer. Each of these detergents had different effects on AlkPase extractibility at 4° C when compared to TX-100 (Fig. 3f–h). Most interestingly, octyl glucoside (OG) dramatically improved the efficiency of the detergent extractibility of AlkPase at 4° C. Octyl glucoside readily solubilized > 90% of neutrophil AlkPase at 4° C.

Stimulated cells:

The temperature-sensitivity of neutrophil AlkPase extraction was independent of the cellular location of this enzyme. Neutrophils isolated from whole blood and purified at 4° C constitutively express ≈ 20–25% of AlkPase activity at the cell surface. Warming the cells to 37° C or stimulation with the chemotactic peptide fMLP induces the translocation of the enzyme from an intracellular pool to the cell surface to ≈ 50% and > 85%, respectively (Fig. 4). In TX-100 solubilization studies conducted on cells previously kept at 4° C, warmed to 37° C, or stimulated with fMLP, the temperature-dependent extractibility with TX-100 was unchanged; there was no observed difference in the solubilization profiles in each of these conditions (Fig. 4). This observation indicates that the microenvironment defining the solubilization behavior of AlkPase was already present in the intracellular stores and was retained upon delivery to the cell surface.

3.2. Translocation of FcRIIIB following cell stimulation:

Immunofluorescence methods were used to assess the distribution of GPI-anchored FcRIIIB in unstimulated and fMLP-stimulated neutrophils. Cryosections (≈ 0.5 μm in

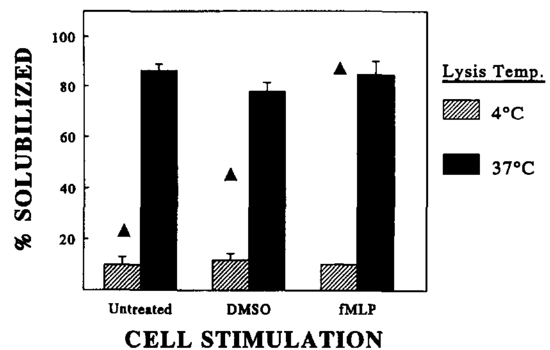

Fig. 4. The temperature-sensitivity of AlkPase extraction with TX-100 is independent of the cellular location of the enzyme. Cells were incubated in PBS$^+$ at 37° C in the presence of chemotactic peptide (fMLP; 10^{-7} M), DMSO (the solvent for fMLP) or left untreated and kept on ice for 15 min. Following the indicated treatments the cells were extracted with HT at 4° C or 37° C; the resultant soluble and insoluble fractions were assayed for AlkPase activity. Warming to 37° C in the presence of DMSO or fMLP induces the up-regulation of AlkPase to the cell surface from ≈ 24% to 50% and > 85%, respectively (▲ indicates the % of AlkPase expressed on the surface of intact cells for each defined treatment). Note that extraction of AlkPase at 4° C and 37° C exhibits similar solubilization profiles and in each case is independent of cell surface expression. $n = 3$.

thickness) of cells in suspension were immunostained to assess the subcellular distribution of FcRIIIB. In unstimulated cells FcRIIIB was localized primarily in numerous cytoplasmic granules with lesser amounts being found on the cell surface. Following cell stimulation with chemotactic peptide the FcRIIIB-positive cytoplasmic granules are largely depleted while cell surface labeling dramatically increases (Fig. 5).

3.3. Solubilization of other neutrophil proteins:

The differential solubilization of GPI-anchored AlkPase led us to examine the extraction behavior of two other GPI-anchored proteins, as well as two transmembrane proteins in neutrophils. Immunoblotting methods were used to examine the effect of temperature on the TX-100 extractibility of the GPI-anchored proteins FcRIIIB and DAF and the transmembrane proteins CD35 and MHC Class I from human neutrophils; the ability of OG to extract these proteins was also determined.

Soluble and insoluble preparation from 4° C and 37° C TX-100 lysed neutrophils were resolved on either 7.5 or 12% SDS-PAGE gels under non-reducing conditions, transferred to nitrocellulose membranes and incubated for the immunochemical detection of FcRIIIB, DAF, CD35 and MHC Class I. In some experiments both unstimulated and fMLP-stimulated cells were used. At 4° C TX-100 only partially solubilizes FcRIIIB (< 45%) and DAF (< 15%) (Fig. 6; lanes 4° C, s) with more than half of each GPI-protein remaining with the insoluble fractions in both unstimulated and stimulated cell preparations (lanes 4° C, i). Similar to AlkPase extraction behavior, raising the lysis temperature to 37° C greatly increased the efficiency of TX-100 solubilization of FcRIIIB and DAF to > 90% (lanes 37° C, s); under these conditions very little protein of either remained insoluble (lanes 37° C, i). On the other hand, this temperature-dependent extraction was not observed with the two transmembrane proteins. Both CD35 and MHC Class I were readily solubilized at 4° C with no differential effect seen when the extraction was carried out at 37° C. It should be noted that MHC Class I resides on the plasma membrane while CD35 is found primarily in an intracellular pool in unstimulated neutrophils but is up-regulated to the cell surface upon stimulation with fMLP [24,25]. Interestingly, on immunoblots probed with anti-FcRIIIB, a small band was detected at ≈ 41 kDa (Figs. 6 and 7, arrowhead). This band likely represents an immature form of FcRIIIB prior to its acquisition of resistance to low temperature detergent extraction [26]. This observation is consistent with the solubilization properties of immature forms of another GPI-anchored protein-placental alkaline phosphatase [13,27].

Similar to our biochemical results for AlkPase, OG readily solubilized FcRIIIB and DAF at 4° C (Fig. 7: OG,

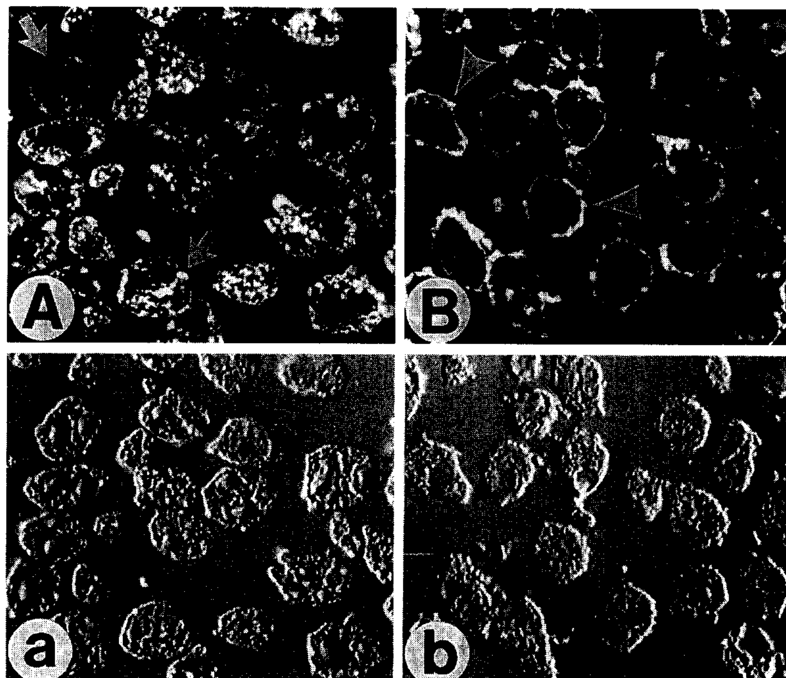

Fig. 5. Immunofluorescence micrographs of 0.5 μm cryosections demonstrating the distribution of FcRIIIB in neutrophils in suspension. Cells purified at 4° C were kept on ice (A) or warmed to 37° C and stimulated by the addition of fMLP (10^{-7} M) for 15 min (B). The cells were then fixed, sectioned and probed with a murine monoclonal antibody to FcRIIIB. Note that in the unstimulated cells (A) FcRIIIB is localized predominately in numerous cytoplasmic granules. Under these control conditions the amount of FcRIIIB expressed on the cell surface is somewhat variable from cell to cell (arrows). Following cell stimulation (B) the FcRIIIB-positive granules are largely depleted while cell surface labeling for the GPI-anchored receptor dramatically increases (arrowheads). Moreover, following stimulation all cells display elevated FcRIIIB at the cell surface. The accompanying differential interference contrast micrographs (a and b) are included to aid in identification of cells. Bar = 5 μm.

s) with < 10% of these proteins remaining with the insoluble fraction (OG, i). As noted previously, TX-100 did not completely solubilize FcRIIIB or DAF at 4° C (TX100, s) with over half of each protein remaining associated with the insoluble fraction (TX100, i). The detergent substitution had no apparent effect upon the extractibility of CD35 or Class I; both transmembrane proteins were efficiently solubilized with TX-100 or OG at 4° C. As noted above, a lower MW band was also detected with anti-FcRIIIB, whose extraction behavior resembled that of the two transmembrane proteins (Fig. 6). It should be noted that on immunoblots of gels run under reducing conditions (i.e., in

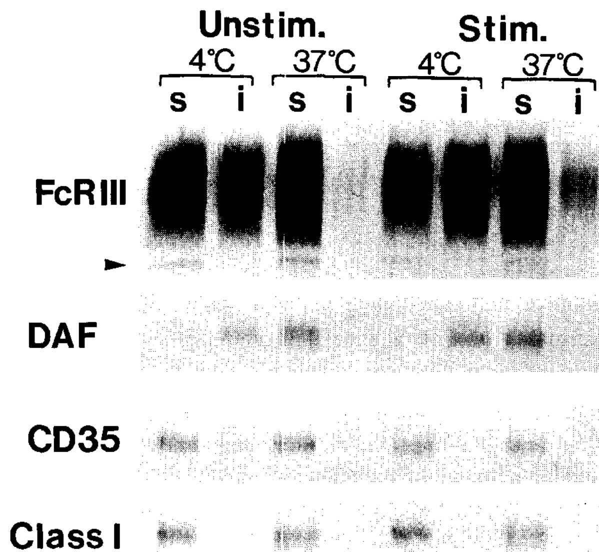

Fig. 6. Immunoblots assessing the temperature-sensitive extraction of other selected neutrophil proteins with TX-100. Fractions (loaded with an equal protein concentration) from TX-100-lysed unstimulated or fMLP-stimulated cells were resolved on 12% SDS-PAGE gels, electroblotted to nitrocellulose membranes and probed with murine monoclonal antibodies to FcRIIIB, DAF, CD35, or MHC Class I. Lanes 4° C, s (soluble) and i (insoluble) show the partitioning of proteins following 4° C extraction in both unstimulated (unstim.) and fMLP-stimulated (stim.) cells. Note that 4° C TX-100 treatment does not completely solubilize GPI-anchored FcRIIIB and DAF, and at least half of each protein remains with the insoluble fraction (4° C, i). In contrast the transmembrane proteins CD35 and Class I are effectively solubilized at 4° C in both unstimulated and stimulated cells as seen in lanes 4° C, s. When the lysis temperature is raised to 37° C the GPI-linked proteins are largely solubilized by TX-100. In this case FcRIIIB and DAF are found predominately in the soluble fraction (lanes 37° C, s) with very little protein observed in the insoluble extract (lanes 37° C, i). Note that raising the lysis temperature to 37° C did not effect the partitioning of CD35 and MHC Class I. Note also that the solubilization profiles are independent of the physiological state of the cell (i.e., unstimulated vs. stimulated). Interestingly the solubilization of the putative immature FcRIIIB (denoted by arrowhead) is not temperature-sensitive and mimics the behavior of the transmembrane proteins. Glycosylation of FcRIIIB accounts for its characteristically broad electrophoretic profile [26]. The relative molecular mass of each protein was calculated to be: FcRIIIB (45–75 kDa), DAF (73 kDa), CD35 (200–230 kDa) and Class I (44 kDa). Representative immunoblots from at least three separate experiments are shown here.

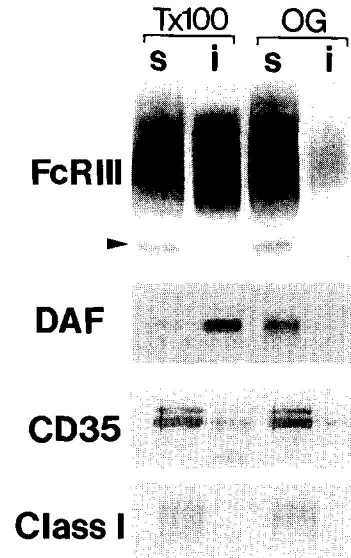

Fig. 7. Immunoblots assessing the solubilization of GPI-anchored FcRIIIB and DAF from neutrophils by OG at 4° C. Fractions (loaded with an equal protein concentration) from cells extracted at 4° C with either TX-100 or OG were resolved on SDS-PAGE 7.5% gels and probed with murine monoclonal antibodies to FcRIIIB, DAF, CD35 or MHC Class I. Solubilization of FcRIIIB and DAF at 4° C with TX-100 is incomplete with the larger proportion of each protein in the insoluble (i) relative to the soluble (s) fraction (also noted in Fig. 6). Substituting OG for TX-100 in the lysis buffer dramatically increases the efficiency of 4° C extraction as seen by the nearly complete extraction of FcRIIIB and DAF (compare OG; s vs. i). Note that the detergent substitution does not effect the extractibility of CD35, Class I or the smaller immature form of FcRIIIB (denoted by arrowhead). The relative molecular mass of each protein was calculated to be: FcRIIIB (45–75 kDa), DAF (73 kDa), CD35 (200–230 kDa) and Class I (44 kDa). Representative immunoblots from at least three separate experiments are shown here.

the presence of β-mercaptoethanol), the monoclonal antibody for DAF failed to detect the antigen and the antibody against FcRIIIB yielded only faint immunostaining.

4. Discussion

Our laboratory identified a novel membrane-bound compartment with unusual secretory properties in human neutrophils that is distinct from the azurophilic and specific granules in morphology and kinetics of up-regulation to the cell surface upon cell stimulation [8]. The cell biological and mechanistic questions raised by these observations led us to examine more closely AlkPase, a protein marker for this compartment. This compartment, as indicated by the marker enzyme AlkPase, is rapidly up-regulated to the cell surface in a stimulus-dependent manner [7,8].

AlkPase is a GPI-anchored protein in human neutrophils. GPI-anchored proteins are a large, growing, and functionally diverse group of polypeptides sharing a common membrane-linking motif through a covalent attach-

ment to membrane glycosyl-phosphatidylinositol. Two features of GPI-anchored proteins are their susceptibility to liberation from the bilayer by the action of phosphatidylinositol-specific phospholipase C (PI-PLC) and their relative resistance to extraction with nonionic TX-100 at low temperatures. In an earlier study we documented the sensitivity of neutrophil AlkPase to release by PI-PLC [28]. In the present study we examined the detergent extraction behavior of neutrophil AlkPase, a second diagnostic feature of GPI-anchored proteins [14]. These studies were complemented by concomitant experiments examining the solubilization properties of two additional neutrophil GPI-linked proteins-FcRIIIB and DAF; all of these results were then compared to the solubilization features of the transmembrane proteins, CD35 and MHC Class I.

Most studies examining the assembly, processing and surface expression of GPI-anchored proteins have been carried out in polarized epithelial cells [12,27,29]. In epithelial cells, GPI-anchored proteins are preferentially sorted to the apical surface and appear to be constitutively expressed. Given the non-polarized nature of neutrophils and the fact that the three GPI-anchored proteins studied here exhibit regulated cell surface expression (i.e., neutrophil AlkPase, FcRIIIB and DAF can be translocated from intracellular stores to the cell surface in a stimulus-dependent manner) [7–10], this system provides a new perspective in the study of GPI-anchored proteins

The resistance to TX-100 extraction at 4° C that we describe for neutrophil AlkPase, FcRIIIB, and DAF is characteristic of GPI-linked proteins. The solubilization properties of GPI-anchored proteins likely result from their interaction with neighboring molecules within the membrane. Membrane-associated glycosphingolipids and sphingomyelin have been shown to be insoluble upon TX-100 extraction at low temperatures [30,31]. Tracking the extraction properties of GPI-anchored placental alkaline phosphatase during its biosynthesis, Brown and Rose [13] identified the *medial-trans* Golgi as the site at which this GPI-linked protein acquires its resistance to low temperature TX-100 extraction. They also demonstrated an association between placental alkaline phosphatase and membrane glycosphingolipids. The association of GPI-anchored proteins with other membrane lipid constituents including glycolipids [32] and cholesterol [19,20] has been noted. In addition, immunocytochemical methods have indicated that the cell surface distribution of the GPI-anchored folate receptor is regulated by membrane cholesterol [19]. The expression of membrane cholesterol and glycosphingolipids in the non-polarized neutrophil is well-documented, the latter having served as cell surface markers for leukocyte lineage and cell differentiation [33–35]. The solubilization behavior of neutrophil GPI-anchored AlkPase, FcRIIIB and DAF studied here may also involve interactions between the lipid-linked proteins and glycolipid/cholesterol complexes.

Solubilization of membrane proteins involves perturbation of the surrounding lipid bilayer and is influenced by the nature of the lipid constituents of a given membrane. The similarities observed upon TX-100 extraction of AlkPase, FcRIIIB and DAF suggests a membrane feature shared by these neutrophil proteins; this common feature may be the association of each with neighboring glycosphingolipids and cholesterol. Such a relationship would offer a mechanism for explaining the temperature-sensitive solubilization observed for neutrophil AlkPase, FcRIIIB, and DAF. In phospholipid bilayers, glycosphingolipids coalesce forming small aggregates or microdomains that are less fluid than neighboring phospholipids [36,37]. As a result, these tightly packed clusters resist the escape of endogenous lipids and intercalation of exogenously added TX-100 monomers at low temperatures. Elevating the lysis temperature to 37° C likely decreases the rigidity of these glycolipid complexes, permitting detergent intercalation and extraction of GPI-anchored proteins. The similar solubilization profiles observed for AlkPase, FcRIIIB, and DAF suggest common microenvironmental features shared by these proteins. Further support for this argument is found upon comparison to the TX-100 solubilization properties of CD35 and MHC Class I. Both of these membrane-spanning proteins displayed temperature-independent extraction, suggesting that the neighboring membrane constituents of GPI-anchored proteins are different from those whose membrane association is defined by a transmembrane polypeptide.

The extraction of AlkPase, FcRIIIB, and DAF by OG at low temperatures also argues in favor of a relationship between GPI-anchored proteins and glycolipids. Octyl glucoside is a nonionic detergent with glycolipid-like features, having a small, hydrophilic carbohydrate head and long lipophilic tail. It is reasonably argued that the structural similarities between OG and membrane glycolipids allow the detergent to readily partition into glycolipid-enriched membrane domains even at low temperatures. This line of evidence once more implicates a relationship between GPI-linked protein and glycolipid clusters. It is worth noting that substitution of OG for TX-100 had no apparent effect upon the extractibility of CD35 and MHC Class I, again suggesting a membrane microenvironment that is unique for GPI-anchored proteins.

An alternative explanation for the differential solubilization of GPI-anchored AlkPase is the potential for interactions with the TX-100 resistant cytoskeleton indirectly through other membrane-associated proteins. However, this seems unlikely since there were no differences observed upon the extraction of cells pretreated with microfilament or microtubule-targeting drugs relative to the untreated control cells. Under all three conditions AlkPase was less soluble at 4° C but readily solubilized when cells were lysed at 37° C.

It should be noted that not all GPI-linked proteins demonstrate identical extraction properties under the same conditions (e.g., the porcine kidney GPI-anchored ectoen-

Table 1
Neutrophil protein partitioning following detergent lysis

Conditions/treatment:	Neutrophil proteins			
	FcRIIIB	DAF	CD35	Class I
Unstimulated cells				
TX-100 extraction				
4° C soluble	44.7% (±3.9) [a]	15.8 (±8.4)	88.3 (±7.8)	91.6 (±5.2)
4° C insoluble	55.3 ($n=7$)	84.2 ($n=10$)	11.7 ($n=9$)	8.4 ($n=5$)
37° C soluble	98 (±2.9)	97.3 (±5.9)	93.9 (±6.1)	95.9 (±2.3)
37° C insoluble	2 ($n=5$)	2.7 ($n=5$)	6.1 ($n=5$)	4.1 ($n=3$)
OG extraction				
4° C soluble	92.5 (±7.8)	93.7 (±2.1)	96.6 (±6.8)	93.2
4° C insoluble	7.5 ($n=4$)	6.3 ($n=4$)	3.4 ($n=4$)	6.8 ($n=2$)
Stimulated cells				
TX-100 extraction				
4° C soluble	42.8 (±10)	9.3 (±7.5)	89.1 (±14.2)	89.6 (±9.3)
4° C insoluble	57.2 ($n=3$)	90.7 ($n=4$)	10.9 ($n=3$)	10.4 ($n=3$)
37° C soluble	92.1 (±4.8)	93.1 (±9.8)	88.1 (±20.6)	91.8
37° C insoluble	7.9 ($n=3$)	6.8 ($n=4$)	11.9 ($n=3$)	8.2 ($n=2$)

[a] Immunoblots were analyzed to quantify the relative protein partitioning following neutrophil detergent lysis. Values represent the percentage (±S.D.) of protein associated with the soluble or insoluble fraction for each given treatment. See Materials and methods for details. ($n=$) indicates the number of blots analyzed.

zymes trehalase, 5′-nucleotidase and renal dipeptidase are found in the 4° C TX-100 insoluble extract at ≈ 45%, ≈ 80% and ≈ 90%, respectively) [14]. In the present study we find that FcRIIIB is not extracted to the same extent as DAF or AlkPase with TX-100 at 4° C (see Table 1 and Fig. 1).

Of particular interest are the studies comparing the protein extractibility from unstimulated and fMLP-stimulated neutrophils. These results indicate that the membrane microenvironment responsible for the solubilization profiles of AlkPase, FcRIIIB, and DAF when expressed at the cell surface is similar when the GPI-anchored proteins predominate in an intracellular form in quiescent cells. Neutrophil AlkPase, FcRIIIB, and DAF are present in an intracellular pool and to a lesser extent on the cell surface in unstimulated cells. The cell surface expression of these proteins can be up-regulated upon activation with stimuli such as chemoattractant peptides [7–10]. While the intracellular store for AlkPase in resting cells has been described, the storage sites for neutrophil FcRIIIB and DAF have yet to be completely resolved at the ultrastructural level. Of note is the fact that CD35 also exists in an intracellular compartment which also displays stimulus-induced surface up-regulation [24,25]; however, we show that it does not have the temperature-sensitive solubilization characteristic of GPI-linked proteins in either location. Results presented here show that the extraction of AlkPase, FcRIIIB, and DAF is independent of the cellular location of these proteins, and lends credence to our postulation that the AlkPase-containing compartment in neutrophils may serve as an intracellular reservoir for other GPI-anchored proteins [8].

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