Biochemical isolation of a membrane microdomain from resting platelets highly enriched in the plasma membrane glycoprotein CD36

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Here we describe the isolation and characterization of a Triton X-100-insoluble fraction isolated from lysates of platelets by flotation in sucrose gradients. Transmission electron microscopy of the insoluble material revealed a heterogeneous population of vesicles ranging in size from 20 to 1000 nm, and Western blot analyses of platelet lysates for the caveolae structural coat protein, caveolin/VIP21, were negative. Biochemical characterization of the Triton X-100-insoluble fraction showed it to be cholesterol-rich, greatly and specifically enriched in the plasma

membrane glycoprotein CD36, and also to contain Src and the Src-related kinase, Lyn. CD36 within this fraction is shown to be palmitoylated, but the fraction itself is not generally enriched in palmitoylated platelet proteins. These results suggest that this fraction represents caveolin-negative, CD36-rich microdomains in the resting platelet membrane. CD36 can form associations with certain Src-related kinases and can signal to activate platelets. These results suggest the possibility that such microdomains are implicated in platelet activation.

INTRODUCTION

The functional interaction of several signalling molecules within the cell requires that they can associate physically, and this event would be facilitated if these molecules were concentrated in spatial associations at the plasma membrane. Fox et al. [1] have propounded the involvement of a membrane cytoskeleton in platelets as an organizing platform for mediating signal transduction. These authors have shown that several signalling molecules are localized to this fraction of platelet lysates and that these translocate simultaneously to the actin cytoskeleton of the platelet after activation [1]. Such translocation, which may be driven by biochemical modifications to the translocating molecule [2–4], are dependent on the functional activation of the major platelet integrin α IIb β 3 [1]. The same biochemical modifications that determine the relocation of signalling molecules might also function in altering the activation state of the protein; it is known from other systems that both phosphorylation of specific amino acid residues and protein acylation can influence cellular partitioning of specific proteins as well as modifying the activation potential of the molecule [5–9]. In addition, the lipid micro-environment can regulate molecular function, and, for example, the ligand-binding capacity of α IIb β 3 is altered in different lipid backgrounds [10].

A cholesterol- and sphingolipid-rich subcompartment of the plasma membrane, potentially involved in signal transduction, has been described in a number of different cell types [11,12]. These are seen as invaginations of the plasma membrane termed caveolae that are 50–100 nm in diameter and are characterized by a 22 kDa structural protein, caveolin/VIP21 [11–13]. Caveolae can be isolated by flotation in sucrose density gradients on the basis of their insolubility in Triton X-100 at 4 °C, where the fraction appears as a light-refractive band that is rich in sphingolipids and cholesterol [11]. In a detailed study of caveolae isolated from endothelium-rich mouse lung tissue, Lisanti et al. [12] identified CD36 as one of only three transmembrane glycoproteins to be found in this fraction in addition to caveolin/VIP21. Further, this membrane fraction was notably enriched in the protein tyrosine kinases p60^{lyn}, p53/56^{lyn}, p62^{c-yes} and p60^{c-src}

[12]. More recent reports have challenged the presumption that the Triton X-100 floating fractions, or rafts, necessarily represent caveolae [14]. Indeed in several cell types, including human lymphocytes and myeloma cell lines [15] and cultured mouse neuroblastoma cells [16], such detergent-insoluble rafts displaying the same buoyant-density characteristics as the reported caveolar fraction have been found in the complete absence of morphologically identifiable caveolae or caveolin/VIP21 protein. However, each of the cell lines examined in this way has been isolated from human or animal tumours, and Koleske et al. [17] observed that oncogenic transformation markedly reduces the cellular levels of caveolin/VIP21. In these cases the nature of the fraction insoluble in cold Triton X-100, isolated *in vitro*, is still an open question: whether these represent specific membrane microdomains enriched in low-melting-temperature lipids and of low fluidity, as has been suggested [14], or conglomerates of lipids and acyl-linked proteins that associate during the isolation procedure [18,19], has not been resolved.

Perhaps surprisingly, despite the prevalence of CD36 in platelets and the co-occurrence of the Src-related tyrosine kinases known to associate with this molecule, no analysis of caveolae or microdomains in platelets has been reported. We have analysed platelets in this way and found that abundant Triton X-100-insoluble rafts can be fractionated from these cells in the complete absence of detectable caveolin/VIP21 protein. Characterization of these insoluble rafts shows them to be greatly and specifically enriched in two surface-membrane glycoproteins, CD36 and GPIb, and also to contain the protein tyrosine kinases p60^{c-src} and p53/56^{lyn}. These results suggest microdomains associated with platelet surface membranes that may play a role in platelet signal transduction.

EXPERIMENTAL

Materials

Sephacrose-2B, Protein-A and deoxyribonuclease 1 were purchased from Pharmacia (Uppsala, Sweden). The cholesterol analysis kit and octylglucoside were from Boehringer-Mannheim

Abbreviations used: biotin-CNHS-ester, biotin amidocaproate *N*-hydroxysuccinimide ester; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; mAb, monoclonal antibody; MBS, Mes-buffered saline.

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(Mannheim, Germany). The bicinchonic acid protein assay kit was from Pierce (Rockford, IL, U.S.A.); streptavidin–biotin–horseradish peroxidase (HRP) and hyperfilm-MP were from Amersham (Bucks., U.K.); ultracentrifuge tubes (cat. no. 344059, 14 mm × 89 mm) were from Beckman (Palo Alto, CA, U.S.A.); [9,10-³H(N)]palmitic acid and enhanced chemiluminescence (ECL) detection reagents were from DuPont (Australia) (North Ryde, NSW, Australia); nitrocellulose was from Sartorius (Göttingen, Germany); biotin amidocaproate *N*-hydroxysuccinimide ester (biotin-CNHS-ester), sucrose, apyrase, dithiothreitol and 2-mercaptoethanol were from Sigma (St. Louis, MO, U.S.A.). All other chemicals were from Ajax (Auburn, NSW, Australia) or BDH (Kilsyth, Victoria, Australia) and were of the highest grade available.

Antibodies

Polyclonal antibodies to p60^{c-src}, p53/56^{lyn} and p62^{c-yes} were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.); GPIIb specific monoclonal antibodies (mAbs) AK1, AK2 and AK3 were a gift from Dr. E. Favalaro (Institute of Clinical and Medical Research, Westmead, NSW, Australia). Of the mAbs specific for CD36, IA7 and IE8 were provided by Dr. A. W. Boyd (Walter & Eliza Hall Institute, Melbourne, Victoria, Australia), VM58 by Dr. M. C. Berndt (Baker Medical Research Institute, Prahran, Victoria, Australia) and 8A6 was a gift from Dr. J. W. Barnwell (NYU Medical Centre, New York, U.S.A.). Polyclonal antibodies to CD36 and αIIbβ3 were also provided by Dr. Berndt and have been described previously [20,21]. An mAb to β3, AP3, and a platelet-irrelevant mAb, OKT4 (anti-CD4), were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The mAbs against actin, tubulin and vinculin were from ICN (Costa Mesa, CA, U.S.A.). An anti-CD9 mAb (FMC56) was provided by Dr. Heddy Zola (Flinders Medical Centre, Adelaide, SA, Australia), and an antibody to the platelet glycoprotein PTA1 has been described previously [22]. Secondary antibodies for immunoblotting were from Bio-Rad (Hercules, CA, U.S.A.). Rabbit anti-mouse IgG was from Dako (Carpinteria, CA, U.S.A.).

Platelet isolation

Fresh human platelets from consenting adult donors were isolated as previously described [23] and resuspended into buffer A (138 mM NaCl, 2.9 mM KCl, 0.5 mM MgCl₂, 12 mM NaHCO₃, 0.3 mM NaH₂PO₄, 10 mM Hepes, pH 7.4, 5.5 mM glucose). Platelet suspensions were allowed to stand at 37 °C for 30 min in the presence of 5 units/ml apyrase to recover after isolation.

[³H]Palmitate labelling of platelet proteins

Washed platelets seeded into Buffer A (2 × 10⁹ platelets/ml) were incubated at 37 °C for 4 h in the presence of [³H]palmitic acid (specific radioactivity 30–60 µCi/mmol) at 400 µCi/ml. After this labelling, prostaglandin E1 was added to 1 µM and platelets were pelleted by centrifugation at 200 *g* for 15 min at room temperature (22 °C). Platelets were lysed and lysates fractionated by density-gradient centrifugation as described below.

Biotin labelling of platelet surface proteins

Biotin-CNHS-ester was added to 50 µg/ml to platelets [(0.5–2) × 10⁹ platelets/ml] suspended in 3.0 ml of borate buffer (10 mM Na₂B₄O₇, pH 8.8, and 150 mM NaCl) and the platelets were left at room temperature (22 °C) for 15 min. Ammonium

chloride was added to 10 mM to stop the reaction and the platelets were washed in buffer A containing 1 µM prostaglandin E1, pelleted by centrifugation [200 *g* for 15 min at room temperature (22 °C)] and lysed before further analysis.

Platelet lysis and density-gradient fractionation

Platelets prepared and manipulated as described above were lysed in ice-cold Triton X-100 lysis buffer [1 % (w/v) Triton X-100, 150 mM NaCl, 25 mM Mes, pH 6.5, and 2 mM PMSF]. All concentrations are final and all subsequent procedures were performed at 4 °C with suitably precooled solutions. Lysates were adjusted to 40 % (w/v) sucrose by the addition of an equal volume of 80 % sucrose in Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, and 150 mM NaCl). A step gradient of 5–30 % (5 % steps) sucrose in MBS was formed above the 40 % homogenate in an ultracentrifuge tube. The gradient was centrifuged at 200 000 *g* at 4 °C for 16 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). Triton X-100-insoluble rafts were revealed by using a vertical beam of high-intensity light, and gradient columns were fractionated into 12 equal volumes taken by pipette from the top (5 % sucrose) of the column. A hard pellet at the bottom of the gradient was washed *in situ* with lysis buffer, and 500 µl of reducing SDS/PAGE sample buffer was added.

Electron microscopy

Insoluble light-refractive material recovered from the sucrose gradient column was concentrated by centrifugation at 15 000 *g* for 15 min at 4 °C after a 3-fold dilution with MBS. To prepare the insoluble material for analysis by transmission electron microscopy, pelleted samples were washed with PBS, fixed with glutaraldehyde/formaldehyde [24], post-fixed in OsO₄, dehydrated with graded acetone and embedded in Spurr's low-viscosity medium [25]. Thin sections (0.3–0.5 µm) were cut and stained with Toluidine Blue, and material was selected by light microscopy for further sectioning. Ultrathin sections (90 nm) were collected on to copper grids, stained with uranyl acetate in 50 % (v/v) ethanol and 0.4 % lead citrate [26] before examination under a Philips CM10 transmission electron microscope.

Protein quantification, immunoblotting and immunoprecipitation

Protein quantification

To determine the quantitative distribution of protein across the density gradient, fractions were analysed for protein with the bicinchonic acid protein assay kit in accordance with the manufacturers' instructions. In some gradient fractions protein was not detectable. For subsequent analysis, where comparison within the gradient was required, a volume equivalent to that taken from the fraction with the least detectable protein was used from these 'protein-free fractions'. An equal volume of 2 × SDS/PAGE sample buffer containing 2-mercaptoethanol was added to aliquots of equivalent protein volumes from each of the fractions (*n* = 12) of the gradient. The insoluble pellet at the base of the gradient could not be adequately solubilized for protein quantification: to permit a comparison between preparations obtained from different experiments, a volume relative to the number of platelets was used for analysis of this insoluble pellet. Samples were separated by SDS/PAGE before being revealed by silver staining or being transferred electrophoretically to nitrocellulose membranes.

Immunoblotting

The distribution of specific proteins was ascertained by immunoblotting of nitrocellulose membranes with detection of antigen by ECL in accordance with the manufacturers' instructions. Biotinylated proteins were detected by probing immunoblots of biotinylated protein fractions with streptavidin-biotin-HRP conjugate before detection by ECL.

Immunoprecipitation

Specific proteins from within gradient fractions were isolated by immunoprecipitation. Insoluble rafts were pelleted as described in the 'Electron microscopy' section, and the pelleted material was resuspended into 1% (w/v) Triton X-100/60 mM octylglucoside in MBS lysis buffer, pH 8.0, and left on ice for 60 min. Lysates were then precleared with Sepharose-2B-coupled rabbit anti-mouse IgG for 2 h at 4 °C. Precleared lysates were incubated with the required precipitating antibody for at least 2 h and an anti-species Sepharose-2B conjugate was added for an additional 30 min. The immunoprecipitated bead-antigen conjugates were washed twice in octylglucoside-containing lysis buffer, reducing SDS/PAGE sample buffer was added and samples were subjected to SDS/PAGE.

RESULTS

Initial characterization of the Triton X-100-insoluble fraction of platelet lysate

Resting human platelets lysed in Triton X-100 were fractionated by density through a step gradient of 5–40% (w/v) sucrose and centrifuged for 16 h at 200000 *g* at 4 °C. At the end of this period an opaque band could clearly be seen at a density of between 10 and 18% sucrose, even in the absence of direct illumination. When viewed in a high-intensity light beam the band was light-refractive, and in some preparations two closely adjacent bands could be resolved. Independent analysis of each band showed identical protein characteristics (D. J. Dorahy and G. F. Burns, unpublished work). The sucrose gradient was fractionated into 12 fractions of 1.0 ml from the top (5% sucrose) of the gradient; the light-refractive band was generally recovered in fractions 3–6 (12–18% sucrose). Analysis of the fractions for protein concentration showed that approx. 97% of total cellular protein was to be found in fractions 9–12 (40% sucrose) and in a pellet of insoluble material recovered at the bottom of the tube. Fractions 3–6 consistently contained less than 3% of total protein (Figure 1A). The rafts of Triton X-100-insoluble proteins isolated from other cell types have been described as being enriched in cholesterol [11]. Analysis of the platelet lysate within the sucrose gradient for cholesterol distribution showed that fractions containing the insoluble raft were greatly enriched in cholesterol (41%). A substantial amount (27%) was also apparent in fraction 1, which contained no detectable protein (Figure 1A). To reveal the light-refractive band at the ultrastructural level, the band was harvested directly from a sucrose gradient, the sucrose diluted and the Triton X-100-insoluble material pelleted by centrifugation at 15000 *g* at 4 °C for 15 min. Transmission electron microscopy of the pellet revealed vesicle-like structures that were heterogeneous in size, ranging from 20 to 1000 nm in diameter (Figure 1B).

Interspersed among these vesicles were occasional amorphous structures that might be lipid droplets (Figure 1B). Thus the light-refractive band is shown to be predominantly Triton X-100-insoluble vesicles containing protein and enriched in cholesterol.

A

B

Figure 1 Characterization of the light-refractive rafts of Triton X-100-insoluble material of resting platelets separated by density flotation through sucrose gradients

(A) Total cholesterol and protein analyses for each of 12 equal-volume fractions taken across the gradient column [ranging from 5–40% (w/v) sucrose]. The insoluble raft (fraction 5) was cholesterol-rich (41%) and contained less than 3% of total platelet protein. These analyses are representative of multiple routine determinations of numerous platelet isolates. (B) Transmission electron microscopic examination of the material within the insoluble raft. Scale bar, 1 µm.

These vesicles did not present the uniform appearance or the characteristic morphology of caveolae. To establish whether this fraction contained the structural coat protein of caveolae, caveolin/VIP21, the fractions from the gradient were tested by immunoblot analysis. No detectable caveolin/VIP21 was present in any of the fractions of the platelet lysate; lysates from MDCK cells served as a positive control, and the antibody identified caveolin/VIP21 from lysates of these cells under the same conditions (results not shown). We then examined each of the fractions from the density gradient for their protein content. Under conditions of equal protein loading (see the Experimental section), silver staining showed fractions 8–12 to contain multiple bands (Figure 2A), the profile of which did not differ greatly from total soluble platelet lysate. In contrast, fractions 3 and 4 were greatly enriched in a protein of approx. 88 kDa and a smaller amount of another band of approx. 140 kDa (Figure 2A). In multiple preparations analysed in this way, the 88 kDa band was consistently abundant, whereas the 140 kDa band varied in amount between experiments but was consistently the second most abundant protein (results not shown). Immuno-

Figure 2 Silver staining and surface biotinylation of fractions obtained from sucrose gradients of Triton X-100 lysates of resting platelets to show enrichment of surface CD36 and GPIb in the cholesterol-rich insoluble fraction

(A) Equal protein loadings of samples containing protein from the gradient column fractions ($n = 12$) subjected to SDS/PAGE then silver-stained, showing the enrichment of specific proteins within the insoluble raft (fractions 3 and 4). Protein bands identified in subsequent analyses are labelled at the left. The positions of molecular-mass markers are indicated at the left in kDa. (B) Distribution of biotinylated platelet surface proteins into the insoluble raft material. Resting platelets (3×10^8 platelets/ml) were labelled with biotin, lysed with Triton X-100 and fractionated through a sucrose gradient column (see the Experimental section). Equal protein loads from each of the fractions containing protein from the 12 linear fractions were separated by SDS/PAGE, transferred to nitrocellulose and probed with streptavidin–biotin–HRP before being revealed by ECL. Insoluble raft material was present in fractions 3–5. (C) Immunoprecipitation and immunoblot of biotinylated proteins from the Triton X-100-insoluble rafts. Insoluble low-density light-refractive material was recovered from a sucrose gradient column, pelleted, solubilized in Triton X-100/octylglucoside (see the Experimental section) and immunoprecipitated for the specific proteins as indicated. Antigen was processed by SDS/PAGE, immunoblotted with streptavidin–biotin–HRP and revealed by ECL. NMS, normal mouse serum. The positions of molecular-mass markers are indicated at the left in kDa.

Figure 3 Immunoblots showing the distribution of major platelet surface glycoproteins, cytoplasmic tyrosine kinases and cytoskeletal elements from Triton X-100 lysates of resting platelets fractionated on sucrose gradients

Resting platelets [$(1-2) \times 10^9$ platelets/ml] lysed in 1% Triton X-100 were fractionated on a sucrose step gradient (5–40%). The centrifuged gradient was divided into 12 equal fractions, protein concentrations were determined for each fraction, and aliquots containing equal quantities of protein from protein-containing fractions were subjected to SDS/PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were immunoblotted for a range of proteins representative of surface-expressed proteins, cytoplasmic tyrosine kinases and cytoskeletal determinants, followed by detection by ECL. P, an insoluble hard pellet recoverable from the base of the gradient (see the Experimental section). The Figure is a compilation of five separate platelet isolates.

precipitations of proteins from this fraction identified the 88 kDa band as CD36, the 140 kDa band as GPIb, and the faint band at 45 kDa as actin (results not shown). Both CD36 and GPIb are surface membrane glycoproteins that are also found in other membrane compartments within the intact platelet [21,27]. To determine whether the CD36 and GPIb were derived from the platelet surface, the sucrose gradient preparation was repeated with lysates of surface-biotinylated resting platelets. Immunoblotting of the fractions for biotin (Figure 2B) revealed that bands in the position of CD36 and GPIb were indeed biotinylated, and their identity was confirmed by immunoprecipitation from this fraction (Figure 2C). In addition, in these biotinylation experiments faint bands were consistently observed at 110, 58, 47 and 40 kDa. The 110 kDa band was identified as the integrin subunit $\beta 3$ (GPIIIa) (Figures 2B and 2C).

The presence of actin within the Triton X-100-insoluble raft suggested the possibility that cytoskeletal elements act as scaffolding molecules around which the insoluble material coalesced. To test this, the Triton X-100-insoluble raft from resting platelets was treated with DNase 1 to depolymerize actin polymers; after this treatment, silver staining of the samples in SDS/PAGE gels showed that there was no significant shift of protein out of the raft, other than a proportion of the initial total actin (results not shown).

To further characterize proteins contained within the insoluble raft and across the complete density gradient, the protein distribution of significant platelet-surface glycoproteins, specific tyrosine kinases and cytoskeletal elements was studied by immunoblotting. CD36 and p60^{c-src} were found to be particularly enriched within the insoluble raft (Figure 3, lanes 3–6) when compared with other protein-containing fractions. In contrast, GPIb, $\beta 3$ (GPIIIa) and CD9 were found predominantly in the soluble fractions (Figure 3, lanes 8–12). In these resting platelets

Figure 4 Fluorograph of gels of [^3H]palmitate-labelled resting platelets lysed in Triton X-100 and fractionated on sucrose gradients, and immunoprecipitants of CD36 from similarly labelled fractions

(A) Resting platelets (2×10^9 platelets/ml) were metabolically labelled with [^3H]palmitate, lysed in Triton X-100 and fractionated through a 5–40% sucrose step gradient. The centrifuged gradient was divided into 12 equal fractions and a protein profile determined (see the Experimental section). Equal protein aliquots from each fraction containing protein were subjected to SDS/PAGE. The gel was treated with Enhance (DuPont) and exposed to autoradiography film for 4 weeks. An 88 kDa band present in the insoluble raft fraction (5) from this preparation is arrowed. The positions of molecular-mass markers are shown at the left in kDa. (B) Fractions from palmitate-labelled platelets were prepared as in (A). Each gradient fraction (1–12) was immunoprecipitated with anti-CD36 (IE8). The samples were washed twice in lysis buffer before being subjected to SDS/PAGE and processed as in (A). The fractions in (B) were obtained from a separate experiment from those in (A) and the position of the majority of the Triton X-100-insoluble raft varied slightly between fractions 3 and 6 in different experiments.

GPIb was the only surface glycoprotein found in association with the hard pellet at the base of the gradient (Figure 3, lane P). p53/56^{lyn} was abundant within the raft material, but the greater proportion of total Lyn protein was distributed between soluble fractions (Figure 3, lanes 8–12). Analysis of the gradient for cytoskeletal determinants showed a relatively low level of distribution of actin into the raft, the presence of a trace of the membrane skeleton protein vinculin, and a complete absence of tubulin from the same fractions (Figure 3, lanes 3–6). All three cytoskeletal proteins were present in the hard pellet (Figure 3, lane P).

It has been reported that for Src-related tyrosine kinases to associate with the Triton X-100-insoluble rafts and caveolae they should be palmitoylated [8], and Mayor and Maxfield [14] have gone so far as to suggest that the physical isolation of proteins within such Triton-insoluble rafts might be an artifact caused by acylated protein non-specifically associating with the sphingolipid and cholesterol contained in this fraction. This seemed unlikely in the present study because numerous platelet proteins are known to be palmitoylated [28], yet the Triton X-100-insoluble fraction is greatly enriched in only very few proteins. To test this directly, resting platelets were labelled with [^3H]palmitate and the lysates fractionated through sucrose as previously described. Analysis of the fractions obtained (Figure 4A) shows that most of the palmitoylated material was located

outside the Triton X-100-insoluble fraction (Figure 4, lanes 9–12). Within the Triton X-100-insoluble fraction there were relatively few bands present, including a single faint band at 88 kDa. This band was identified by immunoprecipitation as palmitoylated CD36 (Figure 4B). In addition, it can be seen in Figure 4B (lanes 8–12) that some CD36 that was palmitoylated was present, but it remained in the Triton X-100-soluble material.

DISCUSSION

Previous studies of transformed human and mouse cell lines have shown the enrichment of several glycoproteins within detergent-insoluble rafts in the absence of caveolin/VIP21 protein or morphologically identifiable caveolae [15,16]. We show here that a similar caveolin/VIP21-negative fraction can be isolated from the membranes of human platelets. Analysis of the fractions obtained by buoyancy separation in sucrose gradients showed this Triton X-100-insoluble material to be greatly enriched in the membrane glycoprotein CD36, and surface-labelling experiments showed that at least some of this material was exposed on the platelet surface. Lisanti et al. [12] obtained sequence data of a number of proteins isolated from the Triton X-100-insoluble rafts fractionated from endothelium-rich mouse lung tissue, one of which was identified as CD36; in addition, immunoblotting of human lung tissue showed CD36 to reside within a comparable detergent fraction. From these results the authors concluded that CD36 is a transmembrane component of caveolae. Our results with platelets show that such biochemical results do not support this conclusion, and the presence or absence of CD36 within caveolae will require an immunohistological study. This finding substantiates the suggestion of Mayor and Maxfield [14] that results obtained from an analysis of such detergent-insoluble complexes do not necessarily relate to the caveolar structure. However, we would not concur with Mayor and Maxfield [14] in their suggestion that the isolation of such fractions from Triton X-100 cell lysates is largely a result of detergent/protein chemistry. Our results suggest a selective partitioning into the insoluble raft of palmitoylated CD36 rather than non-specific associations: in resting platelets most palmitoylated proteins were found in the Triton X-100-soluble fractions isolated in sucrose gradients. This very selective enrichment of a very few membrane proteins suggests that this fraction is representative of compositionally distinct microdomains [29] present within the membrane of intact, resting platelets. Presumably such microdomains would be enriched in specific phospholipids and glycopospholipids that cause them to cluster, perhaps as a result of their differing fluidities [18]; although we have not completed a lipid analysis of this fraction isolated from platelets, it is enriched in cholesterol (Figure 1A) and sphingolipids (D. J. Dorahy, unpublished work). In support of this concept, an immuno-ultrastructural analysis of CD36 on the platelet membrane showed this protein to be clustered into tight groups rather than dispersed evenly over the surface of resting platelets [21].

The composition of such microdomains is likely to vary between different cell types, perhaps regulated by distinct targeting molecules. Thus Lisanti et al. [12] demonstrated the presence of p60^{c-src} in the insoluble rafts of mouse lung tissue, whereas Sargiacomo et al. [11] failed to detect p60^{c-src} in rafts prepared in the same manner from MDCK cells. The latter result was confirmed by Shenoy-Scaria et al. [8] with an alternative method for the isolation of caveolae from this same cell line, and these authors also provided results to demonstrate that palmitoylation of p60^{c-src} was required for localization to caveolae in MDCK cells. These combined results imply a cell-specific mech-

anism of partitioning far more complex than simple detergent chemistry. In addition, in studies with mutated caveolin/VIP21, it has been found that the palmitoylation of caveolin/VIP21 makes no difference to its location within the Triton X-100-insoluble fraction [30]. Whether this is also true of CD36 is not known because the molecular mechanisms that target CD36 to this membrane microdomain have not been investigated. However, it is of some interest in this regard that Rigotti et al. [31] have shown recently that CD36 binds tightly and specifically to anionic phospholipids, including phosphatidylinositol, which would be well represented in this fraction because of the accumulation of GPI-anchored proteins [11,12,14].

Also well represented within the Triton X-100 rafts from platelets is the glycoprotein GPIb. This major surface glycoprotein is linked to the platelet membrane skeleton, a submembranous structure consisting of actin filaments cross-linked by actin binding protein that has been identified by Fox and colleagues as the pellet obtained after ultracentrifugation of platelet lysate after removal of the actin cytoskeleton by low-speed centrifugation [32,33]. This structure has some superficial similarities to the Triton X-100-insoluble rafts described here. Indeed, both GPIb and actin are prominent components of the membrane cytoskeleton of platelets [31,33], and the occurrence of GPIb and actin within the platelet rafts suggests the possibility that these simply represent this well-characterized platelet membrane fraction after its isolation by a different method. Several points of difference between the published results on the membrane skeleton [31,32,34,35] and the Triton X-100-insoluble rafts reported here militate against this possibility. First, despite the abundance and great enrichment of CD36 in the Triton X-100-insoluble rafts, this protein has not been described in the membrane skeleton [31,33]. Secondly, treatment of the insoluble raft with DNase I, to depolymerize filamentous actin, failed to cause the release of GPIb or any other identifiable proteins from the insoluble material, apart from causing the solubilization of some actin. In contrast, treatment of the membrane cytoskeleton in this manner results in the release of GPIb and actin-binding protein [31,36]. Thirdly, vinculin, a 130 kDa protein, is reported as a permanent component of the membrane skeleton [32,34], but was only detectable in trace amounts in our insoluble rafts isolated from resting platelets. Even these traces of vinculin found in the rafts were possibly due to slight activation of the platelets, because we found that the amount of this protein (and also of actin) within the floating fraction was greatly increased after thrombin-mediated platelet activation (D. J. Dorahy, unpublished work). Together these results suggest that the insoluble raft material is not simply derived from the membrane skeleton, and also that the protein associations within this fraction are not the result of coincidence with the presence of filamentous actin. It remains to be established whether or not there is some relationship between the Triton X-100-insoluble rafts isolated by density flotation and the membrane skeleton isolated by pelleting, and it is possible that the rafts represent a subcomponent of membrane that also associates with the membrane skeleton.

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