Tyrosine phosphorylation and translocation of LAT in platelets

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Abstract Platelet aggregation is accompanied by the tyrosine phosphorylation of several proteins including syk. However, some of these proteins are not identified. Recent studies showed that LAT is a syk substrate and is tyrosine phosphorylated during T cell stimulation. In this study, we demonstrated that LAT is present in platelets and is tyrosine phosphorylated in response to ADP- and thrombin-stimulated aggregation. Moreover, LAT, like syk and β_3, translocates to the cytoskeleton during the late stage of thrombin-stimulated irreversible aggregation and not during ADP-stimulated reversible aggregation.

Key words: LAT; Platelet; Phosphorylation; Signaling

1. Introduction

Platelet aggregation is critical for hemostasis and thrombosis. Aggregation of platelets is mediated by the binding of fibrinogen to its integrin receptor $\alpha_{IIb}\beta_3$ when platelets are activated by agonists such as ADP, thrombin or collagen [1]. Platelet aggregation results in tyrosine phosphorylation and activation of several known proteins including syk tyrosine kinase. However, some of these tyrosine-phosphorylated proteins remain to be identified [1]. The tyrosine phosphorylation profile of proteins is markedly reduced in thrombasthenic platelets, which do not aggregate [1], which indicates that tyrosine phosphorylation might play a critical role in platelet signaling.

LAT is a 36–38-kDa transmembrane protein recently cloned from T cells [2]. LAT is a substrate of syk and ZAP-70 in T cells and is tyrosine-phosphorylated in response to T-cell stimulation. LAT is critical for T-cell signaling as two tyrosine to phenylalanine mutations in LAT significantly inhibit T-cell signaling [2]. LAT is not present in B cells, making it a cell specific signaling molecule. Since platelets, like T and B cells, are of hematopoietic origin, we were curious to see whether LAT is one of the unidentified tyrosine-phosphorylated proteins in platelets.

Cytoskeletal rearrangement is necessary for platelet aggregation. A recent study differentiated ADP-mediated reversible aggregation from thrombin-mediated irreversible aggregation in terms of differential translocation of signaling molecules [3]. They observed that RhoA did not translocate to the cytoskeleton during ADP-mediated reversible aggregation, but translocates during thrombin-stimulated irreversible aggregation [3]. RhoA is a member of the Ras family of small GTPases and is known to take part in cytoskeletal rearrangement [4]. These results correlate translocation of signaling molecules with reversible and irreversible aggregation, which are dependent on the differential rearrangement of the cytoskeleton. Thus, the use of ADP and thrombin as agonists under the conditions of reversible and irreversible aggregation could be very useful in identifying translocation of other signaling molecules. We wanted to see whether LAT translocates to the cytoskeleton during platelet aggregation in response to ADP and thrombin stimulation.

Results presented in this paper show that LAT is present in platelets and it is tyrosine phosphorylated during both ADP- or thrombin-stimulated platelet aggregation. Furthermore, this study documents that the translocation of LAT during thrombin-stimulated irreversible aggregation differs from that during ADP-stimulated reversible aggregation. Interestingly, β_3 and syk showed a similar pattern of translocation. The possible implications of these observations are discussed.

2. Materials and methods

2.1. Materials

Monoclonal antibody to β_3, AP-3, used for immunoprecipitation, was obtained from the previously described cell line supplied by Dr. Peter J. Newman, Blood Institute, Milwaukee, WI, USA [5]. Polyclonal antibody to β_3 called Fire and Ice, used for Western analysis, was a gift from Dr. Peter J. Newman [6]. The polyclonal antibodies to LAT and syk used in Western analysis or immunoprecipitation were purchased from Upstate Biotech, NY, USA or Santa Cruz Biotech, CA, USA. The anti-phosphotyrosine monoclonal antibody used for Western analysis was from Upstate Biotech, NY, USA. The Super Signal Chemiluminescent Substrate for the Western detection was purchased from Pierce. HRP-conjugated goat anti-rabbit and anti-mouse secondary antibodies and purified human plasma fibrinogen were purchased from Cal Biochem. Protein G- and protein A/G-conjugated agarose beads were from Santa Cruz and PVDF membrane was from Millipore. Human α-thrombin was a gift from Dr. Frank Church, University of North Carolina, Chapel Hill, NC, USA. ADP was purchased from Chrono-log. All other chemicals were purchased from Sigma.

2.2. Isolation and purification of human platelets

Platelets were purified as described [7] from whole blood of volunteers, who had abstained from using aspirin for at least 10 days. In short, platelet-rich plasma was obtained by centrifugation of the collected blood at $200\times g$ for 25 min at ambient temperature. Platelets were pelleted from the supernatant by centrifugation at $800\times g$ for 20 min at ambient temperature. Platelets were resuspended 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) bovine serum albumin (fraction V, Miles Pentex)) and passed through a Sepharose CL-2B column

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Abbreviations: TCR, T-cell receptor; EGTA, ethylene glycol-bis(β-amino ethyl ether)-*N*,*N*,*N*′,*N*′- tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; PI3kinase, phosphoinositide 3-kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CONA, concanavalin A; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TBST, Tris-buffered saline Tween; RIPA, radioimmunoprecipitation assay buffer; HRP, horseradish peroxidase; TSF, Triton-soluble fraction; RSF, RIPA-soluble fraction

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equilibrated with Tyrode's buffer. The platelets were eluted in the same buffer, were diluted to the desired concentration in Tyrode's buffer with 1 mM Ca^{2+} and 2 mM Mg^{2+} and maintained at 37°C.

2.3. Platelet aggregation

Platelets were incubated at 37°C at a final concentration of 1×10^8 platelets in 0.5 ml in cuvettes in a Chrono-log aggregometer. Aggregation was initiated by stepwise addition of 300 nM human plasma fibrinogen and agonist, 10 μM ADP or 0.5 units/ml thrombin, followed by stirring. Aggregation was monitored as the increase in light transmission [7], and the aggregation reaction was stopped by the addition of 125 μl ice cold 5× Triton lysis buffer (see below).

2.4. Preparation of Triton-soluble fractions and RIPA-soluble fractions

Triton-soluble fractions were prepared as modified from Clark et al. [8] in lysis buffer containing 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 (1×). Unstimulated (platelets incubated at 37°C with no addition and no stirring) or aggregated platelets (500 μl) were added to 125 μl ice-cold 5× lysis buffer, mixed by gentle shaking and kept on ice for 1 h. For experiments with syk and β_3, 500 μl contained 1×10^8 platelets and for experiments with LAT, 500 μl contained 2×10^8 platelets. The sample was centrifuged at $15\,000\times g$ 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. RIPA-soluble fractions were prepared from the Triton-insoluble pellet, which was resuspended in 625 μl of ice-cold 1× RIPA buffer containing 20 mM Tris-HCl, pH 7.4, 158 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 1 mM EGTA, 4 μg/ml leupeptin, 100 μg/ml PMSF, 4 μg/ml aprotinin and 1.2 mM sodium vanadate. Samples were kept on ice for 1 h with occasional vortexing (at least three times). After centrifugation at $15\,000\times g$ for 10 min at 4°C, the supernatant was collected; this is the RIPA-soluble fraction which contains the cytoskeletal proteins. All of the fractions were stored at −70°C.

2.5. Jurkat T-cell extract preparation

Jurkat T cells were suspended at 1×10^8/ml concentration in RPMI 1640 media containing 10% FBS. One set was stimulated with 2 μg/ml CONA for 30 s. No addition was done in the unstimulated set. The cells were centrifuged at 2000 rpm for 5 min, resuspended in 1 ml of ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.4, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EGTA, 4 μg/ml leupeptin, 100 μg/ml PMSF, 4 μg/ml aprotinin and 1.2 mM sodium vanadate and kept on ice for 1 h with occasional vortexing. The lysates were centrifiuged at 10 000 rpm at 4°C for 10 min. The supernatant was collected and was frozen at −70°C until use. 200 μl of each lysate, corresponding to 2×10^7 Jurkat T cells, was immunoprecipitated with 4 μg anti-LAT antibody as described below.

2.6. Immunoprecipitation

Triton-soluble fractions and RIPA-soluble fractions were thawed on ice. Protein G agarose beads were used for LAT immunoprecipitation. Protein A/G agarose beads were used for all other immunoprecipitations. Beads were washed once with 1 ml ice cold 1× PBS. Forty μl of beads were added to each fraction and tumbled for 1 h at 4°C. After centrifugation at $1000\times g$ for 20 s, the precleared supernatant was collected. Primary antibody was added and the sample was tumbled overnight at 4°C. Washed beads (40 μl) were added and the sample was tumbled for 1 h at 4°C. After centrifugation at $1000\times g$ for 1 min, the supernatant was removed. The beads, which retain the immunoprecipitate, were washed four times with 1 ml ice-cold 1× lysis buffer. The washed beads were resuspended in 40 μl 2× reducing SDS-PAGE sample buffer, boiled for 5 min, centrifuged and the supernatants were applied to SDS-PAGE gels [9]. For β_3 immunoprecipitations (Fig. 3B), 7.5 μg of an anti-β_3 monoclonal antibody AP-3, for syk immunoprecipitation (Fig. 3A), 1 μg of a polyclonal antibody (Santa Cruz) and for LAT immunoprecipitation (Figs. 1 and 3C), 4 μg of a polyclonal antibody (Upstate) was used.

2.7. Western blots

Samples in reducing SDS sample buffer were boiled for 5 min, run on 10% SDS-PAGE for syk and β_3 or 7.5% SDS-PAGE for LAT and transferred to PVDF membrane. After blocking with 5% milk TBST containing 50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl and 0.01% Tween-20, the membrane was incubated overnight at 4°C with the primary antibody diluted in 3% milk in TBST. For phosphotyrosine blot, the membrane was blocked with 10% BSA 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 1:25 000 dilution in 3% milk in TBST for LAT detection and at 1:10 000 dilution for syk and β_3 detection. After washing three times with TBST, the signal was developed with the Super Signal Chemiluminescent Substrate (Pierce) for 5 min. For syk immunodetection, 1 μg/ml of the anti-syk polyclonal antibody (Upstate), for β_3 immunodetection, 10 μg/ml of the polyclonal anti-β_3 antibody [6] and for LAT immunodetection, 1 μg/ml of the anti-LAT polyclonal antibody (Upstate) was used.

3. Results

3.1. Tyrosine phosphorylation of LAT in platelets

To show the presence and tyrosine phosphorylation of LAT, we immunoprecipitated LAT from the Triton-soluble fractions of ADP-stimulated and thrombin-stimulated platelets under the conditions of aggregation and from unstimulated platelets, and probed with an anti-phosphotyrosine antibody in an immunoblot. The results shown in Fig. 1A demonstrate that the tyrosine phosphorylation of a 36–38-kDa band was enhanced during ADP- (lane 2) and thrombin- (lane 3) stimulated aggregation (compare with the unstimulated sample in lane 1). These bands co-migrated with the bands in lanes 4 and 5 which represent LAT immunoprecipitates from Jurkat T cells. The tyrosine phosphorylation was enhanced in lane 5 compared to lane 4 showing that stimulation of T cell enhanced LAT tyrosine phosphorylation. The blot was stripped and reprobed with the anti-LAT antibody. Fig. 1B showed that the 36–38-kDa band in all lanes cross-reacted with the anti-LAT antibody. The intensity of the band is similar in lanes 1, 2 representing unstimulated and ADP-stimulated platelets. The intensity in lane 3 was little less which represents thrombin-stimulated platelets. These bands co-migrated with the band in lanes 4 and 5, representing T cells. The intensity of the bands in lane 4 was also similar to that in lane 5. Data from Fig. 1 demonstrated that the tyrosine phosphorylation of LAT was enhanced during ADP- and thrombin-stimulated aggregation (panel A), though the amount of LAT present in unstimulated and ADP-stimulated

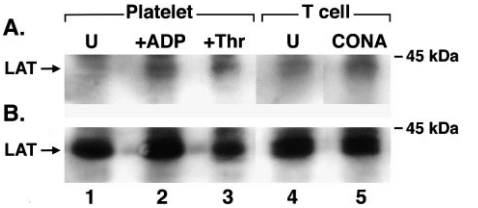

Fig. 1. Tyrosine phosphorylation of LAT during platelet aggregation. Immunoprecipitates prepared with an anti-LAT polyclonal antibody (panel A) were subjected to SDS-PAGE under reducing condition. The blot in panel A was probed with an anti-phosphotyrosine monoclonal antibody. Platelets represent samples made from platelets. The sample in lane U was from unstimulated platelets. Stimulated platelet samples with 300 nM fibrinogen were stirred for 20 s following addition of 10 μM ADP (lane marked +ADP), or 0.5 U/ml thrombin (lane marked +Thr). T cells represent samples made from T cells. The sample in lane U was from unstimulated T cells. T cells stimulated with 2 μg/ml CONA for 30 s (lane marked CONA). The blot in panel A was stripped and probed with the anti-LAT polyclonal antibody (panel B).

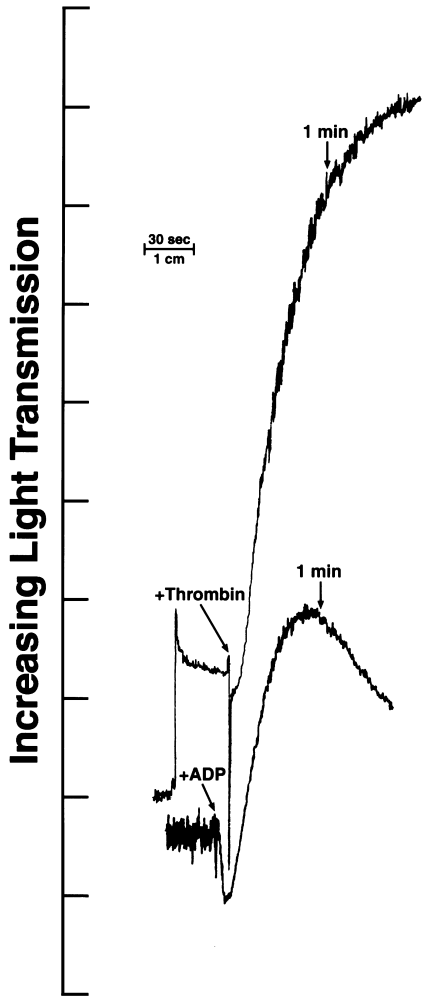

Fig. 2. ADP- and thrombin-stimulated reversible and irreversible aggregation of platelets. Platelets (2×10^8/ml) were incubated at 37°C with 300 nM fibrinogen and aggregation was initiated with either 10 μM ADP or 0.5 units/ml thrombin; additions are indicated by the arrows. Aggregation of stirred samples was measured as an increase in light transmission.

samples was similar (panel B, lanes 1 and 2) and little less in the thrombin-stimulated sample (panel B, lane 3). Similar results were obtained with T cells (compare lanes 4 and 5 in panel A and panel B).

3.2. Translocation pattern that distinguished ADP-stimulated and thrombin-stimulated aggregation

ADP and thrombin stimulation led to reversible and irreversible aggregation, respectively. Representative aggregation curves, shown in Fig. 2, showed that aggregation following ADP stimulation reached a maximum at about 1 min and then fell slowly, while aggregation following thrombin stimulation progressed beyond 1 min. The reversible nature of ADP-stimulated aggregation suggests that the cytoskeleton is not permanently rearranged during this process. Since other signaling molecules implicated in platelet aggregation are known to differentially translocate during ADP-stimulated reversible and thrombin-stimulated irreversible aggregation [3], we examined the translocation of LAT to the cytoskeleton after stimulation with each agonist. We further examined the translocation of syk and β_3 to the cytoskeleton. Syk and β_3 are known to translocate to the cytoskeleton during thrombin-stimulated irreversible aggregation [1]; however, it is not known whether they translocate to the cytoskeleton during ADP-stimulated reversible aggregation.

Samples were prepared, terminating aggregation with the addition of Triton lysis buffer after 20 s and 2 min, time points chosen to distinguish early and late aggregation. Insoluble material was removed from Triton lysates by centrifugation, and a RIPA-soluble fraction was prepared from the pellet, as described in Section 2. Both Triton-soluble and RIPA-soluble fractions were immunoprecipitated with a polyclonal antibody to syk and the immunoprecipitates were analyzed with a different polyclonal anti-syk antibody (see Section 2). As shown in Fig. 3A, a 72-kDa band corresponding to syk was seen in all Triton-soluble fractions (lanes 1–5). In contrast, syk was seen in the RIPA-soluble fractions only when platelets were stimulated with thrombin (lane 10), at a late stage of platelet aggregation (2 min). Interestingly, syk was not seen in the RIPA-soluble fraction from ADP-stimulated aggregation even at 2 min (lane 8).

To examine the distribution of β_3 in these samples, we immunoprecipitated β_3 from the lysates described in Fig. 3A. The immunoblot was probed with a polyclonal antibody to β_3. As shown in Fig. 3B, a band around 110 kDa corresponding to β_3 was observed in all Triton-soluble fractions (lanes 1–5). In the RIPA-soluble fractions, β_3 was only detected in the sample prepared 2 min after thrombin-stimulated aggregation (lane 10).

Similarly, to examine the distribution of LAT in Triton- and RIPA-soluble fractions, we immunoprecipitated LAT.

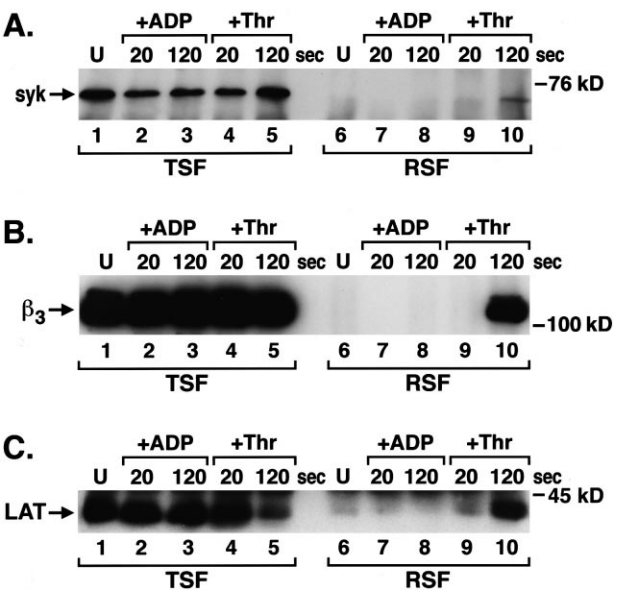

Fig. 3. Distribution of syk, β_3 and LAT following ADP- and thrombin-stimulated aggregation. Triton-soluble and RIPA-soluble samples after 20 s and 2 min of aggregation were immunoprecipitated with a polyclonal antibody to syk (panel A), with a monoclonal antibody to β_3 (panel B), or a polyclonal antibody to LAT (panel C). The immunoprecipitates were subjected to reducing SDS-PAGE and probed with a polyclonal antibody to syk (panel A), with a polyclonal antibody to β_3 (panel B), or with a polyclonal antibody to LAT (panel C). Samples in lanes U were from unstimulated platelets. Stimulated platelet samples with 300 nM fibrinogen were stirred for 20 s or 2 min following addition of 10 μM ADP (lanes marked +ADP), or 0.5 U/ml thrombin (lanes marked +Thr). TSF: Triton-soluble fractions. RSF: RIPA-soluble fractions.

The immunoblot was probed with a polyclonal antibody to LAT. As shown in Fig. 3C, a band around 36–38 kDa corresponding to LAT was observed in all Triton-soluble fractions (lanes 1–5). In the RIPA-soluble fractions, LAT was only detected in the sample prepared 2 min after thrombin-stimulated aggregation (lane 10).

Thus the distribution of LAT correlated with the distribution of syk and β_3 (Fig. 3A, B and C). These data demonstrate that LAT, syk and β_3 translocate to the cytoskeleton in the later phase of aggregation (2 min) in response to thrombin-stimulated irreversible aggregation, but not in response to ADP-stimulated reversible aggregation.

4. Discussion

In this study we have documented the presence of LAT in platelets. LAT was tyrosine phosphorylated when platelets were stimulated with either ADP or thrombin in the presence of fibrinogen under aggregation conditions (Fig. 1). Phosphorylation of LAT during platelet aggregation indicates that LAT might be important in platelet signaling. Since LAT is a substrate of syk in T cells, it will be interesting to determine if LAT is a substrate of syk in platelets. Syk tyrosine kinase has been implicated to play a major role in platelet aggregation as syk −/− mouse platelets aggregate abnormally [10] and syk −/− mice die perinatally due to internal hemorrhaging [11,12]. Further, we have evidence that syk associates with the β_3 subunit of $\alpha_{IIb}\beta_3$ in platelets [13]. Further studies will be necessary to find out whether LAT carries the downstream signaling message starting from β_3 and mediated through syk.

The translocation pattern of LAT was similar to that of β_3 and syk (Fig. 3). None of these molecules translocated to the cytoskeleton during ADP-stimulated reversible and at the early stage (20 s) of thrombin-stimulated irreversible aggregation. On the other hand, all of them translocated at the late stage (2 min) of thrombin-stimulated aggregation. In a recent study, it has been shown that the p85 subunit of PI3kinase-α translocates to the cytoskeleton in response to thrombin-stimulated aggregation with the production of phosphatidyl (3,4)-bisphosphate and phosphatidyl-inositol (3,4,5)-P_3 [3]. LAT associates with the p85 subunit of PI3kinase in T cells [2]. It will be interesting to examine whether there is any functional link between β_3, syk and LAT with PI3kinase-α and RhoA during permanent rearrangement of the cytoskeleton in response to thrombin-stimulated aggregation.

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