

Active Lyn protein tyrosine kinase is selectively enriched within membrane microdomains of resting platelets

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Circulating platelets are primed to respond very rapidly to thrombogenic stimuli, but most platelets complete their lifespan without ever becoming activated. Platelet activation is accompanied by waves of sequential tyrosine phosphorylation thought to involve members of the Src family of protein tyrosine kinases (PTKs). We show here that resting platelets contain highly active pp53/56^{Lyn} PTK within membrane microdomains (rafts) isolated biochemically with or without the use of detergent. This fraction is also greatly enriched in the transmembrane

glycoprotein CD36, known to associate with Lyn PTK, but in transfection studies we could find no evidence to suggest that CD36 affects the distribution or function of Lyn. Upon platelet activation Lyn activity remains constant or diminishes and pp60^{c-src} PTK within this fraction becomes highly activated, indicating the dynamic nature of the membrane microdomains. It is suggested that the function of active Lyn PTK in the resting platelet is to allow prolonged survival of this anucleate cell.

INTRODUCTION

Within the plasma membrane of many cell types, including platelets, there are specialized regions enriched in sphingolipids and cholesterol that also contain a selective enrichment of proteins, including, particularly, proteins engaged in signal transduction [1–6]. In cells expressing caveolin, these membrane microdomains are associated with structural invaginations called caveolae [7,8]. In common with some other haemic cells [4,9–11] platelets lack caveolin but contain biochemically identifiable membrane microdomains [6]. Analysis of a low-density light-refractive raft isolated by sucrose-density-gradient centrifugation of platelets lysed in Triton X-100 showed it to lack the morphological features of caveolae, but to be greatly enriched in cholesterol and to contain only selected membrane glycoproteins.

One such glycoprotein that was greatly and selectively enriched in this fraction was CD36 [6], a surface receptor molecule implicated in signal transduction in both platelets and monocytes [12–16]. Lisanti et al. [17] identified CD36 in caveolin-rich Triton X-100-insoluble rafts from endothelial cell-rich lung tissue and suggested that this finding indicated a caveolar localization for CD36. Clearly the identification of CD36 in the same fraction isolated from lysates of platelets lacking caveolin [6] casts doubt on a caveolar localization for CD36. Membrane microdomains themselves are implicated in cellular signalling [10,11,18,19], and CD36 from platelets has been shown to physically associate with the Src-related protein tyrosine kinases (PTKs) Fyn, Lyn and Yes [16]. In the present study we examined whether any of these PTKs co-localized with CD36 into membrane microdomains and, by use of a transfection system, whether CD36 influenced the subcellular localisation or catalytic activity of Lyn PTK.

Our findings indicate that CD36 partitions into the membrane microdomains of platelets independently of detergent extraction. Unexpectedly, it was found that although Lyn protein was not enriched within the fraction containing membrane microdomains, the kinase activity of Lyn within this fraction was greatly elevated in the presence or absence of calcium. In

transfection studies in COS-7 cells it was found that co-expression of CD36 with Lyn apparently altered neither the subcellular distribution nor the catalytic activity of the PTK. We also show that, in platelets, activation and aggregation induced by thrombin greatly increase the kinase activity of Src PTK within the membrane microdomain fraction, thereby indicating a dynamic nature for this structure.

EXPERIMENTAL

Materials

Prostaglandin E₁ (PGE₁), apyrase, Nonidet P40 (NP-40), leupeptin, soybean trypsin inhibitor, Triton X-100, ATP, iodoacetamide, enolase, β -mercaptoethanol and PMSF were from Sigma (St. Louis, MO, U.S.A.); Sepharose 2B and CL-4B were from Pharmacia (Uppsala, Sweden); Aprotinin was from USB (Cleveland, OH, U.S.A.); Nitrocellulose was from Sartorius (Göttingen, Germany); enhanced-chemiluminescence reagents were from DuPont (Australia); [γ -³²P]ATP was from Bresatec (Adelaide, S.A., Australia); horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Bio-Rad (Hercules, CA, U.S.A.); Rabbit anti-mouse IgG was from Dako (Carpinteria, CA, U.S.A.); bovine thrombin was from Armour Pharmaceutical Co. (Kankakee, IL, U.S.A.); Mes was from ICN (Aurora, OH, U.S.A.); partially purified pp60^{c-src} kinase was from Upstate Biotechnology (Lake Placid, NY, USA); Dulbecco's modification of Eagle's medium (DMEM) was from Trace Biosciences (Castle Hill, NSW, Australia); Hyperfilm-MP was from Amersham (Bucks., U.K.); BSA was from CSL (Parkville, Vic., Australia). All other unspecified chemicals were either from Ajax Chemicals (Auburn, N.S.W., Australia) or BDH (Kilsyth, Vic., Australia).

Polyclonal antibodies to pp60^{c-src} and pp53/56^{Lyn} were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.); anti-phosphotyrosine (PY20) and rabbit anti-caveolin were from Transduction Laboratories (Lexington, KY, U.S.A.); anti- β 3 (AP-3) and anti-CD4 (OKT4) were obtained from the American

Abbreviations used: IVK(A), *in vitro* kinase (assay); mAb, monoclonal antibody; MBS, Mes-buffered saline; pAb, rabbit polyclonal antibody; PGE₁, prostaglandin E₁; PRP, platelet-rich plasma; PTK, protein tyrosine kinase; RAM, rabbit anti-mouse IgG–Sepharose CL-4B beads; NP-40, Nonidet P40; DMEM, Dulbecco's modification of Eagle's medium.

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Type Culture Collection (Rockville, MD, U.S.A.); affinity-purified rabbit antibody to CD36 has been previously described [20].

Preparation of platelet-rich plasma (PRP), isolated platelets and platelet activation

PRP was recovered as described previously [6]. Platelets were isolated from PRP on to an albumin cushion using the method of Timmons and Hawiger [21].

Washed platelets [(0.8–1.0) × 10⁹ in 1.0 ml] were activated by addition of 0.1 unit/ml thrombin and gently rocked by hand in an Eppendorf tube (1.5 ml) at room temperature until aggregation was visible (45–90 s).

Platelet lysis

Washed resting platelets (8 × 10⁸–1 × 10⁹ platelets/ml) and activated samples were rapidly pelleted in a benchtop centrifuge at 4000 *g* for 60 s before addition of ice-cold lysis buffer [1% Triton X-100 in Mes-buffered saline (MBS); 150 mM NaCl/25 mM Mes (pH 6.5)/2 mM PMSF/20 mM iodoacetamide/2 μg/ml aprotinin/10 μg/ml soybean trypsin inhibitor], with or without 10 mM EGTA. Activated samples were homogenized by ≈ 15 strokes of a close-fitting Wheaton manual homogenizer. In some cases platelet samples were disrupted by sonication using a modification of the detergent-free method described by Song et al. [22]. Platelet suspensions in MBS buffer in the absence of Triton X-100 were disrupted using an MSE Soniprep 150 instrument (4 × 15 s pulses; 10 μm probe displacement).

Sucrose-density-gradient fractionation of platelet lysates and sonicates

Platelet lysates and sonicates (2.0 ml) were prepared by lysis or sonication and fractionated by sucrose-density-gradient centrifugation as described previously [6].

Platelet plasma-membrane isolation

Plasma membranes were recovered essentially as described by Barber and Jamieson [23]. Recovered plasma membranes were resuspended into detergent-free lysis buffer (see above) and fractionated through a discontinuous sucrose gradient as described above.

COS-7 cell culture, transfection and lysis

COS-7 cells were routinely maintained in DMEM plus 10% fetal-calf serum. Cells were transfected by electroporation (300 V, 250 μF; Bio-Rad Gene-Pulser) with either pEF-BOS, pEF-BOS.human Lyn, pEF-BOS.human CD36 or both pEF-BOS.human Lyn and pEF-BOS.human CD36. Transfected cells were resuspended into DMEM and maintained for 48 h to allow maximal protein expression. Transfected cells were washed *in situ* with PBS before addition of 2.0 ml of 1% Triton X-100 lysis buffer (see under 'Platelet lysis' above) to the flask for 60 min at 4 °C. Monolayers were scraped using a plastic cell scraper occasionally throughout and at the end of the lysis period. Cell lysate was recovered and processed through a continuous step sucrose gradient as described above.

In vitro kinase assay (IVKA)

Protein kinase activity within equal protein aliquots of each of the sucrose density-gradient fractions isolated from platelets or

COS-7 cells was determined by IVKA as previously described [24]. To assess kinase catalytic activity in gradient fractions, acid-denatured enolase was included in the assay buffer of some experiments. All samples analysed by SDS/PAGE were transferred to nitrocellulose. Phosphorylated proteins were revealed by autoradiography.

Immunoprecipitation of ³²P-radiolabelled insoluble raft material

The identity of major phosphorylated proteins in radiolabelled raft samples from whole platelet lysates were determined in one instance by immunoprecipitation. Kinase assay raft samples were diluted (typically 20-fold) with 1% Triton X-100 lysis buffer (see under 'Platelet lysis') plus 60 mM octyl glucoside. Lysates were precleared with a mixture of Protein A–Sephacel CL-4B and rabbit anti-mouse IgG–Sephacel CL-4B (RAM) beads (2 h end-over-end at 4 °C). Precleared lysates were then immunoprecipitated with a range of specific monoclonal antibodies (mAbs) and rabbit polyclonal antibodies (pAbs). Antigen–antibody conjugates were recovered by incubation with either Protein A (pAbs) or RAM (mAbs) beads. All samples were then washed three times with lysis buffer before addition of sample buffer and analysis by SDS/PAGE.

RESULTS

Selective enrichment of membrane CD36 into low-density light-refractive rafts does not require the presence of detergent

We have demonstrated previously that plasma-membrane CD36 is greatly and selectively enriched in membrane microdomains isolated from resting platelets by sucrose-density-gradient centrifugation of platelets solubilized in Triton X-100 [6]. To determine whether the selective partitioning was dependent upon detergent, we fractionated platelets disrupted by sonication in the complete absence of detergent. Analysis of sucrose density gradients produced from such experiments showed CD36 to be selectively enriched into low-density rafts biochemically and visually identical with those isolated from detergent-lysed platelets (results not shown). We further established, using detergent-free hypotonic lysis of platelets and plasma-membrane isolation, that CD36 derived from the plasma membranes exclusively partitioned into the low-density raft fractions (results not shown).

Triton X-100-insoluble rafts contain highly active ppp53/56^{Lyn} PK

Having established that the Triton X-100 raft material was not an artefact of detergent solubilization and appears to represent a *bona fide* membrane microdomain, this fractionation system was employed to investigate whether any kinase activity preferentially associated with these membrane microdomains. An IVKA was carried out on each of the fractions obtained by sucrose-density-gradient fractionation of platelets lysed in Triton X-100. Autoradiography of SDS/PAGE gels of the gradient profile revealed a very high kinase activity to be present within the insoluble raft material (Figure 1A). The high level of this kinase activity was reflected by the exposure times required to reveal the phosphoproteins (varying between 5 and 10 min in several separate analyses). In contrast, faint phosphoprotein bands outside the Triton X-100 insoluble rafts only became apparent after at least 4 h of exposure and these phosphoproteins were of different *M_r*. The catalytic activity observed across the gradient, analysed using enolase as a substrate in the IVKA reaction, reflected the distribution of phosphoprotein (Figure 1B). Thus ≈ 60% of the total kinase activity was contained within the Triton X-100-insoluble raft material. In separate experiments the distribution

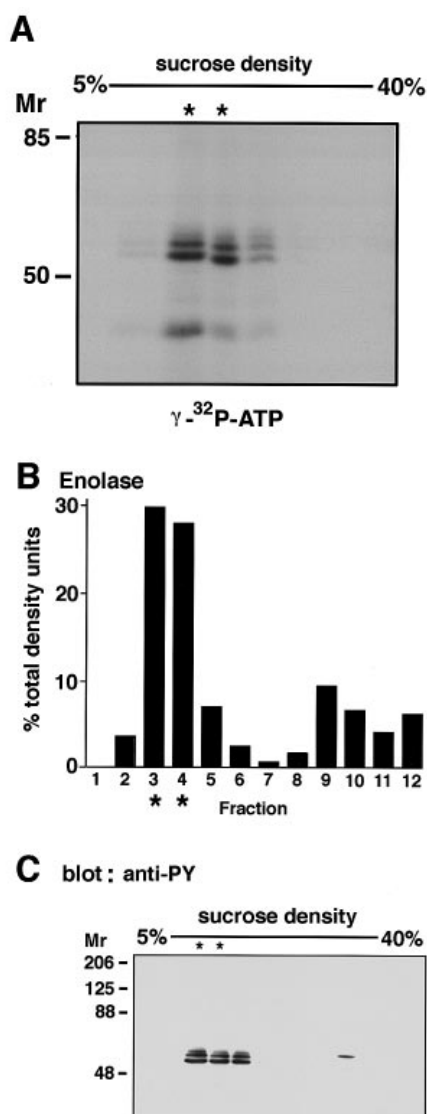


Figure 1 Low-density light-refractive rafts isolated from resting platelets are greatly enriched in kinase activity

(A) Equal protein-gradient fractions ($n = 12$) were subjected to IVKA, analysed by reduced SDS/PAGE and phosphoproteins revealed by autoradiography for 5–30 min. Fractions bearing an asterisk (*) are insoluble raft material. M_r markers are shown at the left (values are $10^{-3} \times M_r$). (B) Tyrosine kinase catalytic activity of gradient fractions was determined by inclusion of acid-denatured enolase in the IVKA, and labelled proteins were processed as described above. Enolase phosphorylation was quantified by NIH Image Software Version 1.57. Data are expressed as percentages of total density units. Fractions bearing an asterisk (*) are insoluble raft material. (C) Equal protein aliquots ($n = 12$) from a continuous sucrose density gradient of resting platelets were trichloroacetic acid-precipitated and analysed by reducing SDS/PAGE, transferred to nitrocellulose and immunoblotted with antibody against phosphotyrosine. Fractions bearing an asterisk (*) are insoluble raft material. All experiments shown were repeated on at least three occasions with essentially the same result.

of phosphotyrosine-containing proteins across the gradient was also analysed by Western blotting. These also showed a great enrichment in the raft material (Figure 1C).

It was considered that the very high kinase activity obtained in the experiments above might be an artefact caused by the isolation procedure, since Earp et al. [25] have reported that Triton X-100 can activate PTKs in the isolated plasma membranes of some cell types. Raft material was therefore isolated

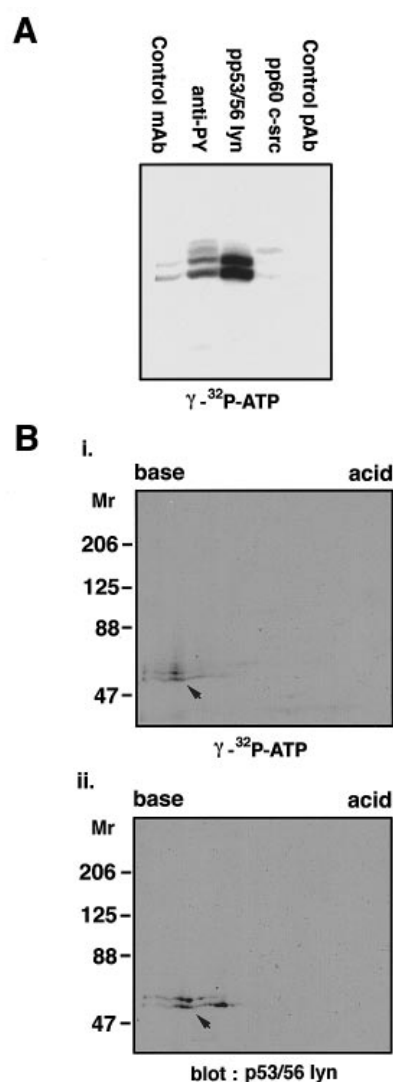


Figure 2 Identification of the major phosphoprotein bands present in the insoluble raft material as pp53/56^{lyn}

(A) Insoluble raft material was subjected to radiolabelled IVKA and diluted with Triton X-100 lysis buffer containing 60 mM octyl glucoside. Equal-volume samples were immunoprecipitated using antibodies against pp53/56^{lyn}, phosphotyrosine and pp60^{c-src}. Precipitated material was analysed by SDS/PAGE, and radiolabelled phosphoproteins were revealed by autoradiography. (B, i and ii) A 1 μ g portion of insoluble raft material from resting platelets was subjected to IVKA, separated by two-dimensional SDS/PAGE and proteins electrophoretically transferred to nitrocellulose. Radiolabelled phosphoproteins were revealed by autoradiography and pp53/56^{lyn} was identified by immunoblotting. M_r markers are shown on the left (values are $10^{-3} \times M_r$). Both experiments shown were repeated on at least two occasions with the same result.

from platelets using the sonication method described above for CD36 analysis and the material analysed by IVKA in the presence or absence of Triton X-100. High kinase activity was found to be enriched within this fraction, and this activity was not influenced by the addition of 1% Triton X-100 (results not shown). The possibility that the enhanced kinase activity was a consequence of either sucrose concentration or an uneven distribution of tyrosine phosphatases was also excluded by reciprocating the sucrose concentrations in the raft fractions and high-density fractions after centrifugation and before the kinase assay and by carrying out the kinase assays in the presence of

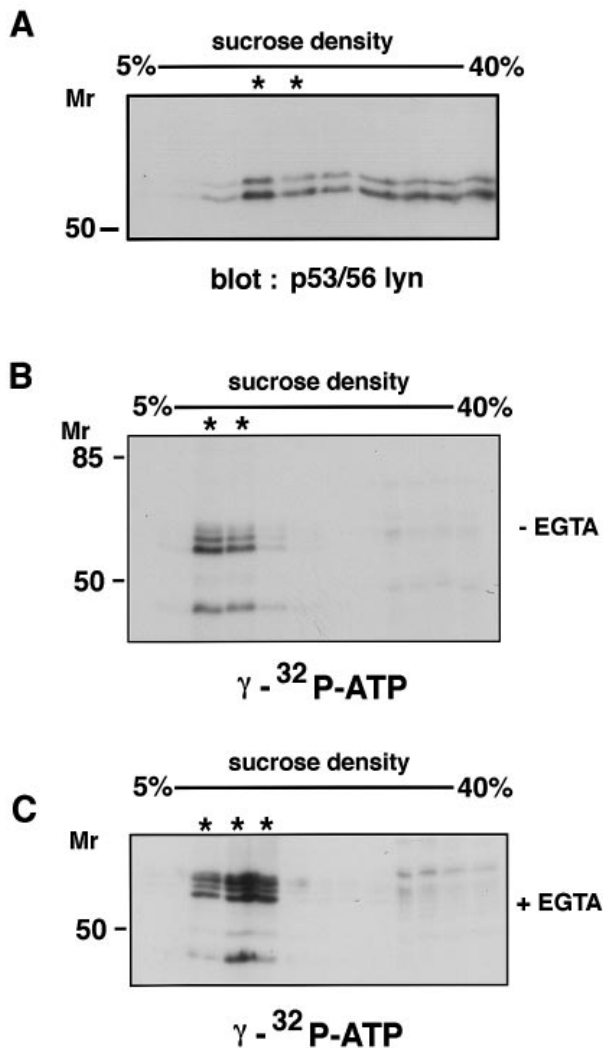


Figure 3 pp53/56^{Lyn} is catalytically enriched, but not selectively partitioned, into the insoluble raft fraction

Equal protein aliquots from sucrose density gradients of two identical donor were analysed for either (A) the distribution of pp53/56^{Lyn} or (B and C) radioactive IVK activity (with or without EGTA). M_r markers are shown at the left of the autoradiographs (values are $10^{-3} \times M_r$). Fractions bearing an asterisk (*) are insoluble raft material. These experiments were repeated for five separate individual donors.

tyrosine phosphatase inhibitors. Kinase activity remained high within the raft fraction, and no induction of kinase activity was seen in the soluble protein fraction regardless of manipulation (results not shown). We also considered the possibility that, in the presence of bivalent cations, tyrosine kinases within this fraction might undergo enhanced autophosphorylation. IVKA of platelet density-gradient fractions from platelets lysed in the presence of EGTA also showed an enrichment of kinase activity across the gradient (see Figure 3C). However, under these conditions it was found that an additional higher- M_r (≈ 60000) band was more prominent than in fractions lysed in the absence of EGTA.

Immunoprecipitation was carried out in an effort to identify the phosphotyrosine bands within the raft material. These experiments suggested that the two major phosphoprotein bands present within this fraction were pp53/56^{Lyn} (Figure 2A). To

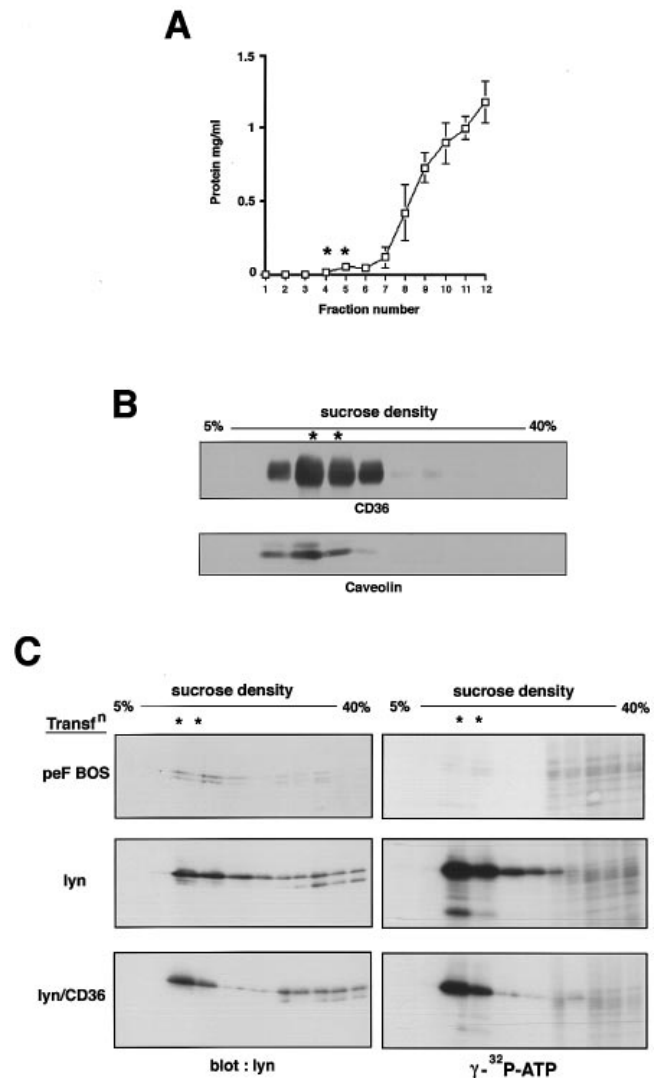


Figure 4 CD36-transfected COS-7 cells selectively partition CD36 into a caveolin-rich insoluble fraction: CD36 does not regulate Lyn distribution or kinase activity in fractionated COS-7 cells

(A) Protein distribution of COS-7 cell lysate fractionated through a continuous sucrose density gradient. Error bars represent the S.D. between cells transfected with pEF.BOS, pEF.BOS.human CD36, pEF.BOS.human Lyn and both pEF.BOS.human Lyn and pEF.BOS.human CD36. Fractions bearing an asterisk (*) are insoluble raft material. (B) COS-7 cells transiently transfected with pEF.BOS.human CD36 were fractionated by sucrose-density-gradient centrifugation and analysed for the distribution of CD36 and caveolin by immunoblotting. Fractions bearing an asterisk (*) are insoluble raft material. (C) COS-7 cells transiently transfected with either pEF.BOS, pEF.BOS.human Lyn or both pEF.BOS.human Lyn and pEF.BOS.human CD36 were lysed and fractionated as described in (A). A radioactive IVKA was performed, and labelled phosphoproteins were analysed by reducing SDS/PAGE. The distribution of Lyn was determined by immunoblotting.

confirm this, a two-dimensional SDS/PAGE analysis was carried out. Western-blot analysis of ³²P-labelled material with an antibody against Lyn demonstrated that the Lyn bands precisely overlaid the major phosphoprotein bands (Figure 2B, i and ii).

CD36 does not influence the distribution or kinase activity of Lyn-transfected COS-7 cells

Having established the identity of two of the phosphoprotein bands within this fraction as Lyn, the distribution of Lyn across

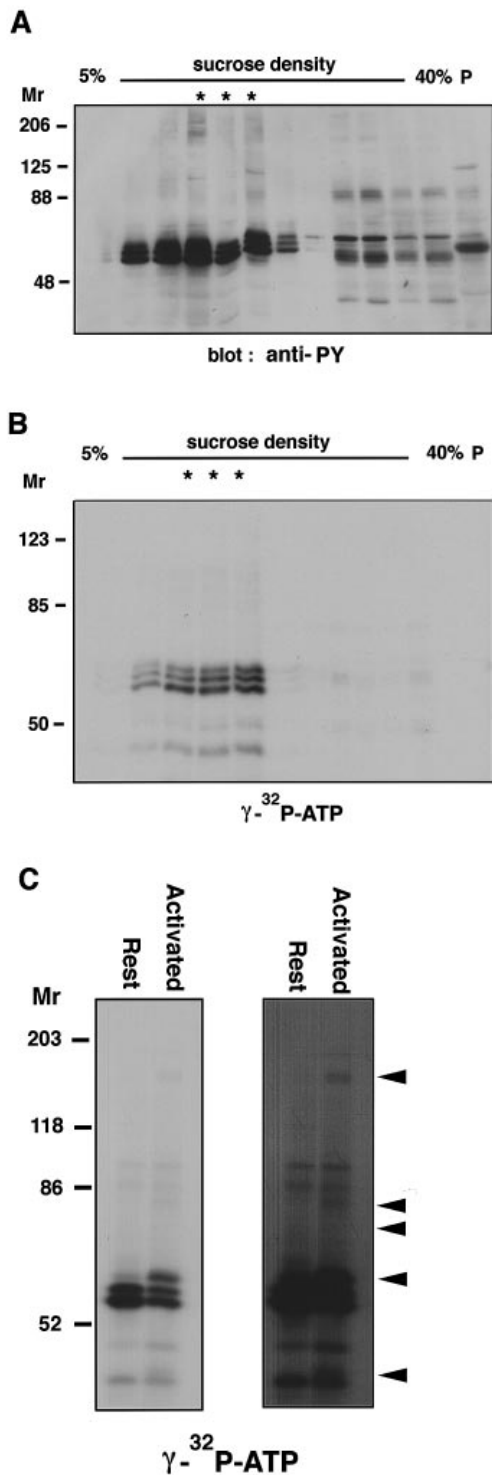


Figure 5 Platelet activation alters the IVK and phosphotyrosine profile of the insoluble raft material, and novel phosphoproteins are evident in raft fractions

(A) Equal protein aliquots from activated, aggregated platelets were recovered from sequential fractions of a continuous sucrose gradient ($n = 12$), a radioactive IVKA was performed and labelled phosphoproteins were analysed by reducing SDS/PAGE and revealed by autoradiography. M_r markers are shown on the left (values are $10^{-3} \times M$). Fractions bearing an asterisk (*) are insoluble raft material. P is a hard insoluble pellet at the base of the column run in total. (B) Equal protein aliquots of trichloroacetic acid-precipitated gradient fractions of activated/aggregated platelets ($n = 12$) were analysed by reducing SDS/PAGE, transferred to nitrocellulose and probed with antibody to phosphotyrosine. M_r markers are shown at the left

the entire gradient was analysed by Western blot. In agreement with our previous findings [6], Lyn was distributed across the gradient and was not enriched within the raft material (Figure 3A); however, IVKA of these same fractions showed that only within the raft material did the Lyn undergo autophosphorylation (Figure 3B) and that this occurred in the presence or absence of bivalent cations (Figure 3C). Lyn has been shown to associate with CD36 in platelets [16] and, since most CD36 is contained within the raft material [6], the active Lyn PTK is possibly associating with CD36 in the membrane microdomains. This raised the possibility that association with CD36 may influence the distribution and/or kinase activity of Lyn. We investigated this by transient transfection of Lyn and CD36 into COS-7 cells. COS-7 cells that lack endogenous CD36 expression (results not shown) were transfected with CD36 and its distribution analysed by sucrose-gradient analysis, exactly as had been carried out on platelet lysates. It was found that, as in platelets [6], the raft material contains a very small proportion of total cellular protein (Figure 4A) and the expressed CD36 was greatly enriched into insoluble, light-refractive raft material (Figure 4B). COS-7 cells also contain endogenous caveolin, and reprobing of the same filters with anti-caveolin antibody showed coincident enrichment of both proteins into the insoluble raft material (Figure 4B). In mock-transfected COS-7 cells immunoblotted for Lyn, endogenous Lyn was detected in very low amounts, and IVKA across the gradient showed little or no phosphoprotein bands. Upon transfection with human Lyn, immunoblotting showed the expressed protein to be distributed across the gradient. IVKA of the same fractions showed that the Lyn within the raft material was highly autophosphorylated (Figure 4C). Co-transfection of the COS-7 cells with both human Lyn and human CD36 did not alter either the distribution or autophosphorylation profile of Lyn (Figure 4C). From these experiments it can be concluded that the localization of Lyn into Triton X-100-insoluble raft material and its increased catalytic activity occurs independently of CD36 in transfected COS-7 cells.

Activation-dependent changes to the kinase profile in membrane microdomains of platelets.

Routine phosphotyrosine profile analysis of resting and activated platelet lysates established that the platelet-isolation procedure did indeed result in the preparation of resting platelets. This assessment was based upon the marked difference between resting and activated platelet phosphotyrosine profiles [26,27]. Because of the greatly increased phosphotyrosine phosphorylation seen after activation of the platelets with thrombin, we decided to analyse the distribution of these phosphoproteins in the gradient system. As shown in Figure 5(A), the additional phosphotyrosine bands were distributed across the gradient with only bands corresponding to those in the position of the Src-related kinases being highly prominent within the Triton X-100-insoluble raft material. In over ten such experiments this light-refractive band obtained from sucrose-density-gradient centrifugation of lysates of activated platelets, lysed in the presence or absence of EGTA,

(values are $10^{-3} \times M$). Fractions bearing an asterisk (*) are insoluble raft material. P is a hard insoluble pellet at the base of the column run in total. The experiment was repeated on five occasions with essentially the same result. (C) Radioactive IVKA was performed on insoluble raft material derived from activated, aggregated pellets, labelled proteins were separated by reducing SDS/PAGE, and phosphoproteins were revealed by autoradiography. Additional phosphoproteins evident after stimulation are arrowed beside a computer-enhanced image. M_r markers are shown at the left (values are $10^{-3} \times M$). These experiments are representative of seven separate experiments using different donors for each analysis.

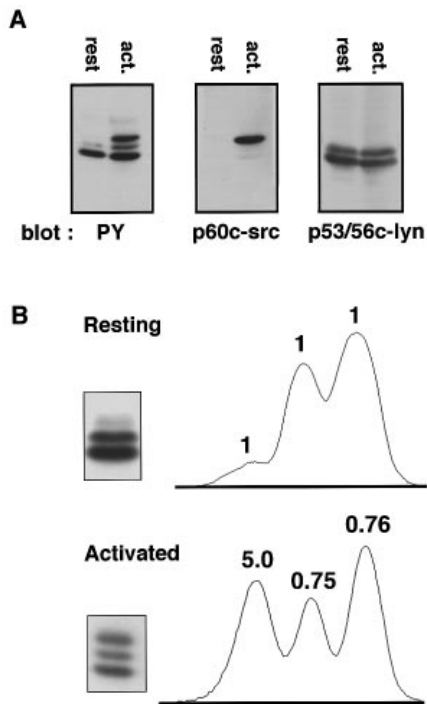


Figure 6 pp60^{c-src} kinase activity is up-regulated in activated platelet rafts and pp53/56^{Lyn} is down-regulated

(A) Activated/aggregated and resting platelets were lysed and fractionated through a discontinuous sucrose gradient. Insoluble raft material was recovered from the 5%/30%-sucrose interface, proteins precipitated, analysed by SDS/PAGE and immunoblotted with antibodies against phosphotyrosine, pp60^{c-src} and pp53/56^{Lyn}. Reactive proteins were detected by enhanced chemiluminescence and revealed by autoradiography. (B) Platelets were processed as described in (A) and a radioactive IVKA was performed on equal protein aliquots of raft material. After analysis by reducing SDS/PAGE and revelation by autoradiography, radiolabelled bands were quantified using NIH Image Software Version 1.57. Numbers above individual peaks represent the change relative to resting peaks where these are given the arbitrary value of 1.0.

was consistently more diffuse and appeared to migrate at a higher density than that obtained from resting platelet lysate (compare Figure 1C with Figure 5A).

Next, the kinase activity across the sucrose gradient of activated platelets was analysed. It was found that after short (5–10 min) exposure of the autoradiograph obtained after IVKA reaction, phosphoprotein bands were largely restricted to the Triton X-100-insoluble raft material (Figure 5B). This profile was similar to that obtained from resting platelets, except that in addition to the two characteristic Lyn bands there was a strong band at $M_r \approx 60000$ (Figures 5B and 5C). Longer exposure of these autoradiographs revealed the presence of several additional phosphoprotein bands that appear to be restricted to activated platelets (Figure 5C). This shift in the profile obtained from activated platelets versus resting platelets confirms that the active Lyn PTK found in the rafts from resting platelets was not an artefact resulting from *in vitro* platelet manipulation.

Src kinase activity is known to increase upon platelet activation [28], and the position of the upper band of the triplet of phosphoproteins seen in the rafts of activated platelets suggested that it might be pp60^{c-src}. That the top band was indeed Src was confirmed by immunoprecipitation (results not shown) and by immunoblotting of resting and activated insoluble raft material (Figure 6A). When platelets were lysed in the presence of EGTA

after activation, this same 60000- M_r Src band also showed markedly greater phosphorylation (results not shown).

When platelet aggregation was allowed to occur after thrombin stimulation, this shift in the raft kinase profile was consistently observed and, in addition to the increased abundance of phosphorylated c-Src in this fraction, there was generally observed to be a slight decrease in the autophosphorylated Lyn (Figure 6B).

DISCUSSION

In this paper we demonstrate that the Lyn PTK contained within the membrane microdomains isolated from resting platelets contains greatly increased catalytic activity. Upon platelet activation, the composition of activated kinases alters such that Lyn activity decreases slightly with a concomitant 5-fold increase in Src activity.

Accumulating evidence argues persuasively that detergent-insoluble low-density complexes are reflections of the native distribution of plasma-membrane proteins representing rafts of membrane microdomains [19] and do not represent an artefact caused by detergent lysis as has been suggested [29]. In the present study we used a detergent-free method [22] to isolate the membrane microdomains of resting platelets, and our findings from this method confirmed our previous results using Triton X-100 in showing that CD36 was greatly and selectively enriched within the floating raft material [6].

The degree of the increased Lyn kinase activity in the platelet rafts is highlighted by the relatively minor representation of Lyn kinase within the raft material compared with the Lyn protein found outside this fraction. Less than 5% of total platelet protein isolates into the raft material [6], but over 60% of detectable catalytic activity is found within this fraction (Figure 3B). Only after platelet activation and aggregation did kinase activity within the membrane rafts change. Src activity was greatly enhanced, whereas Lyn activity was decreased. The implication of this observation is that each enzyme performs a distinct non-redundant role.

Lyn protein was distributed across the gradient with only the catalytic activity being greatly increased within the microdomains. It is possible that the Lyn that partitions into this fraction is physically altered, for example by palmitoylation, in a way that alters its catalytic function. This remains to be investigated. Another possibility is that other proteins or lipids that differentially partition into the membrane microdomains regulate Lyn activation. Despite the demonstrated association of CD36 with pp53/56^{Lyn} [16], we found that CD36 did not influence either the distribution or kinase activity of Lyn in transfected COS-7 cells. However, treatment of the raft material with lipase and some detergents abrogated all kinase activity (D. J. Dorahy and G. F. Burns, unpublished work), suggesting that either the ternary structure of the rafts or an associated molecule(s) displaced by the solubilization procedure maintains Lyn kinase activity. The latter point may be one reason why enhanced kinase activity has not been described in platelet lysates, or indeed in solubilized caveolae from other cell types.

Other likely candidates for the regulation of Lyn kinase activity are the kinases and phosphatases for which Lyn is a substrate. The catalytic activity of Src family kinases is tightly regulated by tyrosine phosphorylation and dephosphorylation [30]. Csk, a non-receptor type PTK, has been shown to phosphorylate the negative regulatory tyrosine at the C-terminus of Src family kinases and to suppress their kinase activity [31–33]. In preliminary work we have found by immunoblotting that the Csk protein does not partition into the Triton X-100-insoluble

raft material, but remains dispersed across the soluble fractions (D. J. Dorahy, unpublished work). However, recent elegant work by Hirao [34] suggests that a more likely candidate for the regulation of CD36-associated Lyn is the Csk homologous kinase Chk. Our findings with platelets reported here appear to be at odds with the work of Rodgers and Rose [11]. These authors found that the Src family member Lck was distributed into both the Triton X-100-insoluble and -soluble fractions when lysates of Jurkat T cells were fractionated through sucrose gradients. However, in this case the Lck within the insoluble fraction contained greatly reduced kinase activity compared with that in the soluble fraction, and evidence was presented to suggest that exclusion of the tyrosine phosphatase CD45 from the Triton X-100-insoluble microdomains prevented Lck within these domains becoming activated. Whether these different results obtained with Jurkat cells reflect true differences between these lymphocytes and platelets, or perhaps variations in the partitioning of active Lck and Lyn, or both, is not clear. However, there may also be technical differences that may account for the variant findings. Rodgers and Rose [11] solubilized their Triton X-100-insoluble raft material in octyl glucoside prior to immunoprecipitation and IVKA; as noted above, we found that solubilization of this fraction obtained from platelets killed the kinase activity of the Lyn. These technical differences may be significant. In a study of the high-affinity IgE receptor (FcεR1) in RBL-2H3 cells, Field et al. [10] found that the receptor had first to partition into detergent-resistant membrane domains before it became tyrosine-phosphorylated by Lyn, and that, in this system, the associations between the FcεR1 and Lyn were highly sensitive to low concentrations of detergent.

An emerging role for active Lyn PTK is as an inhibitor of apoptosis in mature end cells, specifically neutrophils and eosinophils [35,36]. It has also been reported that active Lyn PTK isolated from human neutrophils co-partitions with caveolin in a Triton X-100-insoluble fraction [37]. While platelets have not been recognized as undergoing apoptosis, this form of cell death can occur in anucleate cells [38]. We would suggest, therefore, that the active Lyn PTK contained within the membrane microdomains of resting platelets and reported here may play an intimate role in the survival of circulating platelets, and in this regard it can be noted that Lyn-deficient mice suffer from thrombocytopenia that is not due to primary marrow failure [39].

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