Activation of integrin-β_3-associated syk in platelets

Sibaji SARKAR*, Michael M. ROONEY† and Susan T. LORD*†‡[1]

*Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC 27599, U.S.A., †Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599, U.S.A., and ‡Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC 27599, U.S.A.

Published data suggest that the tyrosine kinase syk participates in platelet signalling through the integrin $\alpha_{IIb}\beta_3$. Our data show an association of syk and integrin β_3 in immunoprecipitates from unstimulated and stimulated platelets. We detected syk in anti-β_3 precipitates and, conversely, β_3 in anti-syk precipitates. *In vitro* kinase assays with anti-β_3 precipitates demonstrated that syk activity was enhanced in ADP-stimulated platelets.

Key words: integrin $\alpha_{IIb}\beta_3$, signal transduction, tyrosine kinase.

INTRODUCTION

Platelet activation is the first step of platelet aggregation. In the presence of an agonist such as ADP, thrombin, collagen or epinephrine, platelets are activated. One of the important consequences of platelet activation is alteration of the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ to allow fibrinogen binding, in a process called 'inside-out' signalling. This signal is probably mediated by phosphorylation, as several cellular kinases, including src, syk, Vav and phosphoinositide 3-kinase, are activated by platelet activation [1–3].

Fibrinogen binding to activated integrin $\alpha_{IIb}\beta_3$ mediates platelet aggregation. The events subsequent to fibrinogen binding are called 'outside-in' signalling. Results from different laboratories have demonstrated that syk and phosphoinositide 3-kinase are activated after the engagement of integrin $\alpha_{IIb}\beta_3$, even in the absence of agonist [4,5]. Syk was activated when fibrinogen was allowed to bind to stirred platelets treated with an integrin-$\alpha_{IIb}\beta_3$-activating antibody, LIBS6 [4]. The role of syk in outside-in signalling has been further documented in CHO cells transfected with integrin $\alpha_{IIb}\beta_3$ and syk. Syk activation in such cells was dependent on fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ [6]. Truncation of the cytoplasmic domain of either the α_{IIb} or the β_3 subunit abolished syk activation [6]. These results suggest that syk is involved in both inside-out and outside-in signalling in platelets.

Results from two other laboratories have suggested a close relationship between integrin engagement, specifically the β subunit, and syk activation. Yan et al. [7] demonstrated the association of integrin β_2 with syk in monocytes, and Gotoh et al. [8] found that antibody-induced cross-linking of integrin β_1 activates syk in myeloid cells.

If syk is involved in integrin-$\alpha_{IIb}\beta_3$-mediated signalling, it should associate with the β_3 subunit of this receptor (henceforth called β_3), at least in the early stages of platelet aggregation. To test this hypothesis, we examined platelet lysates for β_3–syk complexes. Using immunoblot analysis of immunoprecipitates from platelet lysates, we demonstrated that syk associates with β_3 in unstimulated platelets, as well as in platelets stimulated with either ADP or thrombin in the presence of fibrinogen. At present, we do not know whether this association is direct or is mediated by any other intermediary molecule. We performed *in vitro* autokinase assays with β_3 immunoprecipitates and found that autophosphorylation of syk associated with β_3 was enhanced in response to platelet aggregation.

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EXPERIMENTAL

Materials

A monoclonal antibody to β_3, AP-3, used for immunoprecipitation was obtained from a previously described cell line [9] supplied by Dr. Peter J. Newman (Blood Institute, Milwaukee, WI, U.S.A.). A polyclonal antibody to β_3, called Fire and Ice, used for immunoprecipitation and Western analysis was a gift from Dr. Peter J. Newman [10]. The polyclonal antibodies to syk used in Western analysis and immunoprecipitation were purchased from Upstate Biotech and Santa Cruz Biotech respectively. A monoclonal antibody to syk used for immunoprecipitation and the SuperSignal Chemiluminescent Substrate for Western blot detection were purchased from Pierce. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse secondary antibodies and purified human plasma fibrinogen were purchased from Calbiochem. Protein A/G-conjugated agarose beads were from Santa Cruz, and PVDF membrane was from Millipore. Piceatannol was purchased from Biomol Research Laboratories. Human α-thrombin was a gift from Dr. Frank Church (University of North Carolina, Chapel Hill, NC, U.S.A.). ADP was purchased from Chrono-log. [γ-^{32}P]ATP was purchased from NEN. All other chemicals, including isotype-matched control antibodies, were purchased from Sigma.

Isolation and purification of human platelets

Platelets were purified as described [11] from the whole blood of volunteers who had abstained from using aspirin for at least 10 days. Briefly, platelet-rich plasma was obtained by centrifugation of the collected blood at 200 ***g*** for 25 min at ambient temperature. Platelets were pelleted from the supernatant by centrifugation at 800 ***g*** for 20 min at ambient temperature. Platelets were re-suspended in Tyrode's buffer, pH 7.2 [10 mM Hepes, 135 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2% (w/v) BSA (fraction V; Miles Pentex), and passed through a

Abbreviations used: β_3, integrin β_3 subunit; TBST, Tris-buffered saline/Tween; HRP, horseradish peroxidase.

[1] To whom correspondence should be addressed: Department of Pathology and Laboratory Medicine, CB# 7525, Brinkhous-Bullit Bldg, University of North Carolina, Chapel Hill, NC 27599–7525, U.S.A. (e-mail stl@med.unc.edu).

Sepharose CL-2B column equilibrated with Tyrode's buffer. The platelets were eluted in the same buffer and the density was measured using a Coulter Counter. Platelets were diluted to 5×10^8 platelets/ml in Tyrode's buffer containing 1 mM Ca^{2+} and 2 mM Mg^{2+}, and maintained at 37 °C.

Platelet aggregation

Platelets were incubated at 37 °C at a final concentration of 1×10^8 platelets per 0.5 ml in cuvettes in a Chrono-log aggregometer. Aggregation was initiated by the stepwise addition of 300 nM human plasma fibrinogen and agonist (10 μM ADP or 0.5 unit/ml thrombin), followed by stirring. Aggregation was monitored as the increase in light transmission [11], and the aggregation reaction was stopped after 20 s by the addition of 125 μl of ice-cold 5 × lysis buffer (see below).

Preparation of Triton-soluble fractions

Triton-soluble fractions were prepared, as modified from the method of Clark and Brugge [12], in 1 × lysis buffer [20 mM Tris/HCl, pH 7.4, 1% (v/v) Triton X-100, 5 mM EGTA, 4 μg/ml leupeptin, 100 μg/ml PMSF, 4 μg/ml aprotinin and 1.2 mM sodium vanadate]. Unstimulated platelets (incubated at 37 °C with no addition and no stirring) or aggregated platelets (500 μl) were added to 125 μl of ice-cold 5 × lysis buffer, mixed by gentle shaking and kept on ice for 1 h. The sample was centrifuged at 15000 ***g*** for 10 min at 4 °C, and the supernatant was collected; this fraction is the Triton-soluble fraction, which contains both the cytoplasmic and membrane-associated proteins. All fractions were stored at −70 °C.

Immunoprecipitation

Triton-soluble fractions were thawed on ice. Protein A/G–agarose beads were washed with 1 ml of ice-cold PBS. Portions of 40 μl of beads were added to each fraction and tumbled for 1 h at 4 °C. After centrifugation at 1000 ***g*** for 20 s, the precleared supernatant was collected. Primary antibody was added and the sample was tumbled overnight at 4 °C. Washed Protein A/G beads (40 μl) were added, and the sample was tumbled for 1 h at 4 °C. After centrifugation at 1000 ***g*** for 1 min, the supernatant was removed. The beads, which retain the immunoprecipitate, were washed four times with 1 ml of ice-cold 1 × lysis buffer. The washed beads were resuspended in 40 μl of 2 × reducing SDS/PAGE sample buffer [0.125 M Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.1% (w/v) Bromophenol Blue], boiled for 5 min and centrifuged (500 ***g*** for 20 s), and the supernatants were applied to 10% (w/v) polyacrylamide gels for SDS/PAGE [13]. For β_3 immunoprecipitations, 7.5 μg of an anti-β_3 monoclonal antibody, an anti-β_3 polyclonal antibody or a respective isotype-matched control antibody was used. For syk immunoprecipitation, 4 μg of an anti-syk monoclonal antibody, 1 μg of an anti-syk polyclonal antibody or 4 μg of an isotype-matched mouse antibody was used.

Western blots

Samples in reducing SDS sample buffer were boiled for 5 min, run on SDS/10%-PAGE and transferred to a PVDF membrane. After blocking with Tris-buffered saline/Tween [TBST; 50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.01% (v/v) Tween-20] containing 5% (w/v) non-fat dried milk, the membrane was incubated overnight at 4 °C with the primary antibody diluted in 3% (w/v) non-fat dried milk in TBST. After washing three times with TBST, the membrane was incubated for 1 h with goat anti-rabbit secondary antibody conjugated to HRP at 1:10000 dilution in 3% (w/v) non-fat dried milk/TBST. After washing three times with TBST, the signal was developed with the Super Signal Chemiluminescent Substrate (Pierce) for 5 min. Films were exposed for between 10 s and 2 min before being developed as described in the legends to the Figures. For syk immunodetection, 1 μg/ml anti-syk polyclonal antibody was used. For β_3 immunodetection, 10 μg/ml polyclonal anti-β_3 antibody [10] was used.

In vitro kinase assay

The incorporation of radiolabelled phosphate into β_3 immunoprecipitates was measured as described in [14,15], with minor modifications. In brief, after washing with lysis buffer, the immunoprecipitates, prepared as described above, were washed with 1 ml of ice-cold kinase buffer (50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 10 μM sodium vanadate) and resuspended in 15 μl of ice-cold kinase buffer. The kinase reaction was started by the addition of 15 μl of ice-cold kinase buffer containing 4 μM ATP and 10 μCi of [γ-^{32}P]ATP. The mixture was placed at 30 °C in a water bath. After 2 min, the reaction was stopped by centrifugation for 20 s. The supernatant was discarded and the beads were resuspended in 40 μl of 2 × reducing SDS/PAGE sample buffer, boiled for 5 min, pelleted by centrifugation (500 ***g*** for 20 s), and subjected to SDS/10%-PAGE. The proteins were transferred to a PVDF membrane, and an autoradiogram was developed. The membrane was then subjected to Western analysis for syk, as described above. In order to quantify the differences in autophosphorylation, the images in the blots were scanned using the program IS 2000 from Alpha Innotec.

RESULTS

Association of β_3 and syk in platelets

To examine whether the intracellular tyrosine kinase syk associates with β_3, we immunoprecipitated β_3 from Triton-soluble fractions of platelets and analysed the precipitates in immunoblots probed with an antibody to syk. As shown in Figure 1, a 72 kDa protein was detected in immunoprecipitates prepared with either monoclonal antibody AP-3 (Figure 1A, lanes 1–3) or a polyclonal antibody to β_3 (Figure 1B, lanes 1–3). The mobility of this band corresponded to that seen in syk immunoprecipitates (Figures 1A and 1B, lane 4). This co-precipitation of syk was specific, as shown in parallel immunoprecipitations with isotype-matched antibodies (Figure 1C). Comparison of the AP-3 precipitate (lane 3) with the isotype-matched mouse IgG precipitate (lane 1) and of the polyclonal anti-β_3 precipitate (lane 4) with the isotype-matched rabbit IgG (lane 2) showed that the intensity of the 72 kDa band was greater with the integrin-specific antibodies. The 10 s exposure time was chosen to minimize background, and emphasizes the differences between lanes 1 and 2, relative to lanes 3 and 4 (Figure 1C). These parallel immunoprecipitations demonstrated that the 72 kDa protein was specific to the β_3 immunoprecipitates. A Western blot of identical samples run under similar conditions was developed with the secondary HRP-conjugated goat anti-rabbit antibody. No 72 kDa band was detected in this blot (results not shown). These data support the conclusion that the 72 kDa band found in the β_3 immunoprecipitates is syk.

These data demonstrate a specific association of syk and β_3 in anti-β_3 immune complexes prepared from platelet lysates. Further, syk was present in anti-β_3 immune complexes from all

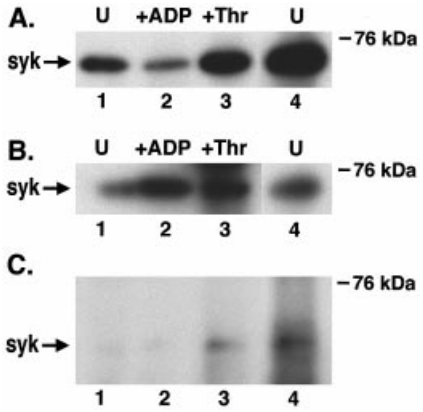

Figure 1 Detection of syk in β_3 immunoprecipitates

Immunoprecipitates prepared with an anti-β_3 monoclonal antibody, AP-3 (**A**, lanes 1–3), an anti-β_3 polyclonal antibody (**B**, lanes 1–3) and isotype-matched control antibodies (**C**, lanes 1 and 2) were subjected to SDS/10%-PAGE under reducing conditions. Blots were probed with a polyclonal antibody to syk and developed for 1 min (**A**), 30 s (**B**) or 10 s (**C**). Samples in lanes 4 (U) were from unstimulated platelets. Stimulated platelet samples containing 300 nM fibrinogen were stirred for 20 s following addition of 10 μM ADP (lanes marked +ADP) or 0.5 unit/ml thrombin (lanes marked +Thr). Control immunoprecipitates were prepared with a polyclonal antibody to syk (lane 4 in **A** and **B**), with isotype-matched mouse IgG1 (lane 1 in **C**), or with rabbit IgG (lane 2 in **C**). Lanes 3 and 4 in (**C**) contain anti-β_3 immunoprecipitates prepared as for lanes U in (**A**) and (**B**) respectively. See the Experimental section for details.

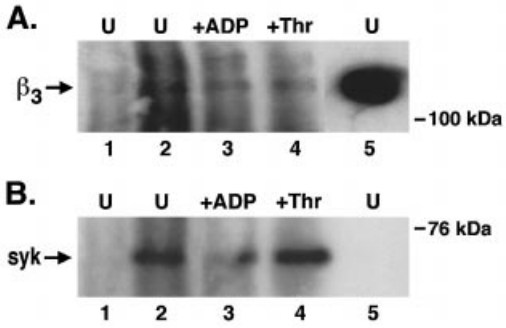

Figure 2 Detection of β_3 in syk immunoprecipitates

Immunoprecipitates prepared with an anti-syk monoclonal antibody (lanes 2–4), an isotype-matched control antibody IgG2$_a$k (lane 1) or the anti-β_3 monoclonal antibody AP-3 (lane 5; one-third loaded) were subjected to SDS/10%-PAGE under reducing conditions. The blot in (**A**) was probed with the anti-β_3 polyclonal antibody and developed for 1 min. Unstimulated platelets (lanes U) and stimulated platelets [10 μM ADP (lanes marked +ADP) or 0.5 unit/ml thrombin (lanes marked +Thr)] were prepared as described in the legend to Figure 1. The blot was then stripped, probed with a polyclonal antibody to syk and developed for 1 min (**B**).

lysates (Figures 1A and 1B, lanes 1–3), demonstrating that this association is not dependent on platelet activation.

To confirm the association of syk with β_3, we performed the reverse analysis. Using similar Triton-soluble fractions, we prepared immunoprecipitates with an anti-syk monoclonal antibody and analysed these on immunoblots probed with the anti-β_3 polyclonal antibody. The blot showed a band around 110 kDa (Figure 2A) in samples prepared from unstimulated (lane 2), ADP-stimulated (lane 3) and thrombin-stimulated (lane 4) platelets. This band was not present in immunoprecipitates with an isotype-matched mouse antibody (lane 1). The mobility of this band corresponded to that in anti-β_3 immunoprecipitates (lane 5). A second immunoreactive band with a slightly lower mobility

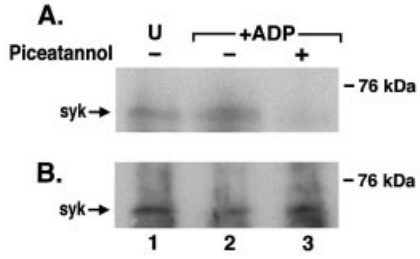

Figure 3 Autophosphorylation of syk in β_3 immunoprecipitates: inhibition by piceatannol

Platelets were incubated in the presence of 40 μg/ml piceatannol or DMSO for 15 min at 37 °C prior to initiating platelet aggregation. β_3 was immunoprecipitated from Triton-soluble fractions with the anti-β_3 monoclonal antibody AP-3. Immunoprecipitates were resuspended in kinase buffer and autokinase activity was measured as described in the Experimental section. The phosphorylated proteins were resolved in SDS/10%-PAGE under reducing conditions, transferred to a PVDF membrane and visualized by autoradiography (**A**). Western analysis with an anti-syk antibody was then performed on the membrane (**B**). Platelets were either unstimulated (U) or were stimulated with 10 μM ADP (see the legend to Figure 1).

was present in the anti-syk samples (Figure 2, lane 2–4). We have not examined this protein further, but we (results not shown) and others [16] have seen a similar band in anti-β_3 immunoblots. We stripped this blot and re-probed it with an anti-syk antibody (Figure 2B), demonstrating that similar amounts of syk were immunoprecipitated from lysates of unstimulated, ADP-stimulated and thrombin-stimulated platelets.

Taken together, the results in Figures 1 and 2 demonstrate that β_3 and syk are associated in platelet lysates. Although the data show an association, they do not distinguish between a direct association and an indirect association mediated by other molecules.

Syk autophosphorylation in β_3 immunoprecipitates

To determine whether the syk associated with β_3 was active, we assayed syk autophosphorylation in β_3 immunoprecipitates from unstimulated and ADP-stimulated platelets. Prior to initiating aggregation, we treated the platelets with the syk-selective inhibitor piceatannol (40 μg/ml in DMSO) or with DMSO for 15 min at 37 °C. This concentration of piceatannol has been shown to inhibit syk [17], but not the src-family kinase lyn. Kinase assays were performed on β_3 immunoprecipitates, the products were separated by SDS/PAGE, and radiolabelled bands were visualized after transfer to a PVDF membrane. As shown in Figure 3, autophosphorylation of a 72 kDa protein was increased following ADP stimulation (compare lane 2 with lane 1), and this stimulation was inhibited by piceatannol (compare lane 3 with lane 2). The inhibition supports the conclusion that the autophosphorylated 72 kDa band is syk. Furthermore, Western analysis of the membrane with an anti-syk antibody demonstrated that these differences were not due to changes in loading. As shown in Figure 3(B), the immunoreactive 72 kDa band was least intense for the ADP-stimulated sample (Figure 3B, lane 2), demonstrating that sample loading minimized the increased autophosphorylation seen with this sample (Figure 3A, lane 2).

To quantify the differences in autophosphorylation, we scanned the X-ray films from Figure 3. The intensity of each band in the autokinase assay was divided by that obtained for each band in the Western analysis. This ratio normalized the autophosphorylation of each band to the amount of the 72 kDa protein. For the unstimulated sample the ratio was 0.25, for the

ADP-stimulated sample it was 0.38 and for the piceatannol-pretreated sample it was 0.072. Thus the ADP-stimulated autophosphorylation was 1.5-fold greater than unstimulated autophosphorylation. The piceatannol-pretreated ADP-stimulated sample showed 5 times less autophosphorylation compared with the untreated ADP-stimulated sample. These results demonstrated that the β_3-associated syk activity was enhanced in response to ADP-stimulated aggregation, and that this stimulation was inhibited by pre-incubation with piceatannol.

DISCUSSION

Our results demonstrate that syk is associated with β_3 in unstimulated platelets, as well as in aggregated platelets. The β_3-associated syk was activated when platelets were stimulated with ADP in the presence of fibrinogen under aggregation conditions. Such an association is similar to the association of syk with antigen receptors in B-cells [18,19], where IgM–syk complexes were found to be associated in anti-IgM or anti-syk immunoprecipitates from unstimulated cells. Ligation of IgM transiently activates syk [18,19], similar to the transient activation of syk following integrin $\alpha_{IIb}\beta_3$ ligation in platelets [1,4,6]. Further examination of our data, comparing Figures 1(A) and 1(B) with Figure 2, indicates that the fraction of syk that co-precipitated with anti-β_3 was larger than the fraction of β_3 that co-precipitated with anti-syk antibody. This result is similar to that reported for the association of a src-family tyrosine kinase, fyn-T, with the T-cell-receptor–CD3 complex. Approximately 20% of fyn-T co-precipitated with anti-CD3 [20], while only 2–5% of CD3 was co-precipitated with anti-fyn antibody [21]. Thus our results recapitulate those obtained for ligand-mediated intracellular tyrosine kinase signalling in both T- and B-cells.

A functional link between the engagement of integrin $\alpha_{IIb}\beta_3$ and syk activation has been demonstrated previously in platelets and transfected CHO cells [4,6]. Although others have tried to demonstrate an intracellular association between this integrin and syk [14], our data are the first to show this complex. The experiments [14] that did not co-precipitate integrin $\alpha_{IIb}\beta_3$ and syk were not described in detail, so it is difficult to identify the experimental differences that accounted for our success. We favour the possibility that our lysis conditions were optimal for co-precipitating this complex. It is well documented that lysis conditions are important in maintaining the association between signalling molecules. Yan et al. [7] were not able to detect the association of syk with either $p^{58c\text{-}fgr}$ or $p^{53/56lyn}$ when neutrophils were lysed in radioimmunoprecipitation assay buffer, but these associations were detected when the cells were lysed in Triton X-100 [7]. Further, these authors were able to co-immunoprecipitate integrin β_2 and syk in Triton lysates of neutrophils [7]. Thus it is reasonable to conclude that β_3 and syk associate in a complex that can be co-precipitated from platelets following lysis with Triton X-100, as described here, but cannot be co-precipitated under different lysis conditions.

Our results demonstrate that the autophosphorylation of β_3-associated syk was enhanced following stimulation under aggregation conditions, indicating that the β_3-associated syk was activated by outside-in signalling. Our data add to the results reported by Gao et al. [6]. They examined CHO cells transfected with integrin $\alpha_{IIb}\beta_3$ and syk, and found that syk activation depended on $\alpha_{IIb}\beta_3$ ligation. As syk did not co-precipitate with $\alpha_{IIb}\beta_3$ in transfected CHO cells [6], we suggest that the syk–β_3 complex is more stable in platelets. This raises the possibility that the association of syk and β_3 is mediated by an intermediary molecule that is present in platelets, and which differs from intermediary molecules in CHO cells.

Further studies are necessary in order to assess the activation of β_3-associated syk in platelets. Nevertheless, the demonstration of an association between β_3 and syk in platelets is a major step towards understanding the role of integrin $\alpha_{IIb}\beta_3$ and syk in platelet signalling, and more generally in describing the role of β integrins in the specialized functions of cells of haematopoietic lineage.

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