Differential role of glycolipid-enriched membrane domains in glycoprotein VI- and integrin-mediated phospholipase Cγ2 regulation in platelets

Peter WONEROW*[1], Achim OBERGFELL†, Jonathan I. WILDE*, Régis BOBE*, Naoki ASAZUMA*, Tomáš BRDIČKA§, Albrecht LEO∥, Burkhart SCHRAVEN¶, Václav HOŘEJŠÍ§, Sanford J. SHATTIL†‡ and Steve P. WATSON*

*Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, U.K., †Department of Vascular Biology, The Scripps Research Institute, La Jolla, CA 92037, U.S.A., ‡Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037, U.S.A., §Institute of Molecular Genetics, Academy of Science of the Czech Republic, 14220 Prague 4, Czech Republic, ∥Institute for Immunology, University of Heidelberg, 69120 Heidelberg, Germany, and ¶Institute for Immunology, Otto-von Guericke-University Magdeburg, 39120 Magdeburg, Germany

The platelet collagen receptor glycoprotein VI (GPVI) and the fibrinogen receptor integrin αIIbβ3 trigger intracellular signalling cascades involving the tyrosine kinase Syk, the adapter SLP-76 and phospholipase Cγ2 (PLCγ2). Similar pathways are activated downstream of immune receptors in lymphocytes, where they have been localized in part to glycolipid-enriched membrane domains (GEMs). Here we provide several lines of evidence that GPVI-mediated tyrosine phosphorylation of PLCγ2 in platelets is dependent on GEM-organized signalling and utilizes the GEM resident adapter protein LAT (linker for activation of T cells). In sharp contrast, although fibrinogen binding to platelets stimulates αIIbβ3-dependent activation of Syk and tyrosine phosphorylation of SLP-76 and PLCγ2, it does not utilize GEMs to promote these responses or to support platelet aggregation. These results establish that GPVI and αIIbβ3 trigger distinct patterns of receptor signalling in platelets, leading to tyrosine phosphorylation of PLCγ2, and they highlight the role of GEMs in compartmentalizing signalling reactions involved in haemostasis.

Key words: immunoreceptor tyrosine-based activation motif (ITAM), linker for activation of T cells (LAT), membrane rafts.

INTRODUCTION

Lipids and proteins in plasma membranes are unequally distributed and form distinct microdomains that are characterized by specific lipid and protein compositions. Glycolipid-enriched membrane domains [GEMs; also known as rafts, DIGs (detergent-insoluble glycosphingolipid-rich membranes) and DRMs (detergent-resistant membrane domains)] have been identified in almost all cells [1,2]. GEMs are rich in glycosphingolipids, saturated phospholipids and cholesterol [3] and are characterized by the absence of most transmembrane proteins but enrichment of glycosyl-phosphatidylinositol (GPI)-anchored proteins and dually acylated intracellular proteins such as Src-family kinases [4]. The few identified transmembrane proteins present in GEMs are usually palmitoylated and include the two transmembrane adapter proteins, LAT (linker for activation of T cells) [5] and PAG/Cbp (phosphoprotein associated with glycosphingolipid-enriched microdomains/Csk-binding protein) [6,7].

There is strong circumstantial evidence for the importance of GEMs in membrane signalling, in particular for cells of the haematopoietic system. For example, signalling through the T-cell antigen receptor (TCR) [8,9], B-cell antigen receptor [10], mast-cell Fcϵ receptor I [11,12] and monocyte Fcα receptor [13] is believed to take place in GEMs. In all these cases, cross-linking of the receptor leads to activation of an Src-family kinase and tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) [14], thereby enabling recruitment and activation of an Syk-family tyrosine kinase [15]. Activation of the Syk-family kinase initiates a series of downstream events that involves recruitment and phosphorylation of adapter proteins, tyrosine kinases and effectors such as phospholipase Cγ (PLCγ) isoforms. In T-cells, GEMs contain LAT, which has been shown to play a critical role in the regulation of PLCγ1 [5,16–18], and they are also enriched in the PLCγ substrate PtdInsP_2 [19]. Intriguingly, however, it is less clear whether the ITAM-coupled immune receptors themselves are localized to GEMs. It appears that the major proportion of the receptor complex may lie outside of these regions and is recruited to them upon activation, facilitated by the coalescing of GEMs into larger structures [20]. The role of GEMs therefore may be to optimize conditions for receptor signalling, while not necessarily being essential for this to occur.

We have recently provided evidence that the extracellular-matrix protein collagen activates platelets through an ITAM-coupled pathway, which shares many of the characteristics of the pathways used by immune receptors [21]. The major receptor underlying excitatory responses to collagen in platelets is a complex of the transmembrane ligand-binding molecule glycoprotein VI (GPVI; structurally belonging, like the immunoreceptors, to the immunoglobulin superfamily) and the Fc receptor γ-chain (FcR γ-chain) [22]. Cross-linking of GPVI leads to phosphorylation of the FcR γ-chain ITAM by the Src kinases Lyn and Fyn and recruitment of Syk [23–25]. Syk regulates a cascade that involves the adapters LAT, Gads and SLP-76, the Tec-family kinase Btk, and phosphatidylinositol 3-kinase, culminating in activation of PLCγ2 and other effectors [26–28]. The snake-venom toxin convulxin and collagen-related peptide (CRP) bind exclusively to GPVI, whereas collagen also binds to other receptors on the platelet surface, including the integrin $\alpha2\beta1$ [29].

Abbreviations used: GPVI, platelet collagen receptor glycoprotein VI; PLCγ2, phospholipase Cγ2; GEMs, glycolipid-enriched membrane domains; LAT, linker for activation of T cells; GPI, glycosyl-phosphatidylinositol; TCR, T-cell antigen receptor; ITAM, immunoreceptor tyrosine-based activation motif; FcR γ-chain, Fc receptor γ-chain; CRP, collagen-related peptide [YGKP*(GPP*)$_{10}$GKP*G, where P* represents hydroxyproline]; mAb, monoclonal antibody; ERK, extracellular-signal-regulated protein kinase; 5-HT, 5-hydroxytryptamine; NP-40, Nonidet P40.

[1] To whom correspondence should be addressed (e-mail peter.wonerow@pharm.ox.ac.uk).

© 2002 Biochemical Society

Although only GPVI and the platelet low-affinity IgG receptor FcγRIIA have been shown to activate platelets through an ITAM-dependent pathway, a number of other platelet receptors utilize some of the proteins involved in this signalling cascade. Of these, an 'outside-in' pathway utilized by the platelet fibrinogen receptor integrin αIIbβ3 (also known as GPIIb-IIIa), is particularly well characterized. Fibrinogen binding to, or cross-linking of, αIIbβ3 induces a level of tyrosine phosphorylation of Syk and SLP-76 comparable with that seen by GPVI. In addition, the αIIbβ3/Syk pathway has been shown to be essential for normal platelet spreading on a fibrinogen-coated surface, a reaction requiring massive rearrangements of the actin cytoskeleton [30–33].

The presence of GEMs in platelets has been described, although their functional relevance is not known [34,35]. However, given the possibility that some of the events following ligation of GPVI and αIIbβ3 may involve these specialized membrane domains, the present study was undertaken to assess their relevance to GPVI and αIIbβ3 signalling. The results indicate that glycolipid-enriched membrane domains participate in platelet activation by GPVI but are not required for signalling through αIIbβ3.

EXPERIMENTAL

Antibodies and reagents

CRP [YGKP*(GPP*)$_{10}$GKP*G (Tana Laboratories, Houston, TX, U.S.A.) in one-letter amino acid code, where P* represents hydroxyproline], was cross-linked via lysine residues as described previously [36]). Convulxin, purified from the venom of *Crotalus durissus terrificus* (South American rattlesnake), was kindly donated by Dr M. Leduc and Dr C. Bon (Unite des Venes, Institut Pasteur, Paris, France). Anti-phosphotyrosine monoclonal antibody (mAb) 4G10, anti-LAT polyclonal antibody and anti-(FcR γ-chain) mAb were from Upstate Biotechnology Inc. (TCS Biological Ltd., Botolph Claydon, Buckingham, Bucks., U.K.). The production of the polyclonal antibodies PAG and SLP-76 and the mAbs CD29, SSA6 and PAC-1 have been described elsewhere [6,37–40]. Mouse CD36 mAb (FA6-152) was from Immunotech, Marseille, France. Cy™5 (a cyanine fluorophore-conjugated secondary antibody) and the FITC-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A. Rabbit polyclonal anti-CD59 antibody was generously provided by Dr Peter Sims (Scripps Research Institute). Anti-PLCγ2 and anti-Syk polyclonal antibodies were from previously described sources [25], and anti-Lyn and anti-Fyn polyclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.); fura 2 acetoxylmethyl ester was from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were from Sigma (Poole, Dorset, U.K.) or previously named sources [25,26,41].

Preparation of platelets, methyl-β-cyclodextrin treatment and platelet aggregation

Human blood was taken from drug-free volunteers on the day of the experiment and drawn into acid citrate/dextrose. Platelet-rich plasma was obtained by centrifugation of the blood samples at 200 ***g*** for 20 min. For depletion of cholesterol, platelets were incubated in platelet-rich plasma with 15 mM or 20 mM methyl-β-cyclodextrin (Sigma) for 1 h at 30 °C. Alternatively, to prevent cholesterol depletion, platelets were incubated with the same concentration of methyl-β-cyclodextrin loaded with cholesterol. Cholesterol loading of methyl-β-cyclodextrin was performed as described elsewhere [42]. Platelets were isolated from platelet-rich plasma by centrifugation at 800 ***g*** for 10 min in the presence of prostacyclin (0.1 μg/ml). The pellet was resuspended in a modified Tyrode's/Hepes buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM Hepes, 5 mM glucose and 1 mM $MgCl_2$, pH 7.3, containing 0.1 μg/ml prostacyclin. The platelets were re-centrifuged at 800 ***g*** for 10 min and resuspended at 3×10^8 cells/ml in Tyrode's/Hepes buffer. Platelet aggregation was performed at 37 °C in an aggregometer with continuous stirring at 1200 rev./min.

GEM and membrane preparations

Resting platelets (2×10^8, in 100 μl of Tyrode's/Hepes buffer) were lysed in 200 μl of ice-cold buffer (20 mM Tris, 150 mM NaCl, 10 mM EDTA, 1 mM PMSF, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, pH 8.3) containing indicated final concentrations of the non-ionic detergents Brij 58, Triton X-100 or Nonidet P40 (NP-40; Sigma). The same buffer was used for the sucrose gradient. The lysate was mixed with an equal volume of 80% (w/v) sucrose, giving a final concentration of 40% sucrose. The lysate (600 μl) was loaded on the bottom of an ultracentrifuge tube and overlaid with 1 ml of 25% sucrose and 500 μl of lysis buffer, both containing the indicated detergent concentrations. For GEM signalling studies, resting or convulxin/CRP-stimulated platelets (2×10^8, in 100 μl Tyrode's/Hepes buffer) were lysed in 200 μl of lysis buffer (containing 3% Brij 58; final concn. 2% Brij 58). The lysate was mixed with an equal volume of 80% sucrose without detergent, giving a final concentration of 40% sucrose and 1% Brij 58 and centrifuged in a sucrose gradient containing 1% Brij 58. The samples were centrifuged at 200000 ***g*** for 2.5 h at 4 °C. Seven fractions, each 300 μl, were taken from the top of the gradient and an equal volume of Laemmli buffer was added [2 × Laemmli (sample) buffer is 4% (w/v) SDS/10% (v/v) 2-mercaptoethanol/20% (v/v) glycerol/50 mM Tris, pH 6.8]. The pellet was resuspended in 300 μl of Laemmli buffer. For immunoprecipitations, the visible-light-scattering band (corresponding to fractions 2 and 3, 'the GEM fractions') was recovered in 300 μl. An equal volume of fractions 6 and 7 was used for immunoprecipitation of 'the soluble fractions'. To both samples, 200 μl of Tyrode's buffer was added to give a final volume of 500 μl. Immunoprecipitations were performed as described below.

For membrane preparations cells were disrupted in lysis buffer containing a low concentration of Triton X-100 (0.01%) to platelet number (2×10^9 platelets/ml) and adjusted to 40% sucrose. The membranes were separated from the cytosol and cytoskeleton by ultracentrifugation in a 40–20%-sucrose gradient (2 h, 200000 ***g***) in the absence of detergent. The membrane fraction was a clearly visible floating fraction on the border of 40–20% sucrose, whereas the cytosolic proteins stayed in the 40%-sucrose fraction and the cytoskeletal proteins were pelleted. The integrity of the membrane fraction was confirmed by the presence of membrane as well as GEM marker proteins (β_1 integrin and LAT respectively) and the absence of cytosolic proteins [extracellular-signal-regulated protein kinase (ERK)].

Adhesion assays

To study tyrosine phosphorylation events in response to ligation to αIIbβ3, platelets (5×10^8) were incubated for 45 min in dishes coated with fibrinogen or BSA in the presence of 2 units/ml apyrase [43]. Dishes coated with fibrinogen were washed twice with PBS to remove non-adherent cells. Platelets adherent to fibrinogen or in suspension over BSA were lysed in ice-cold immunoprecipitation buffer and lysates were subjected to immunoprecipitation assays or used directly for SDS/PAGE.

© 2002 Biochemical Society

Immunoprecipitation and immunoblotting

Resting and convulxin-stimulated platelets (5×10^8/ml, 500 μl) were lysed by adding an equal volume of ice cold IP-buffer [2 % (v/v) NP-40, 20 mM Tris, 300 mM NaCl, 10 mM EDTA, 1 mM PMSF, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 μg/ml pepstatin A, pH 7.3]. Samples obtained from the sucrose gradient centrifugation were lysed using the same buffer in the presence of 2 % (w/v) n-dodecyl β-D-maltoside (a detergent with properties similar to the more familiar n-octyl glucoside) to ensure solubilization of GEMs. Samples were precleared for 1 h at 4 °C with Protein A–Sepharose or Protein G–Sepharose [50 % (w/v) in Tris-buffered saline plus Tween 20 (TBS-T; 20 mM Tris, 137 mM NaCl and 0.1 % (v/v) Tween 20, pH 7.6]. Antibodies were added and samples rotated overnight at 4 °C. Immunoprecipitation of GPVI was performed by incubation of the cell lysates with convulxin and an anti-convulxin antibody. The Sepharose pellet was washed sequentially in lysis buffer and TBS-T before addition of Laemmli sample buffer.

Proteins were separated by SDS/PAGE on 10 % gels and electrically transferred on to PVDF membranes. Membranes were blocked in 10 % (w/v) BSA dissolved in TBS-T. Antibodies were diluted in TBS-T containing 2 % (w/v) BSA and incubated with PVDF membranes for 1 h at room temperature. Membranes were washed in TBS-T after each incubation and developed using an enhanced-chemiluminescence system (ECL®, Amersham Pharmacia Biotech, Cardiff, U.K.).

Measurement of platelet cytosolic Ca^{2+} concentration

Platelets isolated from platelet-rich plasma were resuspended in modified Tyrode's/Hepes buffer to a concentration of 3×10^8 cells/ml and incubated with fura 2 acetoxylmethyl ester (3 μM, 1 h, 30 °C). After being washed in Tyrode's/Hepes buffer, platelets were resuspended at 2×10^8 cells/ml. Stimulation with convulxin (14 nM) or CRP (10 μg/ml) was performed in a PerkinElmer LS50B spectrofluorimeter at 37 °C with agitation in the absence of added Ca^{2+} and presence of 1 mM Arg-Gly-Asp-Ser tetrapeptide to prevent aggregation. The intracellular Ca^{2+} mobilization was calculated as the ratio of fluorescence using excitation at 340 and 380 nm, and emission at 510 nm, (FLWinLab; PerkinElmer).

Measurement of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) secretion

Platelets were labelled with [^{3}H]5-HT (0.5 μCi/ml; NEN Life Science Products, Boston, MA, U.S.A.) at 37 °C for 1 h. Stimulations were performed for 5 min in the presence of apyrase (5 units/ml) with continuous stirring at 1200 rev./min and were terminated by the addition of an equal volume of 6 % (v/v) glutaraldehyde. Supernatants were analysed for [^{3}H]5-HT content by liquid-scintillation spectrometry.

Confocal microscopy

Platelets (1.5×10^7 in 0.5 ml Tyrode's/Hepes buffer) were added to fibrinogen-coated coverslips and incubated for 30 min at 37 °C. Non-adherent platelets were washed away and attached platelets were stimulated with 200 nM PMA to enhance spreading. Cross-linking of platelet surface proteins was performed by incubation for 20 min at 37 °C with the indicated primary and relevant dye-labelled secondary antibodies. Platelets were then fixed, permeabilized and further stained as indicated and examined by confocal microscopy [44]. Co-localization was analysed in a single confocal plane using LaserSharp software (Bio-Rad, Hercules, CA, U.S.A.).

RESULTS

Detergent-sensitive localization of LAT in GEMs from unstimulated platelets

Because the recovery of associated proteins and preservation of GEM structure are dependent on the type of detergent and the detergent/protein ratio [45], several non-ionic detergents were compared for their ability to isolate GEMs and associated proteins from unstimulated platelets. Initial studies focused on the distribution of the adapter LAT and CD59 as markers of the GEM fraction [5,45]. The folate receptor CD59 is a GPI-anchored protein which has been shown to associate with GEMs in different cells [4,45,46]. The soluble fractions contain non-GEM-associated membrane proteins and cytosolic proteins and could be identified by blotting for cytosolic p42/44 ERK. These fractions also contained the integrin β_1-subunit (CD29), which was therefore used as a non-GEM transmembrane marker protein.

In the presence of relatively low concentrations of NP-40 and Triton X-100 (0.1 % in both cases), the majority of LAT was recovered in the GEM fractions, whereas higher concentrations of the detergents caused a selective depletion of the adapter from these fractions (Figure 1). LAT was completely redistributed to soluble fractions in the presence of the higher concentrations of Triton X-100. With higher concentrations of NP-40, LAT was localized to intermediate and soluble fractions, indicating a limited preservation of floating membrane fragments (Figure 1). Similar results were seen for the distribution of CD59 (results not shown). The β_1-integrin subunit and ERK were localized to the soluble fractions at all concentrations of Triton X-100 and NP-40 (Figure 1 and results not shown).

In contrast with the results with NP-40 and Triton X-100, LAT and CD59 were preserved in the GEM fractions over a wide range of detergent concentrations (0.1–3 %) of Brij 58, whereas the β_1-integrin and the cytosolic protein p42/44 ERK were restricted to the soluble fractions. It thus appears that GEMs cannot be solubilized by high concentrations of Brij 58. Nevertheless, approx. 10 % of LAT was found in the soluble fraction at all concentrations of Brij 58. This suggests either that a portion of LAT is not localized to the GEM regions (perhaps because it is not palmitoylated) or that a small proportion of the GEM fraction is susceptible to solubilization by the detergent.

Because of the ability of Brij 58 to preserve GEMs over a wide range of concentrations, it was selected as the detergent of choice for further studies. In addition, a limited number of studies were performed with Triton X-100 at a concentration that preserved GEM integrity.

Protein composition of GEMs from unstimulated platelets

The GEM fractions were characterized further by Western blotting for GEM and non-GEM markers and for proteins known to be or potentially involved in GPVI and integrin αIIbβ3 signalling. Similar results were observed with Brij 58 (Figure 2A) and Triton X-100 (results not shown). A high enrichment of CD59 and the Src-family kinases Lyn and Fyn were found in the GEM fraction along with LAT. In addition, a second transmembrane adapter, known as PAG or Cbp, was exclusively present in this fraction (Figure 2A).

A number of proteins were not detected in the GEM fraction or were present at a low level. This includes c-Src, which does not have the GEM targeting sequence found in Lyn and Fyn, and the β_1-integrin subunit and ERK as described above (Figure 2A). We were not able to detect the presence of many of the cytosolic

© 2002 Biochemical Society

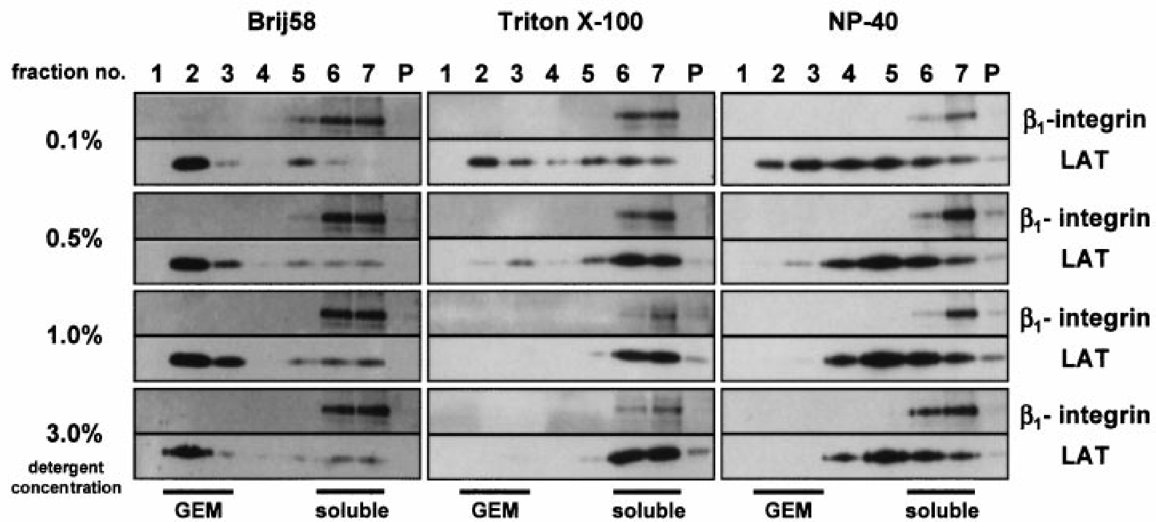

Figure 1 Influence of type and concentration of detergent on the preservation of GEMs

Platelets were lysed in the presence of the indicated concentrations of Brij 58, Triton X-100 or NP-40 and fractionated by sucrose-gradient centrifugation. Seven fractions, each of 300 μl, were taken from the top of the gradient. The pellet (P) was resuspended in 300 μl of Laemmli buffer. Aliquots (25 μl) of each fraction were analysed by SDS/PAGE followed by immunoblotting for LAT and β_1-integrin. Fractions 6 and 7 contain cytosolic and non-GEM-localized membrane proteins. The results are representative of five experiments.

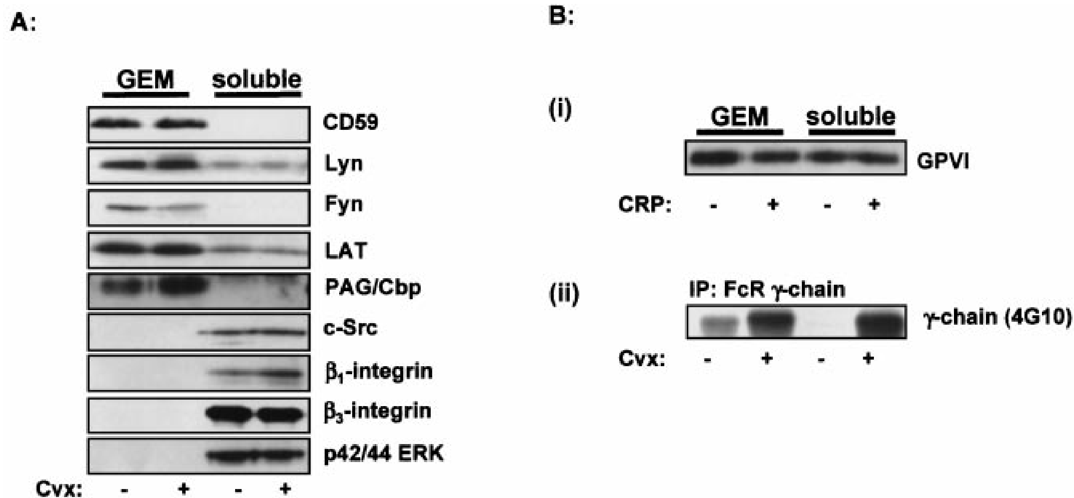

Figure 2 Protein composition of platelet GEMs

Resting and convulxin-stimulated (14 nM for 30 s) platelets were lysed in Brij 58 (2%) and subjected to sucrose-gradient centrifugation. For the detection and immunoprecipitation of GPVI by convulxin labelling, platelets were stimulated with 10 μg/ml CRP. Aliquots (25 μl) of combined fractions 2 and 3 (GEM) and combined fractions 6 and 7 (soluble) were analysed by SDS/PAGE followed by immunoblotting for the indicated proteins (**A** and **B**, i) or indicated proteins were immunoprecipitated (IP) and analysed for tyrosine phosphorylation (4G10) (**B**, ii). Results are representative of between five and ten experiments.

proteins involved in Syk-dependent signalling cascades within GEMs under basal conditions including Syk, Gads, SLP-76 and PLCγ2 (Figure 3B and results not shown).

Tyrosine phosphorylation and protein recruitment in GEMs following platelet stimulation through GPVI

To investigate the role of GEMs in GPVI-mediated activation of PLCγ2, it was important to establish the localization of this collagen receptor. Under basal conditions a slightly greater amount of GPVI could be recovered from the GEM fraction (Figure 2Bi). Stimulation of platelets via GPVI caused no major change in the distribution of the glycoprotein between GEM and soluble fraction. A tyrosine-phosphorylated doublet that is characteristic of the FcR γ-chain was immunoprecipitated from the GEM and the soluble fraction following convulxin stimulation (Figure 2Bii), indicating that GPVI is able to support phosphorylation of the FcR γ-chain in GEM and non-GEM membrane compartments.

Probing for phosphotyrosine revealed that, under basal conditions, a major tyrosine-phosphorylated region of about 50–60 kDa is present in the GEM fraction. In addition, a number

© 2002 Biochemical Society

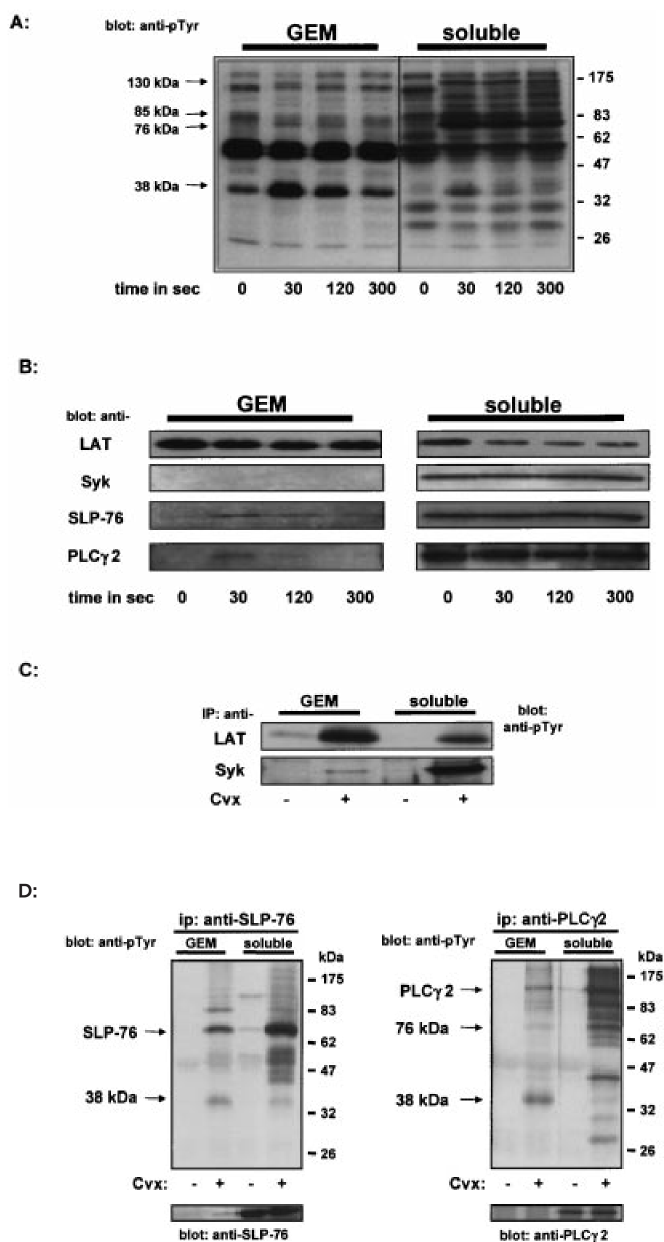

Figure 3 Tyrosine phosphorylation in platelet GEMs

Resting and convulxin-stimulated (14 nM for the indicated time points) were lysed in Brij 58 (2%) and subjected to sucrose-gradient centrifugation. Aliquots (25 μl) of combined fractions 2 and 3 (GEM) and combined fractions 6 and 7 (soluble) were analysed by SDS/PAGE followed by immunoblotting for (**A**) tyrosine-phosphorylated proteins (anti-phosphotyrosine mAb 4G10) or (**B**) for indicated proteins by immunoblotting. (**C**, **D**) Resting and convulxin-stimulated (14 nM for 30 s) platelets were lysed in Brij 58 (2%) and subjected to sucrose-gradient centrifugation. The visible-light-scattering band (corresponding to fractions 2 and 3, GEM) was recovered. An equal volume of fractions 6 and 7, containing the soluble proteins, was used for immunoprecipitation. After adding immunoprecipitation buffer (containing 1% n-dodecyl β-D-maltoside and 1% NP-40 final concn.), LAT, Syk, SLP-76 and PLCγ2 were immunoprecipitated (see the Experimental section). The samples were separated by SDS/PAGE and immunoblotted for tyrosine-phosphorylated proteins. Results are representative of five experiments.

of minor bands can be seen (Figure 3A). The 50–60 kDa region is likely to represent the Src-family kinases Fyn and Lyn, both of which are present in GEMs (Figure 2A) and are phosphorylated under basal conditions. Upon stimulation by convulxin (30–300 s), a 38 kDa band underwent a marked, but transient, increase in tyrosine phosphorylation, which returned to basal levels by 300 s (Figure 3A). Western-blotting and immunoprecipitation studies using a specific antibody identified this band as LAT (Figures 3B and 3C), which has previously been reported to undergo transient tyrosine phosphorylation in convulxin-stimulated platelets [47]. There were also small transient increases in new tyrosine-phosphorylated bands at 76 and 130 kDa in GEMs following stimulation with convulxin for 30 s (Figure 3A), which co-migrate with SLP-76 and Syk, and PLCγ2 respectively. Immunoprecipitation studies confirmed the presence of tyrosine phosphorylated forms of SLP-76 and PLCγ2 in GEMs and demonstrated a transient association with a tyrosine phosphorylated protein of 38 kDa that co-migrates with LAT (Figure 3D). We have previously reported that both SLP-76 and PLCγ2 associate with LAT in convulxin-stimulated platelets [28,47]. Consistent with these observations, a small proportion ($< 10\%$) of PLCγ2 and SLP-76 were transiently recruited to the GEM fraction, with a time course similar to that seen for LAT phosphorylation (Figure 3B). Although we were not able to detect the presence of Syk in GEMs by direct immunoblotting, immunoprecipitation of the kinase revealed the presence of tyrosine phosphorylated Syk in the GEM fraction following convulxin stimulation (Figure 3C).

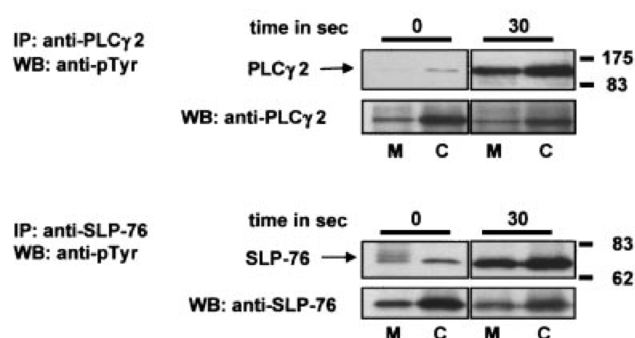

Figure 4 Membrane association of PLCγ2

Membrane and cytosolic fractions were isolated from resting or convulxin (14 nM)-stimulated platelets and PLCγ2 or SLP-76 was immunoprecipitated from the membrane (M) and cytosolic (C) fraction. Samples were separated by SDS/PAGE and immunoblotted for tyrosine-phosphorylated proteins and for PLCγ2 or SLP-76. Results are representative of three experiments.

A far greater number of tyrosine-phosphorylated bands was seen under basal conditions in the soluble fraction, with a particularly prominent band at 60 kDa (Figure 3A). The 60 kDa band co-migrates with c-Src, which is exclusively localized to the soluble fraction (Figure 2A). Upon stimulation, there were increases in bands of 38, 76, 90 and 130 kDa. The band of 38 kDa co-migrates with LAT but underwent a much smaller increase in tyrosine phosphorylation than in the GEM fraction. Immunoprecipitation studies confirmed a limited presence of tyrosine-phosphorylated LAT in the soluble fraction (Figure 3B). In contrast, the majority of tyrosine-phosphorylated forms of Syk, SLP-76 and PLCγ2 were present outside of GEMs, but exhibited a negligible degree of association with the tyrosine-phosphorylated 38 kDa band (Figures 3C and 3D). Cell fractionation revealed that the majority of tyrosine-phosphorylated SLP-76 and PLCγ2 was present in the cytosol (Figure 4). This suggests that either a transient association of these molecules with GEMs takes place following agonist occupancy of GPVI or that the receptor complex dissociates upon cell lysis.

© 2002 Biochemical Society

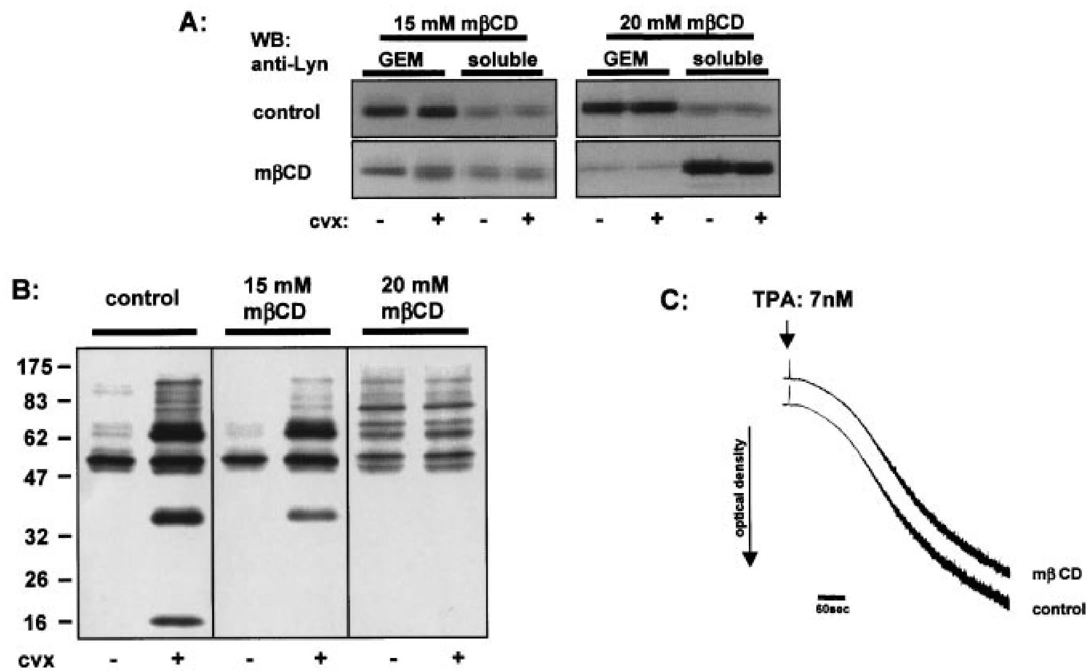

Figure 5 Effect of cholesterol depletion on GEM localization of Lyn and GPVI-induced tyrosine phosphorylation

(**A**) Methyl-β-cyclodextrin (mβCD)-treated or untreated platelets were lysed in Brij 58 (2%) and subjected to sucrose-gradient centrifugation. Aliquots of 25 μl of combined fractions 2 and 3 (GEM) and 6 and 7 (soluble) were separated by SDS/PAGE followed by immunoblotting for Src kinase Lyn. Results are representative of four experiments. (**B**) Methyl-β-cyclodextrin-treated or untreated platelets were stimulated with 14 nM convulxin for 30 s. Lysates were analysed by SDS/PAGE followed by immunoblotting for tyrosine-phosphorylated proteins. (**C**) Methyl-β-cyclodextrin-treated or untreated platelets were prepared as described in the Experimental section and resuspended in modified Tyrode's/Hepes buffer at a concentration of 3×10^8 cells. Aggregation was induced in the absence of added fibrinogen by 7 nM PMA (called 'TPA' on the Figure).

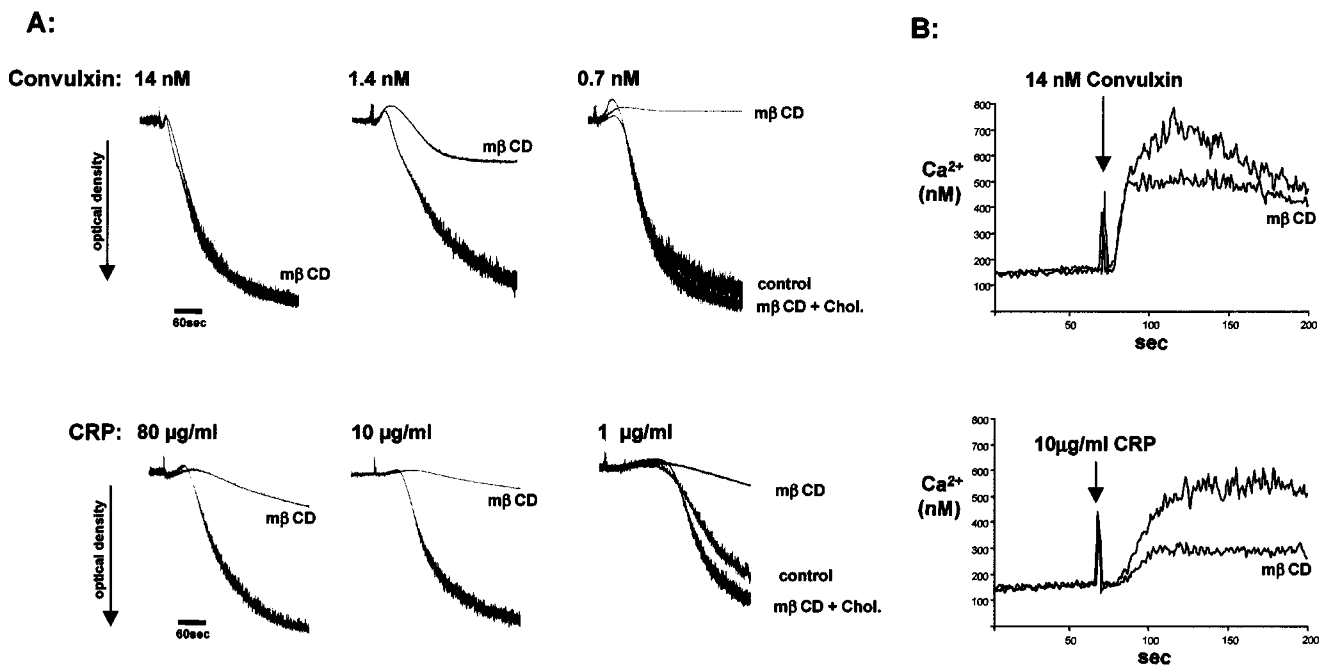

Figure 6 Effect of cholesterol depletion on platelet aggregation and calcium mobilization by GPVI agonists

(**A**) Methyl-β-cyclodextrin (mβCD) [with or without cholesterol (Chol.)] treated or untreated platelets were prepared as described in the Experimental section and resuspended in modified Tyrode's/Hepes buffer at a concentration of 3×10^8 cells. Aggregation was induced by convulxin or CRP at the indicated concentrations. (**B**) Treated or untreated platelets were loaded with the fluorescent Ca^{2+} indicator fura 2 (5 mM, 1 h, 30 °C), washed and resuspended in modified Tyrode's/Hepes buffer at a concentration of 2×10^8 cells. Ca^{2+} mobilization was measured in a spectrofluorimeter after stimulation with 14 nM convulxin or CRP in the presence of 1 mg/ml RGDS (Arg-Gly-Asp-Ser, an integrin αIIβ3 antagonist peptide). Results are representative of four experiments.

© 2002 Biochemical Society

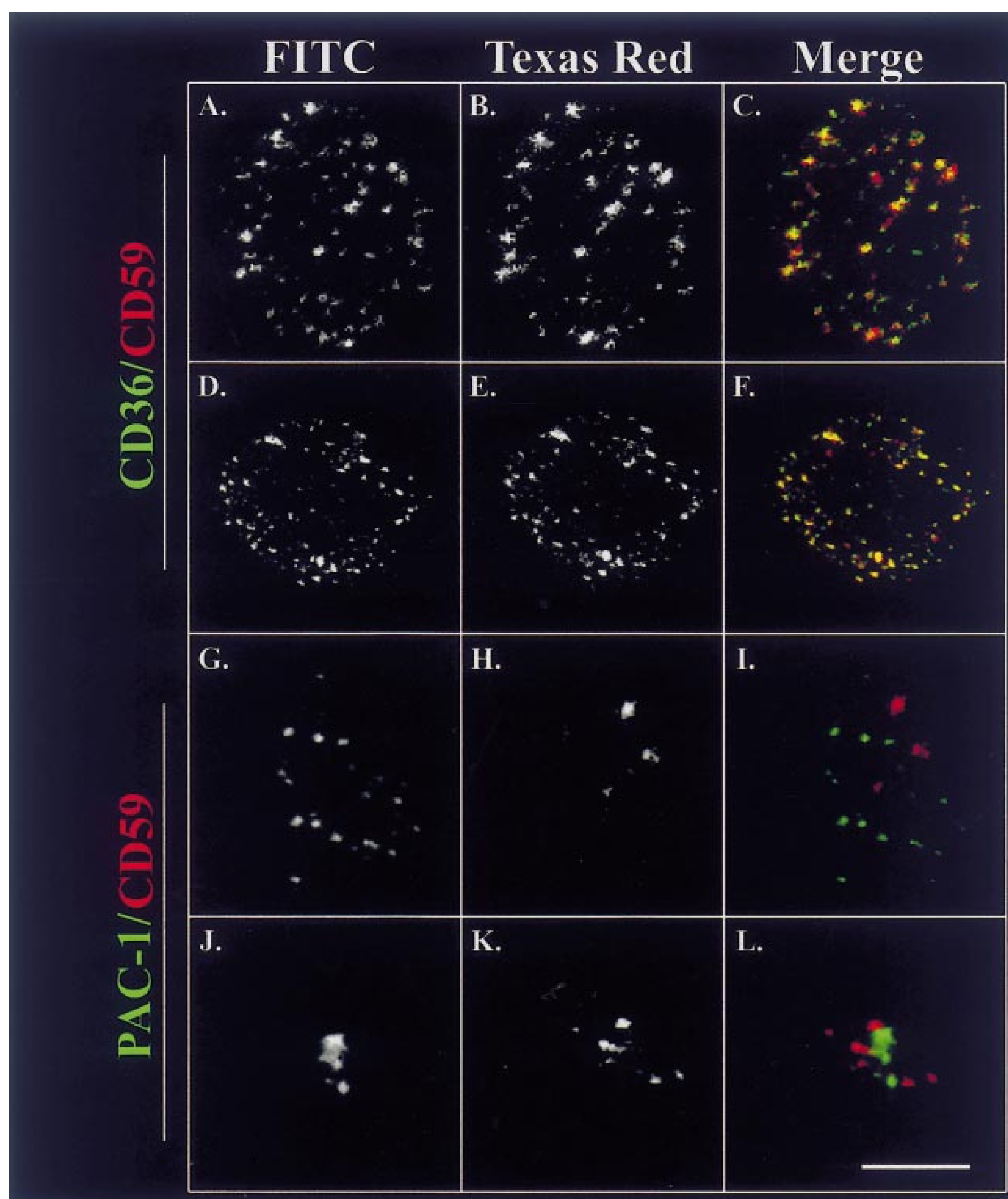

Figure 7 Co-localization of platelet surface proteins

Platelets were attached to fibrinogen-coated coverslips and stimulated with 200 nM PMA to enhance spreading. Cross-linking of platelet surface proteins was performed with primary antibodies directed against CD36 (**A**, **D**) and CD59 (**B**, **E**) or αIIbβ3 (PAC-1, **G**, **J**) and CD59 (**H**, **K**) and dye-labelled secondary antibodies. PAC-1 was used to co-cluster only the activated form of αIIbβ3. Following antibody staining, platelets were fixed, permeabilized and examined by confocal microscopy. Column 1 (FITC) shows the staining for CD36 (**A**, **D**) and PAC1 (**G**, **J**), column 2 (Texas Red) shows the staining for CD59 and column 3 represents the merge of the stainings for CD36/CD59 (**C**, **F**) and PAC1/CD59 (**I**, **L**). For each pair of stainings, two different cells are shown. These are representative pictures of from six to ten experiments. The scale bar represents 10 μm.

To investigate the role of GEMs for GPVI signalling, the cholesterol-lowering agent methyl-β-cyclodextrin was used to disrupt the integrity of these membrane microdomains. Methyl-β-cyclodextrin (20 mM) caused a dramatic displacement of the GEM marker protein Lyn to the soluble fraction (Figure 5A). This treatment also caused an increase in tyrosine phosphorylation of a number of higher-molecular-mass bands, whereas convulxin-induced phosphorylation was completely inhibited (Figure 5B). FACS analysis of methyl-β-cyclodextrin (20 mM)-treated platelets also revealed a marked change in size and granularity (results not shown) although the aggregation response to PMA (Figure 5C) was not altered. For these reasons, functional studies were also performed with platelets incubated with a lower concentration (15 mM) of methyl-β-cyclodextrin, which preserves platelet structure and does not cause a significant increase in tyrosine phosphorylation. Treatment with this concentration of methyl-β-cyclodextrin (15 mM) caused a smaller displacement of Lyn to the soluble fraction of the order of 20 % (Figure 5A) and a strong decrease in convulxin-stimulated tyrosine phosphorylation (Figure 5B). Decreases in phosphorylation were most marked for tyrosine-phosphorylated proteins of 12–14, 38 and 130 kDa, which co-migrate with FcR γ-chain,

© 2002 Biochemical Society

LAT and PLCγ2 respectively (Figure 5B). Treatment with this mild concentration of methyl-β-cyclodextrin (15 mM) inhibited aggregation, dense-granule secretion and Ca^{2+} mobilization following activation of GPVI. Methyl-β-cyclodextrin completely inhibited platelet aggregation to concentrations of convulxin and CRP that were just sufficient to give maximal aggregation (Figure 6A). On the other hand, methyl-β-cyclodextrin had a weak or negligible effect on aggregation to supramaximal concentrations of convulxin, whereas responses to CRP were still inhibited (Figure 6A). Importantly, these effects were not seen if platelets were incubated with methyl-β-cyclodextrin loaded with cholesterol, a condition that prevents membrane cholesterol depletion and GEM disruption (Figure 6A). A greater inhibitory effect of methyl-β-cyclodextrin-treated platelets against CRP relative to convulxin was also apparent for Ca^{2+} mobilization (Figure 6B). Methyl-β-cyclodextrin completely inhibited the release of [^{3}H]5-HT from dense granules to a submaximal concentration of convulxin (1.4 nM), which released 39.3 ± 4.5 % of cellular content, and reduced the response to a ten-fold higher concentration of the toxin from 77.5 ± 3.6 to 19.7 ± 7.2 % ($n = 3$).

Taken together, these results indicate that whereas only a small proportion of proteins involved in GPVI signalling are present in GEMs, agonist occupancy of GPVI induces a specific pattern of tyrosine-phosphorylation events within these membrane microdomains accompanied by recruitment of SLP-76 and PLCγ2. Furthermore, association of the tyrosine-phosphorylated 38 kDa band with SLP-76 and PLCγ2 is restricted to these membrane microdomains, and disruption of GEMs causes inhibition of GPVI-mediated signalling cascades, while aggregation to direct activation of protein kinase C by PMA is not affected.

αIIbβ3 integrin complex signals outside of GEMs

In contrast with the collagen receptor GPVI, the β3-integrin subunit was detected in the soluble fraction following GEM isolation using Brij 58 or Triton-X-100. A trace amount of β3 could be seen in long exposures, which was estimated to be less than 2 % of the cellular level (results not shown). This distribution remained unchanged following adhesion of platelets to the αIIbβ3 ligand, fibrinogen. To examine αIIbβ3 localization using an independent approach, co-localization studies were performed using confocal microscopy. The localization of individual proteins was analysed by cross-linking of antibodies to GEM marker proteins and GEM-excluded proteins. Staining of platelets for CD36 or CD59, both GEM-enriched proteins (Figure 2; [34]), without antibody cross-linking showed an even distribution of both proteins within the cell membrane. Antibody cross-linking caused movement of CD36 and CD59 into clusters, which showed a high degree of co-localization (Figures 7A–7F). Quantification indicated that 63.2 ± 6.2 % ($n = 6$) of CD36 and CD59 stainings were co-localized. On the other hand, despite the high density of αIIbβ3 on the platelet surface, there was little co-localization of CD59 and αIIbβ3 following antibody cross-linking, suggesting that the two proteins are distributed to distinct domains within the membrane (Figures 7G–7L). The degree of co-localization was quantified using an antibody directed against β_3-subunit, SSA6, and an antibody directed against the activated form of αIIbβ3, Pac-1 The degree of co-localization of stainings with CD59 was 13.9 ± 2.9 % ($n = 10$) and 10.0 ± 3.2 % ($n = 8$) for PAC-1 and SSA6 respectively. This is consistent with the biochemical studies described above, which demonstrated the presence of αIIbβ3 in non-GEM structures.

In common with GPVI, αIIbβ3 stimulates intracellular signalling cascades that include tyrosine phosphorylation of Syk and SLP-76 [30,32,33]. We now show that this signalling cascade also leads to tyrosine phosphorylation of PLCγ2. Immunoprecipitation of the phospholipase from fibrinogen-adherent platelets revealed an increase in tyrosine phosphorylation that was similar in magnitude to that induced by convulxin (Figure 8). The observation that αIIbβ3 stimulates tyrosine phosphorylation of Syk, SLP-76 and PLCγ2 suggests that, at least superficially, this cascade is similar to that underlying the regulation of PLCγ2 by GPVI. However, in the case of αIIbβ3 signalling, a tyrosine-phosphorylated band in the region of LAT was not observed in whole lysates (Figure 8) or in immunoprecipitates of LAT, SLP-76 or PLCγ2 (results not shown). Thus, unlike the GPVI pathway, the GEM-resident adapter LAT is not part of the signalling cascade downstream of αIIbβ3.

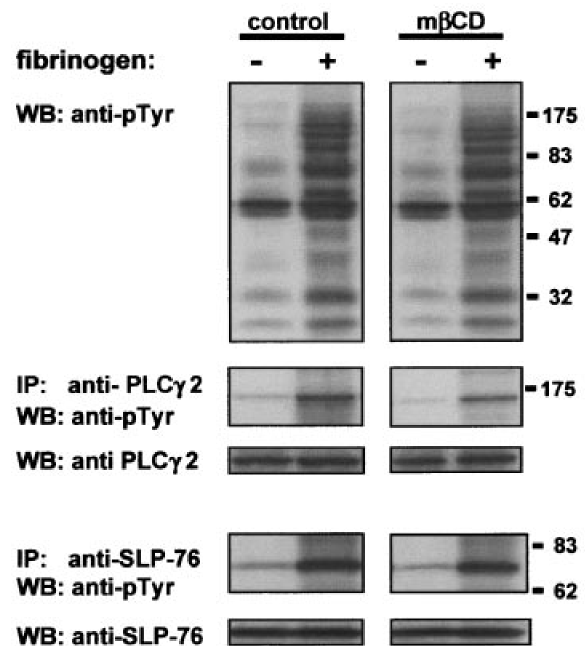

Figure 8 Fibrinogen stimulates tyrosine phosphorylation of PLCγ2

Control and methyl-β-cyclodextrin (mβCD)-treated platelets were placed in dishes coated with fibrinogen or BSA in the presence of 2 units/ml apyrase for 45 min [43]. Dishes coated with fibrinogen were washed twice with PBS to remove non-adherent cells. Platelets adherent to fibrinogen or in suspension over BSA were lysed in ice-cold immunoprecipitation buffer and subjected to immunoprecipitation for PLCγ2 and SLP-76 or used directly for SDS/PAGE and immunoblotted for tyrosine-phosphorylated proteins. Results are representative of three experiments.

These results indicate that activation of PLCγ2 downstream of αIIbβ3 may not be dependent on GEMs. To further address this, we investigated the effects of the mild concentration of methyl-β-cyclodextrin (15 mM) on αIIbβ3-induced tyrosine phosphorylation. In contrast with the results with GPVI signalling, whole cell tyrosine phosphorylation as well as tyrosine phosphorylation of SLP-76 and PLCγ2 induced by adhesion to fibrinogen was not affected by methyl-β-cyclodextrin treatment (Figure 8). These data suggest that αIIbβ3-mediated regulation of PLCγ2 is not dependent on GEMs.

DISCUSSION

This study has provided evidence that GPVI signalling is at least partially dependent on GEMs, whereas signalling by αIIbβ3 is not, despite the fact that several common proteins participate in

© 2002 Biochemical Society

both cascades, including Syk, SLP-76 and PLCγ2. This may help to explain why the two sets of platelet receptors stimulate a similar level of tyrosine phosphorylation of these three proteins despite a much lower level of expression of GPVI compared with the integrin. Thus the role of the GEM may be to facilitate GPVI signalling through the Syk/LAT/SLP-76/PLCγ2 pathway.

In order to define conditions for isolation of platelet GEMs, we used the adapter LAT and GPI-anchored CD59 as marker proteins. Consistent with other studies, we show that recovery of GEM-associated proteins was dependent on the nature and concentration of detergent [45,48]. Whilst GEM integrity could be preserved by low Triton-X-100/platelet or NP-40/platelet ratios, higher detergent concentrations caused complete disruption of this region. Interestingly, higher concentrations of NP-40 preserved a LAT-containing floating membrane domain, which migrated after the GEM fraction, indicating preservation of a qualitatively distinct membrane population, which requires further characterization. In contrast to the results with NP-40 and Triton-X-100, the presence of LAT in GEMs was observed over a wide range of concentrations of Brij 58. Thus the three different detergents gave rise to differing levels of GEM preservation according to their concentration. Brij 58 was the detergent of choice because of its ability to preserve the GEMs over a broad range of concentrations, although a more limited number of studies were performed with Triton X-100 for comparison.

GEMs isolated from platelets were enriched in the GPI-anchored protein CD59, the adapters LAT and PAG/Cbp, and the Src kinases Fyn and Lyn. The integrin β_1 subunit was not detected in the GEM fraction, and there was only a trace amount of the integrin β3-subunit. Two earlier studies have reported the partial presence of β1- and β3-integrin subunits within GEMs in platelets [34,49]. This difference appears to be related to the different conditions used for GEM isolation (type and concentration of the detergent) and serves to illustrate the limitation in relying solely on the use of detergents to define GEM composition. Our observation that β3-integrin is excluded from GEMs was supported by examination of its distribution in spread platelets upon antibody-mediated clustering, the absence of phosphorylation of LAT and the lack of effect of methyl-β-cyclodextrin on protein tyrosine phosphorylation triggered by ligation of αIIbβ3. These separate observations strongly suggest a predominant localization and function of the αIIbβ3 complex outside of GEMs. However, this does not exclude a potential role for GEMs in certain aspects of integrin function, as observed in other cell types [50,51].

The present study provides evidence for a role of GEMs in signalling by the collagen receptor GPVI. The constitutive association of a proportion of GPVI within GEMs contrasts with the situation reported for most other ITAM-coupled receptors, which only become recruited to GEMs following activation [20]. The permanent co-localization of GPVI FcR γ-chain complex and Lyn and Fyn in GEMs, suggests a critical requirement for regulation of Src kinases to prevent unwanted initiation of GPVI signalling. The presence of the Csk regulatory protein PAG/Cbp in these membrane domains may provide a pathway of regulation of Src kinases and thereby initiation of the GPVI signalling cascade as reported in T cells [6,52]. Importantly, we have observed a co-association between PAG/Cbp with the Src kinase regulatory protein, Chk, which is lost upon activation of GPVI (results not shown).

In T cells, tyrosine phosphorylation of LAT has been proposed to facilitate recruitment of a number of proteins to GEMs including SLP-76, Grb2, Gads, Itk and PLCγ1, although the net movement of protein is relatively low [8,53]. It appears that LAT may play a similar role in platelets. Upon activation of GPVI, LAT has been shown to associate either directly or indirectly with Src-family kinases, Gads, Grb2, SLP-76 and PLCγ2 [47]. Moreover, since the majority of LAT is restricted to GEMs (Figures 2 and 3), it follows that these are the sites where these complexes form. In support of this, a small proportion of SLP-76 and PLCγ2 ($< 10\,\%$) was found in association with GEMs upon stimulation.

Further evidence for the involvement of GEMs in platelet activation by GPVI was gained using the cholesterol-binding agent methyl-β-cyclodextrin. Methyl-β-cyclodextrin causes disruption of these domains and inhibition of ITAM-regulated signalling cascades. We used a modest treatment of platelets with methyl-β-cyclodextrin as changes in morphology and protein tyrosine phosphorylation were observed with higher concentrations. Treatment with methyl-β-cyclodextrin inhibited tyrosine phosphorylation, dense-granule secretion and Ca^{2+} mobilization following stimulation of GPVI. This was associated with a reduction in platelet aggregation in response to low but not high concentrations of convulxin. The response to CRP was also inhibited throughout its dose–response curve, which may reflect a weaker stimulatory effect of the peptide. There was no effect of cholesterol-loaded methyl-β-cyclodextrin on GPVI-dependent platelet responses, consistent with an inhibitory effect being mediated through cholesterol depletion. Furthermore, PMA-induced platelet aggregation was not influenced by methyl-β-cyclodextrin, suggesting that this reagent does not have a generalized effect on cellular toxicity.

Although these data support an important role for GEMs in signalling by the collagen receptor GPVI, the presence of the major proportion of many tyrosine-phosphorylated proteins, including Syk, SLP-76 and PLCγ2, within the cytosol raises questions regarding the organization of GPVI signalling. In fact signalling molecules within the GPVI pathway seem to be strongly separated, with LAT, PAG/Cbp, and Fyn/Lyn localized to GEMs, and GPVI, Syk, SLP-76 and PLCγ2 having only limited access. It is possible that this is due to the biochemical isolation procedure for GEMs, which may affect the association of these molecules with GEMs and therefore lead to an underestimation of the amount that is present in these membrane microdomains. The presence and recruitment of a low level of many critical signalling molecules is also a constant finding for immune receptor signalling pathways in other cell systems, despite the use of a variety of detergents and conditions for the analysis of these membrane structures [8,9,54,55]. Alternatively, these results might reflect the fact that many of the signalling molecules undergo only a transient association with GEMs following GPVI activation. This would suggest that recruitment of only a small but critical fraction of these molecules at a particular time may be sufficient for optimal signalling.

It is important to consider whether signalling events mediated downstream of GPVI also take place outside of GEMs. The presence of GPVI and tyrosine phosphorylated FcR γ-chain in these regions, and the presence of a high proportion of phosphorylated proteins outside of GEMs provides indirect evidence that this is the case. However, many of these tyrosine-phosphorylated proteins are localized to the cytosol, possibly having dissociated from GEM and non-GEM membrane structures. The demonstration of a significant level of tyrosine phosphorylation of PLCγ2 in LAT-deficient mice demonstrates the presence of a LAT-independent signalling pathway to the phospholipase. It is important to establish whether this takes place outside of GEMs.

Evidence for the regulation of PLCγ2 downstream of Syk and SLP-76 independent of GEMs is provided here by studies of

© 2002 Biochemical Society

platelet signalling downstream of integrin αIIbβ3. Biochemical isolations revealed that 98% of αIIbβ3 complex was localized outside of GEMs. This was supported by confocal microscopy, which revealed only a small degree of co-localization between GEM-enriched CD59 and αIIbβ3. Additionally, although Syk, SLP-76 and PLCγ2 lie downstream of αIIbβ3 and GPVI, the αIIbβ3 pathway did not utilize the GEM-localized adapter LAT. The absence of an association with GEMs was consistent with the observation that methyl-β-cyclodextrin had no effect on tyrosine phosphorylation, including SLP-76 and PLCγ2, induced by αIIbβ3 in fibrinogen-adherent platelets. Together, these observations suggest a different organization of αIIbβ3-induced PLCγ2 activation that contrasts with the situation for GPVI.

An important new observation made during the course of the present study is that αIIbβ3 signalling causes tyrosine phosphorylation of PLCγ2. This may explain previous reports of increases in intracellular Ca^{2+} following adhesion to fibrinogen [56–58]. Importantly, the present observations demonstrate that the regulation of the phospholipase by the integrin is independent of LAT.

In conclusion, the present study has provided evidence for two distinct patterns of agonist signalling in platelets through GPVI and integrin αIIbβ3, demonstrating dependence and independence from GEMs respectively. While the results highlight the use of common signalling elements by distinct platelet surface receptors, they indicate that compartmentalization of downstream adapters and effectors may account, in part, for marked differences in platelet responses to ligation of GPVI and αIIbβ3. Thus, just as the localization of certain proteins to GEMs is involved in GPVI signalling, the recruitment of signalling molecules to αIIbβ3-based cytoskeletal structures determines platelet responses to fibrinogen.

This work was supported by the Welcome Trust and by grants from the National Institutes of Health (U.S.A.). S.P.W. is a British Heart Foundation Senior Research Fellow. V.H. is also supported by Research Centre project A026 of the Czech Ministry of Education. We are grateful to Stuart Marshall, who helped with the measurement of [^{3}H]5-HT secretion.

REFERENCES

1 Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. Nature (London) **387**, 569–572

2 Brown, D. A. and London, E. (1998) Functions of lipid rafts in biological membranes. Annu. Rev. Cell Dev. Biol. **14**, 111–136

3 London, E. and Brown, D. A. (2000) Insolubility of lipids in Triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). Biochim. Biophys. Acta **1508**, 182–195

4 Horejsi, V., Drbal, K., Cebecauer, M., Cerny, J., Brdicka, T., Angelisova, P. and Stockinger, H. (1999) GPI-microdomains: a role in signalling via immunoreceptors. Immunol. Today **20**, 356–361

5 Zhang, W., Trible, R. P. and Samelson, L. E. (1998) LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity **9**, 239–246

6 Brdicka, T., Pavlistova, D., Leo, A., Bruyns, E., Korinek, V., Angelisova, P., Scherer, J., Shevchenko, A., Hilgert, I., Cerny, J. et al. (2000) Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. J. Exp. Med. **191**, 1591–1604

7 Kawabuchi, M., Satomi, Y., Takao, T., Shimonishi, Y., Nada, S., Nagai, K., Tarakhovsky, A. and Okada, M. (2000) Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature (London) **404**, 999–1003

8 Xavier, R., Brennan, T., Li, Q., McCormack, C. and Seed, B. (1998) Membrane compartmentation is required for efficient T cell activation. Immunity **8**, 723–732

9 Montixi, C., Langlet, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., Chauvin, J. P., Pierres, M. and He, H. T. (1998) Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. EMBO J. **17**, 5334–5348

10 Cheng, P. C., Dykstra, M. L., Mitchell, R. N. and Pierce, S. K. (1999) A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. J. Exp. Med. **190**, 1549–1560

11 Field, K. A., Holowka, D. and Baird, B. (1997) Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains. J. Biol. Chem. **272**, 4276–4280

12 Stauffer, T. P. and Meyer, T. (1997) Compartmentalized IgE receptor-mediated signal transduction in living cells. J. Cell Biol. **139**, 1447–1454

13 Lang, M. L., Shen, L. and Wade, W. F. (1999) Gamma-chain dependent recruitment of tyrosine kinases to membrane rafts by the human IgA receptor Fc alpha R. J. Immunol. **163**, 5391–5398

14 van Oers, N. S., Killeen, N. and Weiss, A. (1996) Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. J. Exp. Med. **183**, 1053–1062

15 van Oers, N. S., Killeen, N. and Weiss, A. (1994) ZAP-70 is constitutively associated with tyrosine-phosphorylated TCR zeta in murine thymocytes and lymph node T cells. Immunity **1**, 675–685

16 Brdicka, T., Cerny, J. and Horejsi, V. (1998) T cell receptor signalling results in rapid tyrosine phosphorylation of the linker protein LAT present in detergent-resistant membrane microdomains. Biochem. Biophys. Res. Commun. **248**, 356–360

17 Lin, J., Weiss, A. and Finco, T. S. (1999) Localization of LAT in glycolipid-enriched microdomains is required for T cell activation. J. Biol. Chem. **274**, 28861–28864

18 Zhang, W., Irvin, B. J., Trible, R. P., Abraham, R. T. and Samelson, L. E. (1999) Functional analysis of LAT in TCR-mediated signaling pathways using a LAT-deficient Jurkat cell line. Int. Immunol. **11**, 943–950

19 Liu, Y., Casey, L. and Pike, L. J. (1998) Compartmentalization of phosphatidylinositol 4,5-bisphosphate in low-density membrane domains in the absence of caveolin. Biochem. Biophys. Res. Commun. **245**, 684–690

20 Langlet, C., Bernard, A. M., Drevot, P. and He, H. T. (2000) Membrane rafts and signaling by the multichain immune recognition receptors. Curr. Opin. Immunol. **12**, 250–255

21 Watson, S. P. and Gibbins, J. (1998) Collagen receptor signalling in platelets: extending the role of the ITAM. Immunol. Today **19**, 260–264

22 Gibbins, J. M., Okuma, M., Farndale, R., Barnes, M. and Watson, S. P. (1997) Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. FEBS Lett. **413**, 255–259

23 Ezumi, Y., Shindoh, K., Tsuji, M. and Takayama, H. (1998) Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets. J. Exp. Med. **188**, 267–276

24 Briddon, S. J. and Watson, S. P. (1999) Evidence for the involvement of p59fyn and p53/56lyn in collagen receptor signalling in human platelets. Biochem. J. **338**, 203–209

25 Quek, L. S., Pasquet, J. M., Hers, I., Cornall, R., Knight, G., Barnes, M., Hibbs, M. L., Dunn, A. R., Lowell, C. A. and Watson, S. P. (2000) Fyn and Lyn phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. Blood **96**, 4246–4253

26 Gross, B. S., Lee, J. R., Clements, J. L., Turner, M., Tybulewicz, V. L., Findell, P. R., Koretzky, G. A. and Watson, S. P. (1999) Tyrosine phosphorylation of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets. J. Biol. Chem. **274**, 5963–5971

27 Pasquet, J. M., Bobe, R., Gross, B., Gratacap, M. P., Tomlinson, M. G., Payrastre, B. and Watson, S. P. (1999) A collagen-related peptide regulates phospholipase Cγ2 via phosphatidylinositol 3-kinase in human platelets. Biochem. J. **342**, 171–177

28 Gross, B. S., Melford, S. K. and Watson, S. P. (1999) Evidence that phospholipase Cγ2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor γ-chain after stimulation of the collagen receptor glycoprotein VI in human platelets. Eur. J. Biochem. **263**, 612–623

29 Watson, S., Berlanga, O., Best, D. and Frampton, J. (2000) Update on collagen receptor interactions in platelets: is the two-state model still valid? Platelets **11**, 252–258

30 Gao, J., Zoller, K. E., Ginsberg, M. H., Brugge, J. S. and Shattil, S. J. (1997) Regulation of the pp72syk protein tyrosine kinase by platelet integrin alpha IIb beta 3. EMBO J. **16**, 6414–6425

31 Shattil, S. J., Kashiwagi, H. and Pampori, N. (1998) Integrin signaling: the platelet paradigm. Blood **91**, 2645–2657

32 Judd, B. A., Myung, P. S., Leng, L., Obergfell, A., Pear, W. S., Shattil, S. J. and Koretzky, G. A. (2000) Hematopoietic reconstitution of SLP-76 corrects hemostasis and platelet signaling through alpha IIb beta 3 and collagen receptors. Proc. Natl. Acad. Sci. U.S.A. **97**, 12056–12061

33 Obergfell, A., Judd, B. A., del Pozo, M. A., Schwartz, M. A., Koretzky, G. A. and Shattil, S. J. (2001) The molecular adapter SLP-76 relays signals from platelet integrin alphaIIbbeta3 to the actin cytoskeleton. J. Biol. Chem. **276**, 5916–5923

© 2002 Biochemical Society

34 Dorahy, D. J., Lincz, L. F., Meldrum, C. J. and Burns, G. F. (1996) Biochemical isolation of a membrane microdomain from resting platelets highly enriched in the plasma membrane glycoprotein CD36. Biochem. J. **319**, 67–72

35 Dorahy, D. J. and Burns, G. F. (1998) Active Lyn protein tyrosine kinase is selectively enriched within membrane microdomains of resting platelets. Biochem. J. **333**, 373–379

36 Morton, L. F., Hargreaves, P. G., Farndale, R. W., Young, R. D. and Barnes, M. J. (1995) Integrin $\alpha2\beta1$-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for $\alpha2\beta1$-independent platelet reactivity. Biochem. J. **306**, 337–344

37 Cerny, J., Stockinger, H. and Horejsi, V. (1996) Noncovalent associations of T lymphocyte surface proteins. Eur. J. Immunol. **26**, 2335–2343

38 Motto, D. G., Ross, S. E., Wu, J., Hendricks-Taylor, L. R. and Koretzky, G. A. (1996) Implication of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production. J. Exp. Med. **183**, 1937–1943

39 Shattil, S. J., Hoxie, J. A., Cunningham, M. and Brass, L. F. (1985) Changes in the platelet membrane glycoprotein IIbIIIa complex during platelet activation. J. Biol. Chem. **260**, 11107–11114

40 Sims, P. J., Ginsberg, M. H., Plow, E. F. and Shattil, S. J. (1991) Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. J. Biol. Chem. **266**, 7345–7352

41 Pasquet, J. M., Gross, B., Quek, L., Asazuma, N., Zhang, W., Sommers, C. L., Schweighoffer, E., Tybulewicz, V., Judd, B., Lee, J. R. et al. (1999) LAT is required for tyrosine phosphorylation of phospholipase cγ2 and platelet activation by the collagen receptor GPVI. Mol. Cell. Biol. **19**, 8326–8334

42 Furuchi, T. and Anderson, R. G. (1998) Cholesterol depletion of caveolae causes hyperactivation of extracellular signal-related kinase (ERK). J. Biol. Chem. **273**, 21099–21104

43 Haimovich, B., Lipfert, L., Brugge, J. S. and Shattil, S. J. (1993) Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. J. Biol. Chem. **268**, 15868–15877

44 Leng, L., Kashiwagi, H., Ren, X. D. and Shattil, S. J. (1998) RhoA and the function of platelet integrin αIIbβ3. Blood **91**, 4206–4215

45 Ilangumaran, S., Briol, A. and Hoessli, D. C. (1997) Distinct interactions among GPI-anchored, transmembrane and membrane associated intracellular proteins, and sphingolipids in lymphocyte and endothelial cell plasma membranes. Biochim. Biophys. Acta **1328**, 227–236

46 Davies, A. and Lachmann, P. J. (1993) Membrane defence against complement lysis: the structure and biological properties of CD59. Immunol. Res. **12**, 258–275

47 Asazuma, N., Wilde, J. I., Berlanga, O., Leduc, M., Leo, A., Schweighoffer, E., Tybulewicz, V., Bon, C., Liu, S. K., McGlade, C. J. et al. (2000) Interaction of linker for activation of T cells with multiple adapter proteins in platelets activated by the glycoprotein VI-selective ligand, convulxin. J. Biol. Chem. **275**, 33427–33434

48 Surviladze, Z., Draberova, L., Kubinova, L. and Draber, P. (1998) Functional heterogeneity of Thy-1 membrane microdomains in rat basophilic leukemia cells. Eur. J. Immunol. **28**, 1847–1858

49 Miao, W. M., Vasile, E., Lane, W. S. and Lawler, J. (2001) CD36 associates with CD9 and integrins on human blood platelets. Blood **97**, 1689–1696

50 Green, J. M., Zhelesnyak, A., Chung, J., Lindberg, F. P., Sarfati, M., Frazier, W. A. and Brown, E. J. (1999) Role of cholesterol in formation and function of a signaling complex involving alphavbeta3, integrin-associated protein (CD47), and heterotrimeric G proteins. J. Cell Biol. **146**, 673–682

51 Krauss, K. and Altevogt, P. (1999) Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. J. Biol. Chem. **274**, 36921–36927

52 Torgersen, K. M., Vang, T., Abrahamsen, H., Yaqub, S., Horejsi, V., Schraven, B., Rolstad, B., Mustelin, T. and Tasken, K. (2001) Release from tonic inhibition of T cell activation through transient displacement of C-terminal Src kinase (Csk) from lipid rafts. J. Biol. Chem. **276**, 29313–29318

53 Bunnell, S. C., Diehn, M., Yaffe, M. B., Findell, P. R., Cantley, L. C. and Berg, L. J. (2000) Biochemical interactions integrating Itk with the T cell receptor-initiated signaling cascade. J. Biol. Chem. **275**, 2219–2230

54 Millan, J., Qaidi, M. and Alonso, M. A. (2001) Segregation of co-stimulatory components into specific T cell surface lipid rafts. Eur. J. Immunol. **31**, 467–473

55 Arcaro, A., Gregoire, C., Boucheron, N., Stotz, S., Palmer, E., Malissen, B. and Luescher, I. F. (2000) Essential role of CD8 palmitoylation in CD8 coreceptor function. J. Immunol. **165**, 2068–2076

56 Brass, L. F. (1985) Ca^{2+} transport across the platelet plasma membrane. A role for membrane glycoproteins IIB and IIIA. J. Biol. Chem. **260**, 2231–2236

57 Pelletier, A. J., Bodary, S. C. and Levinson, A. D. (1992) Signal transduction by the platelet integrin αIIb β3: induction of calcium oscillations required for protein-tyrosine phosphorylation and ligand-induced spreading of stably transfected cells. Mol. Biol. Cell. **3**, 989–998

58 Heemskerk, J. W., Hoyland, J., Mason, W. T. and Sage, S. O. (1992) Spiking in cytosolic calcium concentration in single fibrinogen-bound fura-2-loaded human platelets. Biochem. J. **283**, 379–383

Received 21 January 2002/1 March 2002; accepted 9 April 2002

© 2002 Biochemical Society