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Dual Mechanism of Protein-Tyrosine Phosphorylation in Concanavalin A-Stimulated Platelets

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Abstract Treatment of human platelets with the lectin Concanavalin A (Con A) resulted in the tyrosine phosphorylation of several proteins with molecular masses 65, 80, 85, 95, 120, 135, and 150 kDa. These proteins were divided in two groups: the first group included the 65-, 85-, 95-, and 120-kDa bands, which were tyrosine phosphorylated also in thrombin-stimulated platelets; the second group (80-, 135-, and 150-kDa bands) included proteins whose tyrosine phosphorylation was exclusively promoted by Con A, but not by thrombin. Members of the second group were rapidly dephosphorylated when the lectin was displaced from the cell surface by methyl α-D-mannopyranoside. Pretreatment of intact platelets with the prostacyclin analog iloprost, inhibited Con A-induced tyrosine phosphorylation of the first group of proteins, but had no effect on the tyrosine phosphorylation of the proteins of the second group. Succinyl-Con A, a dimeric derivative of the lectin, which binds to the platelet surface but does not promote clustering of the receptor, did not induce tyrosine phosphorylation of the second group of proteins, although phosphorylation of some members of the first group was observed. Our results demonstrate the presence of two different mechanisms leading to protein-tyrosine phosphorylation in Con A-stimulated platelets, and identify a new signal transduction pathway, promoted by the clustering of membrane glycoproteins, that produces tyrosine phosphorylation of specific substrates. This new pathway may be activated by platelet interaction with multivalent ligands, such as adhesive proteins, during adhesion, spreading, and aggregation.

Key words: signal transduction, membrane glycoproteins, tyrosine kinases, clustering, adhesive proteins, aggregation, adhesion

Human platelets possess several tyrosine-specific protein kinases. These include the cellular src gene product ($pp60^{src}$) and the related proteins $pp61^{fyn}$, $pp54^{lyn}$, $pp58^{lyn}$, $pp62^{yes}$, $pp61^{hck}$, and $pp72^{syk}$ [Golden et al., 1986; Horak et al., 1990; Zhao et al., 1990; Huang et al., 1991; Otha et al., 1992]. $pp60^{src}$ is very abundant in platelets (about 0.2% of the total cell proteins) [Golden et al., 1986], is located in the plasma membrane and in the membrane of dense granules [Golden et al., 1986; Rendu et al., 1989], and interacts with the cytoskeleton in thrombin-aggregated platelets [Grondin et al., 1991; Horvath et al., 1992], and with the α-subunit of the heterotrimeric G-protein G_i in epinephrine-stimulated platelets [Torti et al., 1992a]. In resting platelets, $pp61^{fyn}$, $pp54^{lyn}$, $pp58^{lyn}$, and $pp62^{yes}$ are physically associated with the membrane glycoprotein IV [Huang et al., 1991], which is the receptor for the adhesive protein thrombospondin [Asch et al., 1987]. In thrombin-activated platelets, the same kinases are associated with the $p21^{ras}$ GTPase activating protein [Cichowski et al., 1992].

The involvement of tyrosine kinases in platelet function is supported by the evidence that specific tyrosine kinases inhibitors, such as genistein and tyrphostin, inhibit agonist-induced platelet aggregation and secretion [Ashai et al., 1992; Guinebault et al., 1993]. On the other hand, it is known that platelet stimulation with thrombin or collagen induces the rapid tyrosine phosphorylation of several cellular proteins with molecular masses ranging from 50 to 140 kDa [Ferrell and Martin, 1988; Golden and Brugge, 1989; Nakamura and Yamamura, 1989]. Among these proteins, only three have been identified so far: the $p21^{ras}$ GTPase activating protein, the focal adhesion kinase ($pp125^{FAK}$), and vinculin

Abbreviations used: Con A, Concanavalin A; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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[Torti and Lapetina, 1992; Lipfert et al., 1992; Vostal and Shulman, 1993].

Similarly, very little is known about the mechanism coupling agonist binding to platelets and intracellular tyrosine kinase activation. Some studies revealed the existence of at least two different signal transduction pathways. The first one is directly activated by the binding of the agonist to its receptor, and leads to a rapid protein-tyrosine phosphorylation, probably through the action of intracellular messengers, such as cytoplasmic Ca^{2+} [Ferrell and Martin, 1989; Golden et al., 1990; Vostal et al., 1991]. The second pathway is not directly promoted by the agonist itself, but is controlled by the agonist-induced fibrinogen binding to the membrane glycoprotein IIb–IIIa complex, and by platelet aggregation [Ferrell and Martin, 1989; Golden et al., 1990]. This second signaling pathway indicates that membrane glycoproteins other than the agonist receptors, may promote intracellular protein-tyrosine phosphorylation upon binding of specific ligands.

Platelets interact with adhesive proteins such as fibrinogen, fibronectin, laminin, thrombospondin during both aggregation and adhesion [Akiyama et al., 1990]. Since these adhesive proteins are multivalent ligands, they often induce cross-linking of the receptor in intact cells. This has been clearly demonstrated to occur during platelet aggregation, when the binding of fibrinogen induces the clustering of the glycoprotein IIb–IIIa complex [Isenberg et al., 1987]. In other cells, clustering of membrane receptors plays an important role in the mechanism of activation of intracellular tyrosine kinases. In T cells, for example, cross-linking of the CD4 and CD8 surface proteins by monoclonal antibodies increases the activity of the tyrosine kinase $p56^{lck}$ [Veillette et al., 1989].

Plant lectins are useful tools to investigate the effect of the clustering of membrane glycoproteins. In platelets, the lectin Concanavalin A (Con A), which is specific for gluco- and mannopyranoside-containing glycoproteins, binds mainly to the glycoprotein IIb–IIIa complex, and induces cross-linking of this receptor [Fitzgerald et al., 1985; Kakaiya et al., 1988]. In this study, we investigated the ability of Con A to induce protein-tyrosine phosphorylation in human platelets. Results demonstrate the existence of a signal transduction pathway initiated by the clustering of membrane glycoproteins and leading to the tyrosine phosphorylation of specific intracellular proteins.

MATERIALS AND METHODS

Materials

Concanavalin A, human thrombin, and methyl α-D-mannopyranoside were purchased from Sigma (St. Louis, MO). Succinyl-Con A was from Vector Laboratories, Inc. (Burlingame, CA). Iloprost was from Schering (Milano, Italy). Sepharose CL-2B was from Pharmacia (Cologno Monzese, Italy). Triton X-100 was from Pierce (Milano, Italy). Nitrocellulose membrane was from Schleicher & Schuell (Dassel, Germany). Anti-phosphotyrosine antibodies were obtained from UBI (Lake Placid, NY). Peroxidase-conjugated antibodies and prestained molecular weight markers were obtained from Bio-Rad (Segrate, Italy). Enhanced chemiluminescence substrate and Hyperfilm ECL were from Amersham (Milano, Italy). All other reagents were of analytical grade.

Platelet Preparation and Stimulation

Blood was drawn from healthy volunteers using citric acid–citrate–dextrose as anticoagulant. Platelets were isolated by gel filtration on 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) as described [Sinigaglia et al., 1989]. Platelet count was adjusted at 1×10^9 cells/ml with the same buffer. Platelet samples were equilibrated at 37°C and in some experiments incubated with 10 μM iloprost for 30 min. Stimulation was performed with 100 μg/ml ConA, 1 U/ml thrombin or 100 μg/ml succinyl-Con A for the indicated time without stirring. Aliquots were withdrawn at the times indicated in the figure legends, rapidly dissociated with 2% SDS, and boiled for 10 min. Some experiments were performed in siliconized cuvettes placed in an aggregometer with or without stirring as indicated. Aggregation was monitored continuously and reactions were stopped by addition of 2% SDS.

Electrophoresis and Immunoblotting

Samples were rendered 0.5% dithiothreitol, 10% glycerol, 0.01% bromophenol blue, and proteins were separated by SDS–PAGE on 7.5% acrylamide gels and transferred to nitrocellulose. Nitrocellulose membranes were blocked overnight with 6% bovine serum albumin (BSA), and then incubated with the antiphosphotyro-

sine antibodies (1:1,000 dilution) for 2 h. Washing, incubation with the peroxidase-conjugated antibodies, and detection of the immunoreactive proteins with the chemiluminescence reaction were performed as described [Torti et al., 1992b].

Platelet Cytoskeleton Preparation

Cytoskeleton and Triton X-100-soluble fraction were prepared as described [Torti et al., 1993], except that 2 mM Na_3VO_4 was included in the lysis buffer containing Triton X-100.

RESULTS

Gel-filtered platelets were incubated with 100 μg/ml Con A for different times, and aliquots were solubilized with SDS and boiled. Following electrophoresis and Western blotting, samples were probed with antiphosphotyrosine antibodies. Figure 1 shows the time dependent protein-tyrosine phosphorylation induced by Con A in human platelets. Some tyrosine-phosphorylated proteins were detected in resting platelets, the most evident with molecular masses 60, 65, and 120 kDa. The strong reactivity to antiphosphotyrosine antibodies of the 60-kDa band (probably $pp60^{src}$) was not apparently modified by Con A. However, the phosphotyrosine content of the 65- and 120-kDa bands rapidly increased upon addition of Con A. Moreover, the lectin induced tyrosine phosphorylation of several other proteins with molecular masses 35, 80, 85, 95, 130, and 135 kDa. Protein-tyrosine phosphorylation induced by Con A became evident only after 20 s from the addition of the agonist, and there was no evidence for dephosphorylation events during the next 2 min.

Dephosphorylation of specific proteins, could, however, be stimulated by the addition of the haptenic sugar methyl α-D-mannopyranoside (Fig. 2). Platelets were treated with Con A, and then, when the lectin-induced protein-tyrosine phosphorylation had reached the maximal level, 25 mM methyl α-D-mannopyranoside was added. Aliquots of the samples were withdrawn during the following 5 min and analyzed by immunoblotting with the antiphosphotyrosine antibodies. In parallel, samples of Con A-treated platelets were incubated for 5 and 10 min in the absence of the haptenic sugar. As shown in Figure 2, some tyrosine-phosphorylated proteins underwent rapid dephosphorylation when the lectin was removed from the plasma membrane by the addition of methyl α-D-mannopyranoside. These proteins included the 80-, 135-, and 150-kDa bands. By contrast, there was no dephosphorylation of the 65- and 120-kDa proteins and only a slight reduction of the phosphotyrosine content of the 85-kDa band. Dephosphorylation of the 80-, 135-, and 150-kDa proteins was not due to the prolonged time of incubation, since these proteins were still highly tyrosine phosphorylated in platelets stimulated with Con A for 10 min. This indicates that Con A-induced tyrosine phosphorylation of these proteins is an event that persists as long as the lectin remains bound to its receptor(s).

Tyrosine phosphorylation of the 80-, 135-, and 150-kDa proteins by Con A did not require platelet aggregation. As shown in Figure 3, these

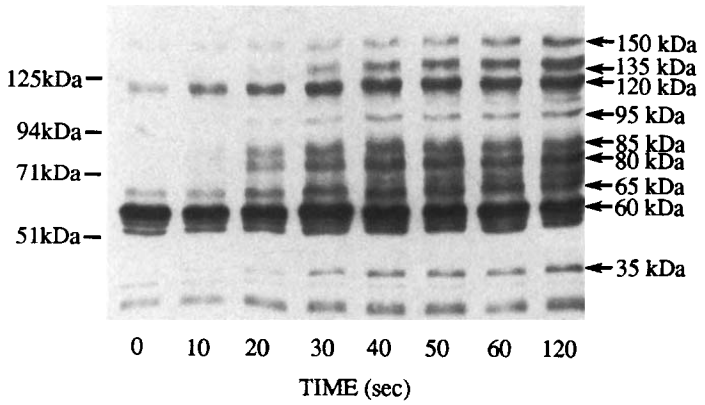

Fig. 1. Time course of protein-tyrosine phosphorylation induced by Con A. Gel-filtered platelets were incubated at 37°C with 100 μg/ml Con A without stirring for the indicated times. Samples were then analyzed by immunoblotting with the antiphosphotyrosine antibodies. The positions of molecular weight markers are indicated on the left. Arrows on the right indicate the molecular masses of the main tyrosine-phosphorylated proteins.

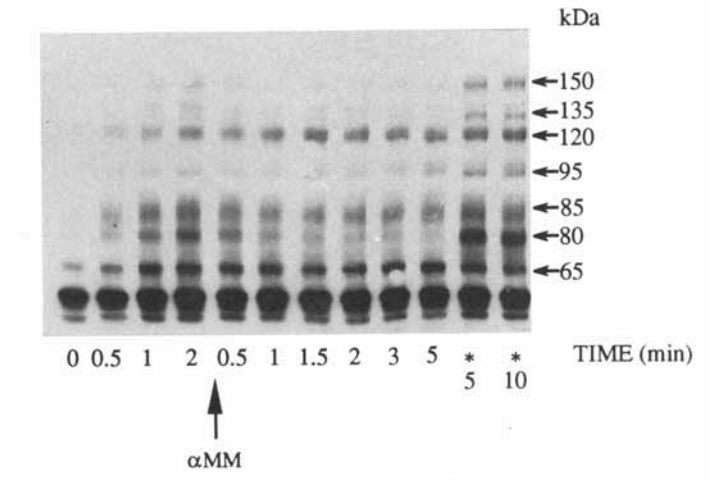

Fig. 2. Effect of methyl α-D-mannopyranoside. Platelets were incubated with 100 μg/ml Con A and aliquots were withdrawn at 30, 60, and 120 s from the addition of the agonist. After 2 min, 25 mM methyl α-D-mannopyranoside (αMM) was added, and aliquots were withdrawn during the following 5 min. In parallel, two platelet samples were incubated with Con A without methyl α-D-mannopyranoside for 5 and 10 min, respectively (indicated with *). All the samples were then processed for immunoblotting with the antiphosphotyrosine antibodies.

tyrosine-phosphorylated proteins were equally detected both in Con A-stimulated and Con A-aggregated platelets (Fig. 3, lanes B and C). Moreover, platelets that had been stimulated with Con A still underwent aggregation when stirred after removal of the lectin from the cell surface and consequent dephosphorylation of the 80-, 135-, and 150-kDa proteins. However, in this case, aggregation did not restore tyrosine phosphorylation of these proteins (Fig. 3, lanes D and E). This indicates that the effects of Con A are directly related to the binding of the lectin to the platelet surface.

We next compared the protein-tyrosine phosphorylation induced by Con A and thrombin. As shown in Figure 4, Con A- and thrombin induced tyrosine phosphorylation of a common set of proteins including the 65-, 85-, 95-, and 120-kDa bands. However other proteins were tyrosine phosphorylated in Con A- but not in thrombin-stimulated platelets: among these are the 80-, 135-, and 150-kDa bands. These proteins were not tyrosine-phosphorylated by thrombin even when platelets were stirred and aggregation occurred (data not shown). Thrombin-induced protein-tyrosine phosphorylation can be inhibited by high levels of intracellular cAMP [Pumiglia et al., 1990]. This is also shown in Figure 4, where cAMP was increased by pretreatment of platelets with the prostacyclin analog iloprost. In Con A-stimulated platelets, iloprost induced inhibition of tyrosine phosphorylation of some proteins, all of which belonged to

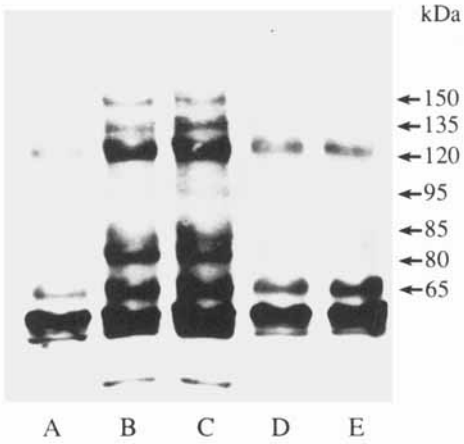

Fig. 3. Effect of platelet aggregation on Con A-induced protein-tyrosine phosphorylation. Platelet samples were incubated at 37°C in an aggregometer and then stimulated with 100 μg/ml Con A with or without stirring as indicated below. Platelet aggregation was recorded continuously. Reactions were stopped by addition of 2% SDS, and samples were immunoblotted with antibodies to phosphotyrosine. **A:** Resting platelets. **B:** Platelets stimulated with Con A for 2′ without stirring. **C:** Platelets stimulated with Con A for 2′ with stirring. **D:** Platelets stimulated with Con A for 2′ without stirring and then treated with 25 mM methyl α-D-mannopyranoside. **E:** Platelets stimulated with Con A for 2′ without stirring, then treated with 25 mM methyl α-D-mannopyranoside and then stirred. Aggregation was observed with samples C and E but not with samples A, B, and D

the set of proteins, which were also tyrosine-phosphorylated by thrombin (as the 85- and 95-kDa bands). However, tyrosine phosphorylation of the 80-, 135-, and 150-kDa bands, which was specifically induced by Con A, but not by

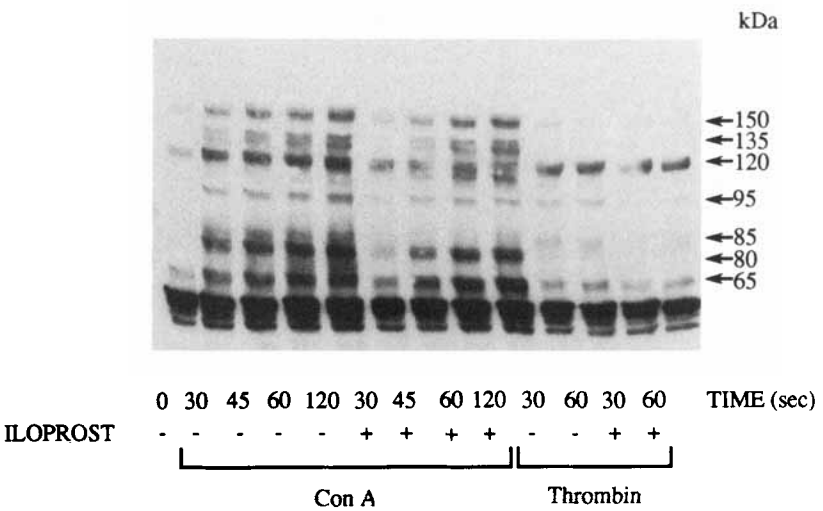

Fig. 4. Effect of iloprost on Con A- and thrombin-induced protein-tyrosine phosphorylation. Platelet samples were incubated at 37°C in the presence or in the absence of 10 μM iloprost for 30 min, and then stimulated with either 100 μg/ml Con A or 1 U/ml thrombin for the indicated times. Proteins were then separated by SDS–PAGE on 7.5% acrylamide gel and analyzed by immunoblotting with the antiphosphotyrosine antibodies.

thrombin, was unaffected by the treatment of platelets with iloprost.

Con A is a multivalent ligand, able to induce clustering of its receptor(s). The role of the cross-linking of membrane glycoproteins in Con A-induced protein-tyrosine phosphorylation was analyzed using succinyl-Con A, a derivative of the native lectin that binds to the same glycoproteins but cannot induce clustering of the receptor [Gunther et al., 1973]. As shown in Figure 5, succinyl-Con A is a less efficient stimulator of protein-tyrosine phosphorylation than the native lectin. In succinyl-Con A-treated platelets we observed tyrosine phosphorylation mainly of the 65- and 120-kDa proteins. However, proteins, whose tyrosine phosphorylation was specifically induced by Con A but not by thrombin, such as the 80- and 135-kDa bands, were not detected in platelets stimulated with succinyl-Con A.

The subcellular distribution of the tyrosine phosphorylated proteins in Con A- and thrombin-stimulated platelets was examined after cell lysis with Triton X-100. Cytoskeleton and Triton X-100-soluble material were prepared and probed with the antiphosphotyrosine antibodies (Fig. 6). In Con A- and thrombin-stimulated platelets, the 85-kDa proteins were mainly associated with the cytoskeletal fraction. The 120-kDa tyrosine-phosphorylated protein was also recovered in the cytoskeleton from thrombin-stimulated platelets. A tyrosine-phosphorylated protein with a similar, but slightly smaller molecular mass

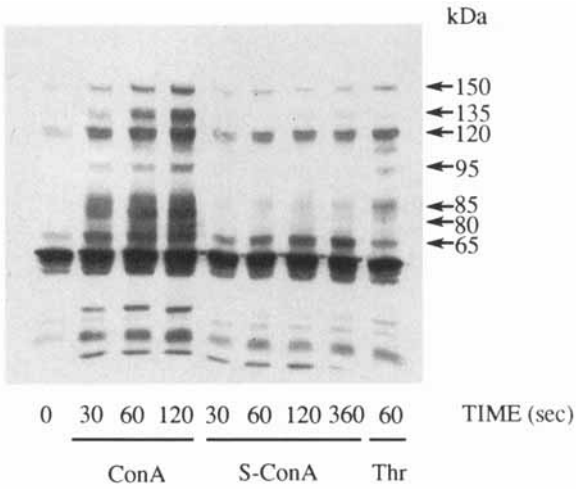

Fig. 5. Protein-tyrosine phosphorylation by Con A and succinyl-Con A. Platelet samples were incubated with 100 μg/ml Con A or 100 μg/ml succinyl-Con A (S-Con A) for the indicated times. Stimulation with thrombin (1 U/ml) was for 1 min. Samples were immunoblotted with antibodies to phosphotyrosine.

was constantly detected in the cytoskeletal from Con A-stimulated platelets. Although it is most likely that this band corresponds to the 120-kDa protein detected in the total cell lysate, it cannot be ruled out that it represents a different protein, which is specifically phosphorylated by Con A, but is masked by the 120-kDa band when total platelet lysate was analyzed. The cytoskeleton from Con A-treated cells also contained the 135- and 150-kDa proteins. However, the same proteins, especially the 150-kDa band, were also

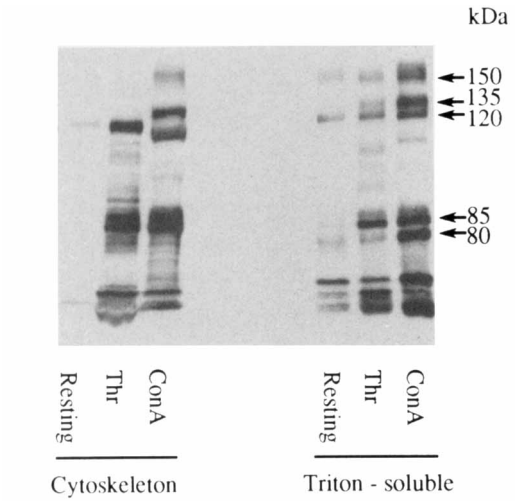

Fig. 6. Analysis of the cytoskeletal-associated tyrosine-phosphorylated proteins. Resting, Con A- and thrombin-stimulated platelets were lysed with Triton X-100. Cytoskeleton and Triton X-100-soluble faction (Triton-soluble) were prepared and analyzed by immunoblotting with the antiphosphotyrosine antibodies. Stimulation was with 100 μg/ml Con A for 2 min or with 1 U/ml thrombin for 1 min. Proteins from the same number of cells were loaded on each lane.

detected in the Triton X-100-soluble material. By contrast, the 80-kDa tyrosine-phosphorylated protein in Con A-treated platelets was exclusively found in the Triton X-100-soluble fraction, indicating that it did not associate with the cytoskeleton.

DISCUSSION

Stimulation of human platelets with physiological agonists, such as thrombin or collagen, produces tyrosine phosphorylation of several proteins [Ferrell and Martin, 1988; Golden and Brugge, 1989; Nakamura and Yamamura, 1989]. The lectin Con A behaves as a strong platelet agonist, as it induces phospholipase C activation, intracellular Ca^{2+} mobilization, secretion, and aggregation [Patscheke et al., 1978; Torti et al., 1992c; Ramaschi et al., 1993]. In this study, we show that Con A also stimulates protein-tyrosine phosphorylation in human platelets. The kinetics of this event are quite different from that reported for other platelet agonists. In the case of thrombin, for instance, tyrosine phosphorylation of some proteins is very rapid and is evident within 5–10 s of agonist treatment [Ferrell and Martin, 1988; Golden and Brugge, 1989; Nakamura and Yamamura, 1989]. Tyrosine phosphorylation induced by Con A is detected only after 20 s of stimulation. This latency is comparable to that observed for Ca^{2+} mobilization and phospholipase C activation [Torti et al., 1992c; Ramaschi et al., 1993]. Another lectin, wheat germ agglutinin, was reported to induce protein-tyrosine phosphorylation in human platelets, with kinetics more similar to that of thrombin than of Con A [Inazu et al., 1991]. Moreover, some proteins, tyrosine phosphorylated by thrombin or wheat germ agglutinin, are rapidly dephosphorylated [Ferrell and Martin, 1988; Golden and Brugge, 1989; Nakamura and Yamamura, 1989; Inazu et al., 1991], but none of the proteins that are tyrosine phosphorylated by Con A undergoes dephosphorylation within 10 min of stimulation.

By comparing the action of thrombin and Con A, we identified two groups of tyrosine-phosphorylated proteins in Con A-stimulated platelets. The first group included mainly the 65-, 95-, and 120-kDa bands, which represent substrates tyrosine phosphorylated by both agonists. In all the experiments the immunoreactivity of these proteins to the antiphosphotyrosine antibodies was greater in Con A- than in thrombin-stimulated platelets, indicating that Con A is a stronger stimulator of tyrosine phosphorylation than thrombin. It should be noted that stimulation with Con A and thrombin was performed without stirring and in the absence of platelet aggregation. It is known that aggregation further stimulates protein-tyrosine phosphorylation induced by thrombin [Ferrell and Martin, 1989; Golden et al., 1990]. However, we constantly found that the immunoreactivity of the tyrosine-phosphorylated proteins in thrombin-aggregated platelets was lower than that observed in Con A-activated platelets (data not shown). The second group of tyrosine-phosphorylated proteins was composed by substrates that were phosphorylated in platelets stimulated with Con A, but not with thrombin. This group included the 80-, 135-, and 150-kDa bands. Tyrosine phosphorylation of these proteins was not observed in thrombin-treated platelets even when aggregation occurred (data not shown).

In Con A-treated platelets, the two groups of proteins were tyrosine phosphorylated through different signal transduction pathways, as indicated by the results shown in Figures 2, 4, and 5. The addition of the haptenic sugar to Con A-treated platelets, when tyrosine phosphorylation had reached the maximal level, removed the lectin from the platelet surface and caused the rapid dephosphorylation of proteins of the second but not of the first group of proteins. This

indicates that tyrosine phosphorylation of the 80-, 135-, and 150-kDa proteins requires the persistent binding of Con A to the plasma membrane. On the other hand, tyrosine phosphorylation of the first group of proteins is produced by the transient interaction of Con A with the membrane glycoproteins. We can hypothesize that in one case the signal for activation of tyrosine kinases (or inhibition of tyrosine phosphatases) is maintained as long as the lectin remains bound to the plasma membrane, while in the other case, it is transmitted from the lectin receptor to other intracellular messengers. These intracellular messengers must also be generated by thrombin, since the first group of proteins are tyrosine phosphorylated, with different kinetics, both in Con A- and in thrombin-stimulated platelets (Fig. 4). This hypothesis is supported by the different effect of cAMP on the tyrosine phosphorylation of the two groups of proteins.

It is known that cAMP inhibits protein-tyrosine phosphorylation induced by thrombin [Pumiglia et al., 1990], probably by interfering with the agonist-induced generation of second messengers, such as inositol phosphates and cytoplasmic Ca^{2+} [Siess, 1989]. In our experiments, the inhibitory effect of cAMP on thrombin-induced tyrosine phosphorylation was particularly evident for the 85- and 95-kDa proteins. Also in Con A-stimulated platelets, the tyrosine phosphorylation of these proteins was also inhibited by the cAMP-increasing agent iloprost, indicating that they are phosphorylated through a mechanism similar to that operating in thrombin-stimulated platelets. However, iloprost had no effect on the Con A-induced tyrosine phosphorylation of the second group of proteins (80-, 135-, and 150-kDa bands).

The selective tyrosine phosphorylation of these proteins required the clustering of membrane glycoproteins. The succinyl-Con A, that binds to platelets with the same specificity of the native lectin but is not able to induce clustering of the receptor [Gunther et al., 1973] did not induce tyrosine phosphorylation of the 80-, 135-, and 150-kDa proteins. In succinyl-Con A-treated platelets we observed tyrosine phosphorylation of the 65- and 120-kDa proteins, which belong to the first group of proteins and are regulated through a different mechanism. Since the clustering of membrane glycoproteins induced by Con A can be reversed by removing the lectin from the platelet surface, the selective dephosphorylation induced by methyl α-D-mannopyranoside (Fig. 2) reflects the involvement of receptor cross-linking in the tyrosine phosphorylation of the second group of proteins.

All the presented experiments were performed in the absence of extracellular calcium, despite the fact that calcium plays a role in regulating protein-tyrosine phosphorylation in human platelets [Vostal et al., 1991] and that both thrombin and Con A can induce calcium influx across the plasma membrane [Siess, 1989; Ramaschi et al., 1993]. However, we found that the presence of 2 mM extracellular $CaCl_2$ neither modified the protein-tyrosine phosphorylation induced by Con A, nor induced the phosphorylation of the 80-, 135-, and 150-kDa proteins in thrombin-stimulated platelets (data not shown).

None of the tyrosine-phosphorylation proteins in Con A-stimulated platelets have been identified so far. Since Con A induces a strong cytoskeletal reorganization [Wheeler et al., 1985], in preliminary experiments we analyzed the distribution of the 80-, 135-, and 150-kDa tyrosine phosphorylated proteins between the Triton X-100-soluble and -insoluble fractions. The 135-kDa band was equally distributed between cytoskeleton and Triton X-100-soluble material, the 150-kDa band, although detectable in the cytoskeleton, was mostly Triton X-100 soluble in activated cells, and the 80-kDa protein was completely Triton X-100 soluble and did not associate with the cytoskeleton. The different subcellular distribution of these proteins may facilitate their identification.

In conclusion we have found two different signal transduction mechanisms, aggregation-independent, leading to tyrosine phosphorylation of different substrates in Con A-stimulated platelets. The first one is similar, if not identical, to that initiated by thrombin, but the second one is peculiar to Con A, and is mediated by the clustering of membrane glycoproteins. The identity of the receptor whose clustering by Con A induces protein-tyrosine phosphorylation is unknown. The main platelet receptor for Con A is the glycoprotein IIb–IIIa complex, which is also cross-linked upon binding of the lectin [Fitzgerald et al., 1985; Kakaiya et al., 1988]. However, we found that tyrosine phosphorylation of the 80-, 135-, and 150-kDa proteins by Con A occurs, albeit at a lower extent, in platelets from patients affected by Glanzmann thrombasthenia, which lack glycoprotein IIb–IIIa complex (data not shown). Thus the fibrinogen receptor

does not seem to be essential for this effect. This is also supported by the data presented in Figure 3, which shows that platelet aggregation upon removal of the lectin from the cell surface is not able to restore tyrosine phosphorylation of the 80-, 135-, and 150-kDa proteins. However, several adhesive proteins that interact with platelets are multivalent ligands, and can mimic the action of Con A through other integrin receptors such as the $\alpha_v\beta_3$ or the β_1 integrins. Therefore, our results might be of significant relevance, since clustering of membrane glycoproteins normally occurs during platelet aggregation, adhesion, and spreading.

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