

Clustering of Integrin α_{IIb}-β_3 Differently Regulates Tyrosine Phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} in Concanavalin A-stimulated Platelets

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Summary

Tyrosine phosphorylation of the non-receptor tyrosine kinases pp72^{syk} and pp125^{FAK} and of the γ2 isoform of phospholipase C (PLCγ2) in human platelets stimulated with the lectin Concanavalin A was investigated. Concanavalin A induced the rapid tyrosine phosphorylation of pp72^{syk} and PLCγ2 with a similar kinetics, while tyrosine phosphorylation of pp125^{FAK} occurred in a later phase of platelet activation. When compared with other platelet agonists, Concanavalin A revealed to be at least as potent as collagen in inducing tyrosine phosphorylation of PLCγ2 and pp125^{FAK}, while tyrosine phosphorylation of pp72^{syk} induced by the lectin was much stronger than that induced by thrombin or collagen. Concanavalin A-induced tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} was not dependent on platelet aggregation as it occurred normally even in the absence of sample stirring and when fibrinogen binding to integrin α_{IIb}-β_3 was inhibited by the peptide RGDS. Tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} required the binding of the lectin to the platelet surface, but was not observed in platelets treated with succinyl-Concanavalin A, a derivative of the lectin that interacts with the same receptors but does not promote clustering of membrane glycoproteins. Moreover, the aggregation-independent tyrosine phosphorylation of pp125^{FAK} and pp72^{syk} induced by Concanavalin A required the expression of integrin α_{IIb}-β_3 on the platelet surface as it was strongly inhibited in platelets from patients affected by Glanzmann thrombasthenia. By contrast, tyrosine phosphorylation of PLCγ2 occurred normally also in thrombasthenic platelets stimulated with Concanavalin A. These results demonstrate that, even in the absence of aggregation, the clustering of integrin α_{IIb}-β_3 induced by Concanavalin A on the platelet surface directly promotes tyrosine phosphorylation of pp72^{syk} and pp125^{FAK} and provide further evidence that the oligomerization of the fibrinogen receptor promoted by its natural ligand during platelet aggregation may be responsible for the tyrosine phosphorylation of these proteins induced by physiological agonists.

Introduction

Stimulation of human platelets with physiological agonists such as thrombin or collagen induces the rapid tyrosine phosphorylation of several intracellular substrates through the activation of different non-receptor tyrosine kinases including members of the Src family, pp72^{syk} and pp125^{FAK} (1). These processes are regulated by at least two different signaling pathways. The first one is directly activated by the binding of the agonist to its membrane receptor and leads to the rapid tyrosine phosphorylation of a group of substrates that includes p120GAP, vinculin, pp72^{syk} and SHP-1 (2-6). The second pathway is initiated by the agonist-induced exposition of ligand binding site on integrin α_{IIb}-β_3, and requires fibrinogen binding and platelet aggregation. Incubation of platelets with inhibitors of fibrinogen binding to integrin α_{IIb}-β_3 and aggregation prevents the agonist-induced tyrosine phosphorylation of several proteins, including the tyrosine kinase pp125^{FAK} and other unidentified substrates with molecular weight of 84 and 95-97 kDa (7-9). Similarly, thrombin-induced tyrosine phosphorylation of these substrates is not observed in platelets from patients affected by Glanzmann thrombasthenia which lack integrin α_{IIb}-β_3 and fail to aggregate (7-9). Although these results indicate a regulatory role for integrin α_{IIb}-β_3 and platelet aggregation on protein-tyrosine phosphorylation in platelets, the exact mechanisms coupling these surface events with the activation of intracellular kinases are not completely known. It is known that binding of soluble fibrinogen induced by an anti β_3-antibody in the absence of platelet stimulation, as well as integrin α_{IIb}-β_3-mediated adhesion of platelets to fibrinogen-coated surfaces, leads to the tyrosine phosphorylation of some substrates, including the tyrosine kinase pp72^{syk}, indicating that these processes are directly regulated by integrin α_{IIb}-β_3 in the absence of aggregation (5, 10-12). However, tyrosine phosphorylation of other proteins, such as pp125^{FAK}, occurs exclusively when adherent platelets begin to spread or when occupancy of integrin α_{IIb}-β_3 by soluble fibrinogen is followed by platelet aggregation (9, 10, 13).

Platelet aggregation is a complex process, which is initiated by fibrinogen binding to integrin α_{IIb}-β_3, and evolves through a number of post-fibrinogen binding events. These include clustering of integrin α_{IIb}-β_3, binding of other adhesive proteins (like thrombospondin) to their membrane receptors, increased actin polymerization, and interaction of integrin α_{IIb}-β_3 and other membrane glycoproteins with the intracellular cytoskeleton (14-17). The exact contribution of each of these events to the aggregation-dependent protein-tyrosine phosphorylation induced by physiological agonists cannot be evaluated by the use of inhibitors of fibrinogen binding or thrombasthenic platelets, since these approaches interfere with the first event of platelet aggregation (fibrinogen binding to platelets) and, thus, prevent all in all the post-fibrinogen binding events associated with aggregation.

As an alternative model to investigate the role of integrin α_{IIb}-β_3 in protein-tyrosine phosphorylation processes, we used the lectin Concanavalin A (ConA). ConA is a potent platelet agonist, able to induce

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phospholipase C activation, secretion, and aggregation (18, 19). However, this lectin displays some peculiar features. ConA binds to several mannose-containing glycoproteins, but its main receptor on the platelet surface is the integrin α_{IIb}-β_3 (20). Moreover, by binding to platelet surface, ConA promotes, even in the absence of aggregation, events that are usually promoted by physiological agonists only during aggregation. For instance, ConA triggers the clustering of integrin α_{IIb}-β_3 and its interaction with the intracellular cytoskeleton (19, 21). We have recently shown that ConA also induces the rapid tyrosine phosphorylation of several proteins in platelets by a mechanism independent of aggregation, but mediated by the clustering of membrane glycoproteins (22).

In this work we investigated the role of clustering of integrin α_{IIb}-β_3 on the lectin-induced tyrosine phosphorylation of two tyrosine kinases differently regulated in thrombin- or collagen-treated platelets, pp72^{syk} and pp125^{FAK}. Agonist-induced tyrosine phosphorylation of pp72^{syk} occurs rapidly and is also promoted by the interaction of fibrinogen with integrin α_{IIb}-β_3, while pp125^{FAK} is tyrosine phosphorylated exclusively upon complete platelet aggregation (5, 9, 10-13). Therefore, the two kinases are representative of the two pathways regulating protein-tyrosine phosphorylation in platelets involving integrin α_{IIb}-β_3. Moreover, we also analysed the lectin-induced tyrosine phosphorylation of the γ2 isoform of phospholipase C (PLCγ2). We had previously shown that ConA stimulates a phospholipase C activity in platelets by a mechanism independent of GTP-binding proteins (18). PLCγ2 is activated by tyrosine phosphorylation, thus it may represent a candidate responsible for the lectin-induced hydrolysis of membrane phosphoinositides. Moreover, it has been reported that in platelet stimulated with collagen, PLCγ2 is tyrosine phosphorylated by activated pp72^{syk} (23). The results indicate that pp72^{syk}, PLCγ2 and pp125^{FAK} are all tyrosine-phosphorylated in platelets treated with ConA and suggest a link between these events and the clustering of integrin α_{IIb}-β_3 on the platelet surface.

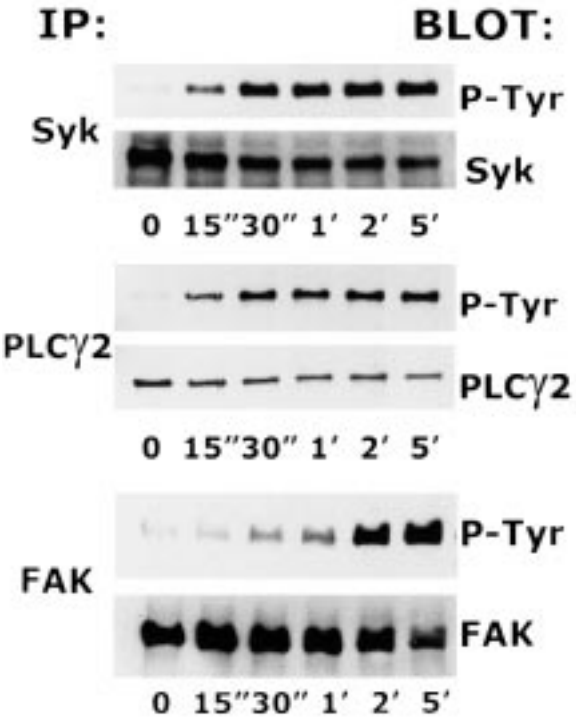

Fig. 1 Time course of pp72^{syk}, PLCγ2 and pp125^{FAK} tyrosine phosphorylation in ConA-stimulated platelets. Gel-filtered platelets were stimulated with 100 μg/ml concanavalin A at 37° C without stirring for the indicated time. Samples were then lysed, and pp72^{syk}, PLCγ2 and pp125^{FAK} were immunoprecipitated with specific antibodies. Immunoprecipitates were then analysed by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr). Each blot was then reprobed with the same antibody used for the immunoprecipitation (anti-pp72^{syk}, anti-PLCγ2 and anti-pp125^{FAK}), as indicated on the right, to assess the amount of proteins loaded onto each gel lane

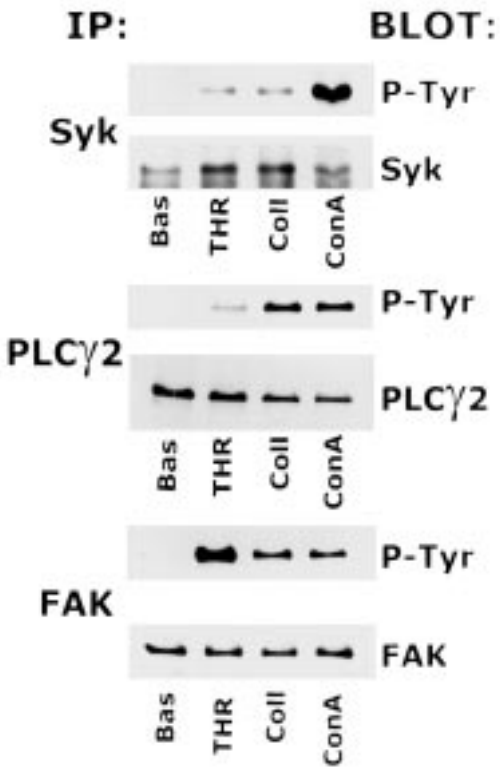

Fig. 2 Comparison of tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA, thrombin and collagen. Gel-filtered platelets were treated with buffer (Bas), or stimulated with 0.6 U/ml thrombin (THR), 20 μg/ml collagen (Coll) or 100 μg/ml concanavalin A (ConA) for 2 min at 37° C. Stimulation with thrombin and collagen was performed under constant stirring and caused full platelet aggregation, while stimulation with concanavalin A was without stirring. Tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} was analysed by immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr). Blots were then stripped and reprobed with the same antibody used for the immunoprecipitation, as indicated on the right

Materials and Methods

Materials

Concanavalin A, thrombin, methyl α-D-mannopyranoside, RGDS peptide were purchased from Sigma. Succinyl-Concanavalin A was from Vector Laboratories, Inc. Collagen was from Chrono-Log Corporation. Sepharose CL-2B was from Pharmacia. Nitrocellulose membrane was from Costar. Anti-pp72^{syk}, anti-PLCγ2, and anti-pp125^{FAK} polyclonal antisera were from Santa Cruz Biotechnology. Anti-phosphotyrosine antibodies were obtained from UBI. Peroxidase-conjugated antibodies and prestained molecular weight markers were obtained from Bio-Rad. Enhanced chemiluminescence substrate and Reflection film were from Du Pont-NEN. All other reagents were of analytical grade.

Platelet Preparation and Stimulation

Blood was drawn from healthy volunteers using citric acid-citrate-dextrose as anticoagulant. The platelet rich plasma (PRP) was obtained by centrifuging the whole blood at 120 g for 10 min at room temperature. PRP was then centrifuged at 300 g for 10 min, and the platelet pellet was resuspended in a small volume (0.5-1 ml) of autologous plasma. Platelets were then isolated by gel-filtration on a 10 ml column of Sepharose CL-2B and eluted in Hepes buffer (10 mM Hepes, 137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.4). Platelet count was adjusted at 1×10^9 cells/ml with the same buffer. Thrombasthenic platelets were prepared from freshly drawn blood from two patients affected by type I Glanzmann thrombasthenia, provided by prof. Edoardo Rossi (Transfusion Unit, Hospital L. Sacco, Milan) as described above. In parallel, platelets from healthy donors were prepared and used as control. Samples of gel-filtered platelets (0.5 ml) were equilibrated at 37 °C, and, in some experiments, incubated with 1 mM RGDS peptide or 25 mM methyl-α-D-mannopyranoside for 2 min. Stimulation was performed with 100 μg/ml ConA or 100 μg/ml succinyl-ConA for the indicated time without stirring. Some samples were stimulated with 0.6 U/ml thrombin or 20 μg/ml collagen under constant stirring for 2 min. In order to analyse the protein-tyrosine phosphorylation in total cell lysate, samples were lysed with an equal volume of SDS-sample buffer 2X (100 mM Tris/HCl pH 8.0, 20% glycerol, 50 mM dithiotreitol, 4% SDS, 0.02% bromophenol blue) and boiled 3 min.

Immunoprecipitation

Platelet stimulation was stopped by addition of 1 volume of immunoprecipitation buffer 2X (100 mM Tris/HCl pH 7.4, 200 mM NaCl, 2 mM EGTA, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM phenylmethylsulphonyl fluoride, 2 mM Na_3VO_4, 2 mM NaF, 2% Nonidet P40, 0.5% sodium deoxycholate). Samples were vortexed, placed on ice for 10 min and then centrifuged at 13,000 g for 5 min. Lysates were incubated with 1 μg (10 μl) of anti-pp72^{syk}, anti-PLCγ2 or anti-pp125^{FAK} polyclonal antisera, or control serum, for 2 h at 4 °C. Protein A-Sepharose (80 μl of a 50 mg/ml stock suspension) was then added and samples were incubated for 45 min at 4 °C. Immunocomplexes were recovered by brief centrifugation, washed three times with 1 ml of immunoprecipitation buffer 1X, and finally resuspended in SDS-sample buffer 1X (50 mM Tris/HCl pH 8.0, 10% glycerol, 25 mM dithiothreitol, 2% SDS, 0.01% bromophenol blue). Samples were boiled 5 min and proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels, and then transferred to nitrocellulose.

Immunoblotting

Nitrocellulose membranes were blocked overnight with 6% bovine serum albumin (BSA) in washing buffer (50 mM Tris/HCl pH 7.4, 0.2 M NaCl, 1 mg/ml polyethylene glycol 20,000, 1% BSA, 0.05% Tween 20) at 4 °C, and then incubated with the anti-phosphotyrosine antibodies (1:1,000 dilution in washing buffer) for 2 h at room temperature. Membranes were then extensively rinsed with several changes of washing buffer, and then incubated with the peroxidase-conjugated secondary antibodies (1:20,000 dilution in washing buffer) for 45 min. Membranes were then washed and the immunoreactive proteins were detected with a chemiluminescence reaction. Nitrocellulose filters were then stripped by incubation in 62.5 mM Tris/HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol at 50 °C for 30 min. Upon extensive washes, membranes were blocked with 6% BSA and reprobed with anti-pp72^{syk}, anti-PLCγ2 or anti-pp125^{FAK} (1:200 dilution in washing buffer) to verify the amount of immunoprecipitated protein loaded onto each gel lane. Reported experiments are representative of at least four experiments.

Results

ConA Induces Tyrosine Phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK}

We have previously shown that the lectin ConA induces the tyrosine phosphorylation of several unidentified substrates in human platelets (22). By immunoprecipitation experiments followed by immunoblotting with anti-phosphotyrosine antibodies we found that the treatment of gel-filtered human platelets with 100 μg/ml ConA under non-stirring conditions induced a time-dependent tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} (Fig. 1). In some of the experiments performed in this study a low level of phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} was observed even in resting platelets, and may reflect a partial activation of the highly reactive gel-filtered cells due to sample handling. The kinetics of ConA-induced tyrosine phosphorylation of pp72^{syk} and PLCγ2 was very rapid, being already evident after 15 s and reaching the maximal level within 30 s after the addition of the agonist. By contrast, ConA-induced tyrosine phosphorylation of pp125^{FAK} was more delayed, as it was detected between 30 s and 2 min after stimulation. In the kinetic experiments shown in Fig. 1, a decrease in the amount of immunoprecipitated proteins with time was evident. Although we do not have a clear explanation for this effect, it may indicate that the tyrosine phosphorylation of the analysed substrates could be even higher than that observed.

We next compared the ability of ConA to induce tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} with that of the strong platelet agonists thrombin and collagen. Gel-filtered platelets were stimulated at 37 °C with 0.6 U/ml thrombin or 20 μg/ml collagen under constant stirring, or with 100 μg/ml ConA, without stirring, for 2 min. Thrombin and collagen were used at concentrations able to induce, under our experimental conditions, maximal platelet aggregation (data not shown). Fig. 2 shows that tyrosine phosphorylation of pp72^{syk} induced by ConA was much stronger than that promoted by thrombin or collagen, which showed similar effects. In agreement with previously reported results (23-25), we also found that collagen was more potent than thrombin in inducing tyrosine phosphorylation of PLCγ2. In this case, the effect of ConA was identical to that of collagen. Moreover, ConA and collagen induced tyrosine phosphorylation of pp125^{FAK} to a similar extent, although the maximal effect was observed in thrombin-stimulated platelets. Thus, ConA revealed to be a potent stimulator of tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK}, and the effects of the lectin were similar or even higher than those of the strong platelet agonists collagen and thrombin.

Tyrosine Phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} Induced by ConA Is Independent of Platelet Aggregation, but Requires the Clustering of Membrane Glycoproteins

In the experiments described, stimulation of platelets with ConA was performed in the absence of stirring to prevent platelet aggregation. However, to confirm that tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by the lectin was really independent of aggregation and to investigate the possible role of fibrinogen binding to integrin α_{IIb}-β_3, we analysed the tyrosine phosphorylation of these substrates in platelet treated with 100 μg/ml ConA without stirring in the absence and in the presence of 1 mM RGDS peptide. Fig. 3 shows that tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA was completely independent of fibrinogen binding to integrin α_{IIb}-β_3 and platelet aggregation, as it was unaffected by incubation with the RGDS peptide.

It is known that ConA binds to several membrane glycoproteins on the platelet surface, including integrin α_{IIb}-β_3, and promotes the clustering of these receptors (21, 26). The role of lectin binding to platelet membrane glycoproteins on the tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} was investigated using gel-filtered platelets stimulated with ConA in the presence of the aptenic sugar methyl-α-D-

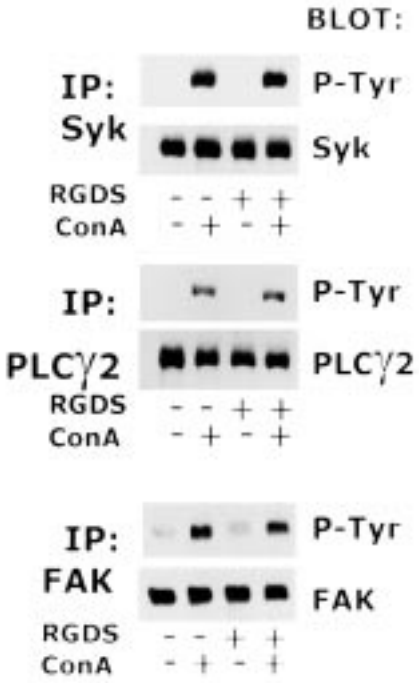

Fig. 3 Effect of RGDS peptide on tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA. Gel-filtered platelets were incubated at 37° C in the absence or in the presence of 1 mM RGDS peptide and then treated with buffer or with 100 μg/ml concanavalin A for 2 min without stirring. The extent of tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} was analysed on the immunoprecipitated proteins by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr). Blots were then reprobed with anti-pp72^{syk}, anti-PLCγ2 or anti-pp125^{FAK}, as indicated on the right

mannopyranoside, while the involvement of the clustering of the ConA receptors was evaluated using the dimeric derivative succinyl-ConA. Fig. 4 shows that tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA was totally inhibited by the methyl-α-D-mannopyranoside, indicating that these events were specific and mediated by the interaction of the lectin with the platelet surface. Moreover, Fig. 4 also shows that succinyl-ConA, a dimeric derivative of the lectin that binds to the same glycoproteins but does not induce clustering of the receptors (27), did not stimulate tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK}. Therefore, tyrosine phosphorylation of these substrates induced by ConA was strictly dependent on the clustering of membrane glycoproteins on the platelet surface.

Analysis of Tyrosine Phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} in Thrombasthenic Platelets

Since integrin α_{IIb}-β_3 is the main platelet receptor of ConA and is clusterized upon the binding of the lectin (20, 21), we investigated its role on the tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA using platelets from patients affected by Glanzmann thrombasthenia. Thrombasthenic platelets do not bind fibrinogen and fail to aggregate in response to physiological agonists like thrombin. However, since tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA was totally independent of platelet aggregation, thrombasthenic platelets are useful tools to highlight the direct involvement of integrin α_{IIb}-β_3. Two different patients (M.L. and C.S.) affected by type I Glanzmann thrombasthenia were analysed and similar results were obtained. Fig. 5 compares the pattern of protein-tyrosine phosphorylation in normal and thrombasthenic platelets stimulated with thrombin or ConA. In thrombasthenic platelets treated with thrombin the tyrosine phosphorylation of some proteins observed in normal cells, such as the 95 kDa band, was totally prevented, while the intensity of others was strongly reduced. This finding is in agreement with previously reported results and it has been related to the lack of aggregation of thrombin-stimulated thrombasthenic platelets. The pattern of protein-tyrosine phosphorylation in thrombasthenic platelets treated with ConA was more similar to that of normal platelets. However, the intensity of some bands revealed with anti-phosphotyrosine antibodies was clearly decreased. This suggested that the aggregation-independent tyrosine phosphorylation of some proteins induced by ConA was affected by the absence of integrin α_{IIb}-β_3 on the platelet surface.

By immunoprecipitation with specific antibodies, we next analysed the tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA in thrombasthenic platelets. Results are reported in Fig. 6. ConA-induced tyrosine phosphorylation of pp72^{syk} was observed also in thrombasthenic platelets, but it was strongly reduced (more than 50%) when compared to that of normal cells, indicating a partial involvement of integrin α_{IIb}-β_3 in this event. By contrast, tyrosine phosphorylation of PLCγ2 induced by ConA in platelets lacking integrin α_{IIb}-β_3 occurred normally and was identical to that observed in control platelets. Finally, the tyrosine phosphorylation of pp125^{FAK}, which was observed in normal platelets, was hardly detectable in thrombasthenic cells, indicating that the lack of integrin α_{IIb}-β_3 dramatically affected the ability of the lectin to induce tyrosine phosphorylation of this kinase.

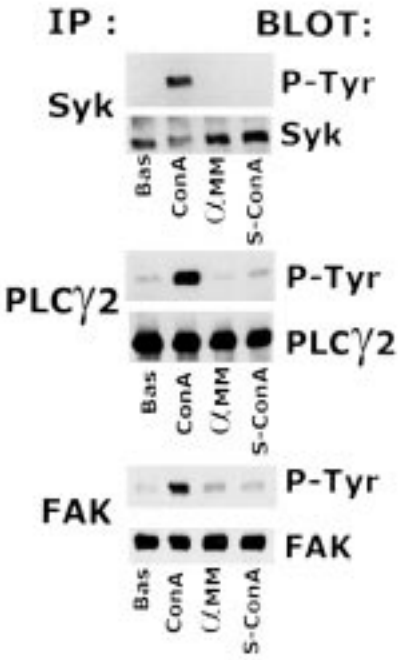

Fig. 4 Tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} requires the lectin-induced clustering of membrane glycoproteins. Gel-filtered platelets were treated with buffer (Bas), or stimulated with 100 μg/ml concanavalin A (ConA), 100 μg/ml ConA in the presence of 25 mM methyl-α-D-mannopyranoside (αMM), or 100 μg/ml succinyl-concanavalin A for 2 min (S-ConA). pp72^{syk}, PLCγ2 and pp125^{FAK} were then immunoprecipitated as indicated on the left, and analysed by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr). Blots were then reprobed with the same antibody used for the immunoprecipitation, as indicated on the right

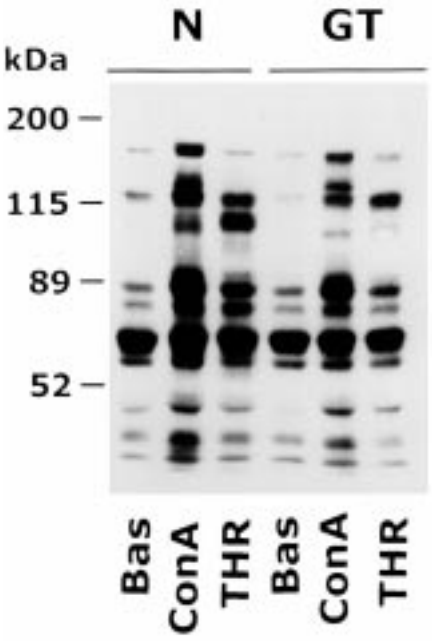

Fig. 5 Protein-tyrosine phosphorylation induced by ConA and thrombin in normal and thrombasthenic platelets. Gel-filtered normal platelets (N) and platelets from a patient affected by Glanzmann thrombasthenia (GT) were treated with buffer (Bas), 100 μg/ml concanavalin A (ConA) without stirring, or 0.6 U/ml thrombin (THR) under constant stirring for 2 min at 37° C. Platelets were directly lysed with SDS-sample buffer, boiled, and subjected to SDS-PAGE on a 5-15% polyacrylamide gradient gel. Proteins were then transferred to nitrocellulose and tested with antibodies against phosphotyrosine. The positions of molecular weight markers are reported on the left

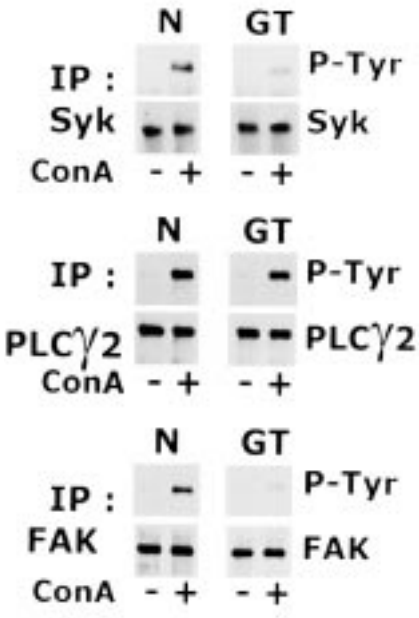

Fig. 6 ConA-induced tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} in thrombasthenic platelets. Normal (N) and thrombasthenic (GT) platelets were incubated at 37° C in the absence (-) or in the presence (+) of 100 μg/ml ConA without stirring for 2 min. Platelets were lysed and immunoprecipitated with anti-pp72^{syk}, anti-PLCγ2 or anti-pp125^{FAK} antisera, as indicated on the left. The immunoprecipitated proteins were analysed by immunoblotting with antiphosphotyrosine (P-Tyr) and then reprobed with the same antiserum used for the immunoprecipitation, as indicated on the right

Discussion

In this work we have demonstrated that the lectin ConA induces tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} in human platelets and that these events are independent of platelet aggregation, but are differently regulated by the clustering of integrin α_{IIb}-β_3. We had previously shown that ConA is a potent stimulator of tyrosine phosphorylation of several proteins in platelets, but none of the phosphorylated substrates had been identified (22). Here we showed that the tyrosine kinase pp72^{syk} represents a major substrate for tyrosine phosphorylation induced by ConA. The content of tyrosine-phosphorylated pp72^{syk} in ConA-treated platelets was found to be several fold higher than that measured in thrombin- or collagen-aggregated platelets. This suggests that pp72^{syk} may play an important role in the mechanisms of platelet activation by this lectin. The kinetics of tyrosine phosphorylation of pp72^{syk} induced by ConA is consistent with this hypothesis, since it is very rapid and occurs within 15 s from the addition of the agonist. Thus, tyrosine phosphorylation of pp72^{syk} is one of the earliest events occurring upon treatment of platelets with ConA, and precedes other signaling processes like phospholipase C activation and Ca^{2+} mobilization which have been described to occur between 30 s and 2 min (18). Moreover, since tyrosine phosphorylation of pp72^{syk} is concomitant with the stimulation of its catalitic activity (4, 5), it is likely that this tyrosine kinase is responsible for some of the other previously described events of tyrosine phosphorylation.

We have also shown that ConA induces the tyrosine phosphorylation of PLCγ2 with a kinetics very similar to that of pp72^{syk}. It has been reported that the tyrosine kinase pp72^{syk} is responsible for the phosphorylation of PLCγ2 in collagen-stimulated platelets (23). It is likely, therefore, that the similar kinetics of tyrosine phosphorylation of the two proteins reflects the fact that PLCγ2 is a substrate for pp72^{syk}. It is known that the γ isoforms of phospholipase C are activated by tyrosine phosphorylation (28). Although PLCγ1 does not undergo tyrosine phosphorylation in stimulated platelets (2), phosphorylation and activation of PLCγ2 has been proposed to play a major role in phosphatidylinositol (4,5) bisphosphate hydrolysis promoted by collagen (23-25, 29). The evidence that ConA induces tyrosine phosphorylation of PLCγ2 is in agreement with our previous work indicating hydrolysis of phosphatidylinositol(4,5)bisphosphate and production of IP_3 in ConA-stimulated platelets (18). Interestingly, we had previously shown that phospholipase C activation by ConA occurred through a mechanism independent of GTP-binding proteins (18). The results reported in this work suggest that PLCγ2 may represent the isoenzyme responsible for phosphoinositides hydrolysis in ConA-stimulated platelets.

In platelets treated with physiological agonists like thrombin, the tyrosine phosphorylation of several proteins requires fibrinogen binding to integrin α_{IIb}-β_3 and platelet aggregation (7, 8). For instance, it has been reported that thrombin-induced tyrosine phosphorylation of pp125^{FAK} was totally inhibited by preventing platelet aggregation and was not observed in platelets from patients affected by Glanzmann thrombasthenia, which lack integrin α_{IIb}-β_3 (7, 9, 13). Similarly, tyrosine phosphorylation of pp72^{syk}, which occurs independently of platelet aggregation in thrombin-stimulated platelets, is potentiated by fibrinogen binding to integrin α_{IIb}-β_3 and cell aggregation (4, 5), suggesting that two different signal transduction pathways, aggregation-dependent and aggregation-independent, regulate this process. In this work we

have demonstrated that tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA is totally independent of fibrinogen binding to integrin α_{IIb}-β_3 and platelet aggregation, as it occurs normally in platelets stimulated with the lectin both in the absence of stirring and in the presence of the RGDS peptide. This makes ConA unique among the platelet agonists, and, to our knowledge, represents the first evidence for a full tyrosine phosphorylation of pp125^{FAK} induced by a soluble ligand in the absence of platelet aggregation. The ability of ConA to induce, even in the absence of platelet aggregation, events that in thrombin-treated platelets are normally observed only during aggregation is a tipical feature of this lectin. We have recently demonstrated, for instance, that activation of the phosphatidylinositol 3-kinase, which is largely dependent on aggregation in thrombin-stimulated platelets, occurs through an aggregation-independent mechanism in ConA-treated platelets (30). This peculiarity of ConA may be due to the tetrameric binding properties of lectin, which induces topographic changes on the cell surface reproducing events occuring during aggregation. ConA binds to mannose-containing glycoproteins, and promotes the clustering of its receptors (21, 26). It is known that integrin α_{IIb}-β_3 is the main ConA receptor on platelet surface and undergoes oligomerization upon the binding of the lectin (20, 21). It is likely, however, that other glycoproteins recognized by ConA are clusterized upon binding of the tetrameric lectin, and this may lead to the formation of heterogeneous glycoproteins clusters on the platelet surface. Similarly, in thrombin-stimulated platelets which undergo aggregation, fibrinogen binding promotes clustering of integrin α_{IIb}-β_3, and other membrane glycoproteins (such as GP IV and CD9) are brought into these clusters either by their specific ligands (i.e. thrombospondin) or by spacial interaction with integrin α_{IIb}-β_3 (14, 15, 31) Thus, similar heterogeneous clusters are formed both in thrombin-aggregated and in ConA-stimulated platelets.

Here we showed that the aggregation-independent tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} induced by ConA was mediated by the formation of such glycoproteins clusters on the platelet surface. In fact, none of the analysed proteins was tyrosine phosphorylated in platelet treated with the succinyl-ConA, a chemical derivative of the lectin, that binds to the same glycoproteins on the cell surface but is unable to promote clustering of the receptors (27).

Since the integrin α_{IIb}-β_3 is the main receptor which is clusterized by ConA, we analysed the contribution of this particular event on the tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125^{FAK} by using platelets from patients affected by Glanzmann thrombasthenia, which lack integrin α_{IIb}-β_3. Analysis of tyrosine phosphorylation of pp72^{syk} and pp125^{FAK} in thrombasthenic platelets stimulated with thrombin has already been reported and, together with the results obtained with normal platelets stimulated in the presence of the RGDS peptide, led to the conclusion that these events were partially or totally regulated by fibrinogen binding to integrin α_{IIb}-β_3 and platelet aggregation. However, it should be noted that the use of thrombasthenic platelets stimulated with thrombin does not really provide new insights into the direct involvement of integrin α_{IIb}-β_3 itself in addition to the results obtained with the RGDS peptide. In fact, integrin α_{IIb}-β_3 may partecipate to the aggregation-dependent tyrosine phosphorylation induced by thrombin by two different mechanisms. On one hand it is possible that its engagement by fibrinogen during aggregation represents the signal leading to the activation of tyrosine kinases. In this case, the inhibition of fibrinogen binding or the absence of integrin α_{IIb}-β_3 would prevent the generation of such a signal. On the other hand, however, it is also possible that, upon fibrinogen binding, integrin α_{IIb}-β_3 initiates platelet aggregation, and that, in a later phase of this process, other membrane glycoproteins, which may be subsequently engaged, act as the real promoter of tyrosine kinase activation. In this case, integrin α_{IIb}-β_3 cannot be considered directly responsible for the analysed events, but the treatment with RGDS peptide, which block aggregation, or the use of thrombasthenic platelets, which lack integrin α_{IIb}-β_3 and fail to aggregate, will result in an inhibition identical to that observed in the former hypothesis.

The experimental model of platelets stimulated with ConA may help to clarify this problem. In this case, in fact, pp72^{syk}, PLCγ2 and pp125^{FAK} are tyrosine phosphorylated independently of both platelet aggregation and other events related to aggregation and are not affected by the peptide RGDS. Therefore, the analysis of thrombasthenic platelets exclusively highlights the possible direct role of integrin α_{IIb}-β_3.

Here we showed that tyrosine phosphorylation of pp72^{syk} induced by ConA was partially inhibited in thrombasthenic platelets. Thus, we conclude that the clustering of integrin α_{IIb}-β_3 represent an event that directly promotes tyrosine phosphorylation of pp72^{syk}. This is in agreement with previous results showing that binding of fibrinogen to integrin α_{IIb}-β_3 induced by anti-β_3 antibodies was able to trigger activation of pp72^{syk} (5). A direct link between integrin α_{IIb}-β_3 and pp72^{syk} has been also recently documented by experiments using CHO cells transfected with the two proteins and induced to adhere to integrin ligands (12). However, the evidence that in thrombasthenic platelets tyrosine phosphorylation of pp72^{syk} is reduced, but not abolished, indicates that, in addition to integrin α_{IIb}-β_3, the clustering of other membrane receptors mediates the activation of this kinase. Possible candidates includes the FcγIIA receptor, whose oligomerization is known to induce tyrosine phosphorylation of pp72^{syk}, and the GP VI, which mediates tyrosine phosphorylation of pp72^{syk} in collagen-stimulated platelets (32, 33). In this regard it is interesting to note that collagen shows some similarities with ConA, as it also represents a multimeric ligand able to bind to different membrane receptors and to promote oligomerization of membrane components.

In contrast to pp72^{syk}, we found that tyrosine phosphorylation of PLCγ2 induced by ConA in thrombasthenic platelets was identical to that observed in normal cells. In the light of the lack of effect of succinyl-ConA, we conclude that, in this case, the clustering of membrane glycoproteins different from integrin α_{IIb}-β_3 mediates the tyrosine phosphorylation of PLCγ2 induced by the lectin. On the other hand, since PLCγ2 is a substrate for the kinase activity of pp72^{syk} (23), it is also possible that the amount of pp72^{syk} activated through the integrin α_{IIb}-β_3-independent pathway in thrombasthenic platelets is sufficient to promote a maximal phosphorylation of PLCγ2.

Tyrosine phosphorylation of pp125^{FAK}, which occurs through an aggregation-independent mechanism in ConA-stimulated platelets, was almost totally prevented in thrombasthenic platelets. Therefore, in this case, clustering of integrin α_{IIb}-β_3 is essential for tyrosine phosphorylation of pp125^{FAK}. It is interesting to note that in thrombin-stimulated platelets tyrosine phosphorylation of pp125^{FAK} is an event strictly dependent on aggregation (9, 13). Our data enstablish a direct link between integrin α_{IIb}-β_3 clustering and pp125^{FAK} phosphorylation, and suggest that a similar effect may be promoted, under physiological conditions, by fibrinogen-triggered oligomerization of its receptor during aggregation.

In conclusion, our data demonstate that in ConA-stimulated platelets, the clustering of integrin α_{IIb}-β_3 on the cell surface is sufficient to trigger tyrosine phosphorylation of pp72^{syk} and pp125^{FAK}. Since clustering of integrin α_{IIb}-β_3 occurs during platelet aggregation, our results suggest a possible mechanism underlying aggregation-dependent phosphorylation of these kinases in platelet stimulated with physiological agonists.

Acknowledgements

We thank prof. Edoardo Rossi (Transfusion Unit, Hospital L. Sacco, Milan, Italy) for providing blood samples of thrombasthenic patients, and Marco Bellaviti (Department of Biochemistry, University of Pavia, Italy) for technical assistance in the preparation of the figures. This work was supported by grants from the Ministero dell' Università e della Ricerca Scientifica e Tecnologica (MURST, Progetti di Ricerca di Interesse Nazionale), and Consiglio Nazionale delle Ricerche (CNR, Target Project Biotechnology).

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Received April 29, 1998 Accepted after revision September 22, 1998