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EPINEPHRINE INDUCES ASSOCIATION OF pp60^{src} WITH $G_{i\alpha}$ IN HUMAN PLATELETS

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SUMMARY. Using specific antibodies against the α subunit of the inhibitory GTP-binding protein G_i, we analyzed the association of $G_{i\alpha}$ with other cellular components in human platelets. Three tyrosine phosphorylated proteins with molecular mass of 63, 58, and 55 kDa were specifically associated with $G_{i\alpha}$ in resting platelets. Stimulation of platelets with epinephrine, but not with thrombin, induced an increase of the reactivity of the 63- and 55-kDa proteins to anti-phosphotyrosine antibodies on western blotting. By in vitro kinase assay we found that epinephrine induced the association of kinase activity with $G_{i\alpha}$ and that the 63-kDa protein was phosphorylated by this activity. The association of kinase activity with $G_{i\alpha}$ in epinephrine-stimulated platelets paralleled the association of pp60^{src} with $G_{i\alpha}$, as detected by western blotting analysis using specific anti-pp60^{src} monoclonal antibodies. The interaction of pp60^{src} with $G_{i\alpha}$ may play a role in the mechanism of platelet activation by epinephrine or in the epinephrine-induced potentiation of the action of other platelet agonists.

Several heterotrimeric GTP-binding proteins have been identified in human platelets (1). Among these the α subunits of the family termed G_i is the only recognized substrate for the pertussis-toxin-catalyzed ADP-ribosylation. Three forms of $G_{i\alpha}$, with molecular masses of 40-41 kDa, are present in platelets; they are referred to as $G_{i\alpha\text{-}1}$, $G_{i\alpha\text{-}2}$, and $G_{i\alpha\text{-}3}$, and share about 90% sequence homology (2). $G_{i\alpha\text{-}2}$ is the predominant isoform in platelets (2). G_i proteins mediate the inhibition of adenylate cyclase (3). Agonists such as thrombin and epinephrine cause a reduction of the level of intracellular cAMP through a pertussis-toxin-sensitive mechanism. Therefore, both thrombin and α_2-adrenergic receptors are coupled with G_i in human platelets. Despite this, there are several differences in the signal transduction pathways initiated by the two agonists. Platelet stimulation with thrombin is associated with rapid hydrolysis of membrane-bound inositol phospholipids, intracellular Ca^{2+} mobilization and protein kinase C activation (4). None of these events occur in

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Abbreviations: G_i, inhibitory GTP-binding protein; $G_{i\alpha}$, subunit of G_i, SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride.

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response to epinephrine (5,6). Epinephrine can induce activation of the Na^+/H^+ antiporter, fibrinogen binding, and platelet aggregation, through a mechanism that is still largely unknown (7-9). On the other hand, it is well known that subthreshold concentrations of epinephrine can potentiate the stimulatory effects of other agonists such as thrombin, through a mechanism independent from Na^+/H^+ antiporter activation (10,11). Moreover, epinephrine can reverse the desensitization of thrombin-induced Ca^{2+} mobilization and phospholipase C activation caused by prolonged stimulation. This effect is mediated by the α_2-adrenergic receptor and probably involves G_i (6,12).

Some nondividing cells, such as red blood cells and platelets, possess high level of tyrosine kinase activity (13). In human platelets the most abundant tyrosine kinase is $pp60^{src}$, which represents about 0.2-0.4% of the total proteins (14). Several other Src-related tyrosine kinases are expressed in platelets: Fyn (61 kDa), Hck (61 kDa), Lyn (54 and 58 kDa) (15-17). Agonists such as thrombin and collagen can induce rapid tyrosine phosphorylation of several platelet proteins, demonstrating that at least some of the tyrosine kinases are activated during platelet stimulation (14, 18, 19). A role for the platelet membrane glycoprotein IIb-IIIa complex in the agonist-induced tyrosine phosphorylation has been demonstrated (20,21). So far, $pp60^{src}$ and the $p21^{ras}$ GTPase activating protein are the only proteins that have been recognized to be tyrosine phosphorylated upon platelet stimulation with thrombin (18, 22, 23). However, the role of tyrosine phosphorylation and the mechanism of agonist-induced tyrosine kinase activation in human platelets are still unknown. Recently, some interesting associations have been reported. Yes, Fyn and Lyn are associated with the membrane glycoprotein IV in resting platelets (17). In thrombin-stimulated platelets Src and Fyn associate with the phosphatidylinositol 3-kinase (24), and Yes, Fyn and Lyn have been found associated with the $p21^{ras}$ GTPase activating protein (22).

In the present study we demonstrate that tyrosine phosphorylated proteins are specifically associated with $G_i\alpha$ in resting platelets. This association is increased by epinephrine but not by thrombin. We identified one of the tyrosine phosphorylated proteins associated with $G_i\alpha$ in epinephrine-stimulated platelets as the tyrosine kinase $pp60^{src}$.

MATERIALS AND METHODS

Materials. Human α-thrombin was a gift from Dr. John Fenton II, Division of Laboratories and Research, New York State Department of Health, Albany, NY. Anti-phosphotyrosine antibodies were obtained from Upstate Biotechnology Inc. Anti-$pp60^{src}$ monoclonal antibodies were from Oncogene Sciences. Octyl-β-D-glucopyranoside was purchased from Calbiochem. [γ-^{32}P]ATP was from ICN. Nitrocellulose membranes were from Schleicher and Schull. Protein A-Sepharose was from Pharmacia. Prestained molecular weight markers were from Bio-Rad. Epinephrine and all other reagents were from Sigma.

Platelet preparation and $G_{i\alpha}$ immunoprecipitation. Blood was obtained from healthy volunteers using ACD (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid) as anticoagulant, and centrifuged at 180 x g for 20 min at room temperature. The platelet-rich plasma was incubated with 1 mM aspirin at 37°C for 20 min, and then platelets were prepared by gel-filtration using Hepes buffer (10 mM Hepes, pH 7.4; 137 mM NaCl; 2.9 mM KCl; 12 mM $NaHCO_3$) as previously described (25). Platelet suspensions (10^9 cells/ml, 0.25 ml samples) were incubated at 37°C and, in some cases, stimulated with 10 nM thrombin or 100 μM epinephrine for 30 sec. Platelets were lysed by adding an equal volume of immunoprecipitation buffer (50 mM Tris, pH 7.4; 0.1 M NaCl; 0.2% BSA, 0.25 mM PMSF, 1 mM Na_3VO_4) containing 1.5% octyl-β-D-glucopyranoside. Samples were vortexed and placed on ice for 30 min. The insoluble material was removed by centrifugation at 12,000 x g for 10 min. The

supernatant was diluted with immunoprecipitation buffer to obtain a final octyl-β-D-glucopyranoside concentration of 0.413%, and then precleared with protein A-Sepharose (50 µl of a 50 mg/ml stock solution) at 4°C for 30 min. The precleared supernatants were used for further immunoprecipitations. $G_{i\alpha}$ was immunoprecipitated using specific antibodies produced against the synthetic peptide KENLKDCGLF, and subsequently affinity-purified as previously described (26). Typically 15 µl of the antibody preparation was used for each sample. In some experiments, the anti-$G_{i\alpha}$ antibodies were incubated with the synthetic peptide KENLKDCGLF (15 µl antibodies: 100 µg peptide) at 4°C before the immunoprecipitation, in order to neutralize the antibodies. Immunoprecipitations were carried out for 2 h at 4°C. Protein A-Sepharose was added and samples were incubated for 30 min at 4°C. The immunocomplexes were recovered by brief centrifugation, washed three times with 50 mM Tris (pH 7.4), 0.2 M NaCl, 1 mM Na_3VO_4, and finally resuspended in SDS-sample buffer (60 mM Tris, pH 8.0; 2% SDS; 2% dithiothreitol; 10% glycerol; 0.01% bromophenol blue). Samples were boiled 10 min before being subjected to SDS-PAGE.

Immunoblotting. This was performed as previously described (27).

Kinase assay. The immunoprecipitates on the protein A-Sepharose beads were resuspended in 50 µl of 10 mM Tris (pH 7.4), 5 mM $MgCl_2$, 1 mM Na_3VO_4, 1 µM ATP and 20 µCi [γ-^{32}P]ATP for 15 min at 37°C. The reaction was stopped by the addition of 450 µl of cold immunoprecipitation buffer. The immunoprecipitates were recovered by centrifugation and washed twice with 50 mM Tris (pH 7.4) 0.2 M NaCl, 1 mM Na_3VO_4. Samples were solubilized with SDS-sample buffer and subjected to SDS-PAGE followed by autoradiography.

RESULTS AND DISCUSSION

Association of $G_{i\alpha}$ with phosphotyrosine proteins in human platelets

The α subunit of G_i was immunoprecipitated from platelet lysates using specific antibodies. The immunoprecipitated material was subjected to SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies (Fig. 1A). Four tyrosine phosphorylated proteins were identified in the $G_{i\alpha}$ immunoprecipitate. By comparison with known protein markers, the molecular weights of these proteins were 63, 58, 55, and 36 kDa. To test the specificity of these associations similar immunoprecipitations were carried out using anti-$G_{i\alpha}$ antibodies neutralized by preincubation with the immunizing peptide KENLKDCGLF. Under these conditions no detectable $G_{i\alpha}$ was present in the immunoprecipitated material, as confirmed by western blotting analysis using anti-$G_{i\alpha}$ antibodies (Fig. 1B). When this sample was analyzed on western blotting with anti-phosphotyrosine antibodies only the 36 kDa protein was detected (Fig. 1A). These observations indicated that the 36 kDa phosphotyrosine protein was non-specifically recovered in the immunocomplex, and demonstrated that the phosphotyrosine-containing proteins of 63, 58, and 55 kDa were specifically associated with $G_{i\alpha}$ in resting platelets.

We then analyzed the effect of thrombin and epinephrine, platelet agonists whose receptors couple to G_i, on the association of phosphotyrosine proteins with $G_{i\alpha}$. Platelets were stimulated with 10 nM thrombin or 100 µM epinephrine for 30 sec, and then were lysed and immunoprecipitated with anti-$G_{i\alpha}$ antibodies or anti-$G_{i\alpha}$ antibodies neutralized by preincubation with the immunizing peptide. As shown in Fig. 1A no significant differences in the intensity of the 63, 58 and 55 kDa proteins were detected after thrombin stimulation. However, when $G_{i\alpha}$ was immunoprecipitated from epinephrine-stimulated platelets, an increase of the reactivity of the 63 and 55 kDa proteins to anti-phosphotyrosine antibodies was evident. No phosphotyrosine proteins, except for the non-specifically recovered 36 kDa, were present when immunoprecipitations were blocked by the immunizing peptide (Fig. 1A). Similar results were obtained after 60 seconds of stimulation with thrombin or epinephrine (not shown). When the same samples were blotted with the $G_{i\alpha}$ antibodies

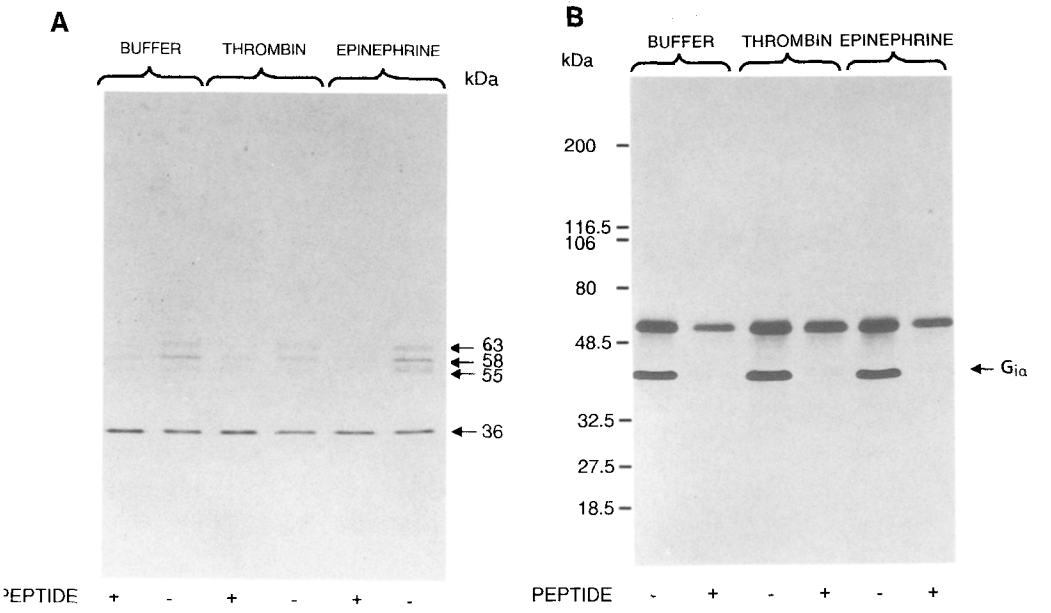

Figure 1. Association of Phosphotyrosine proteins with $G_{i\alpha}$. Platelets were incubated with 10 nM thrombin, 100 µM epinephrine or an equal volume of buffer for 30 sec at 37°C and then lysed. Samples were immunoprecipitated with anti-$G_{i\alpha}$ antibodies or anti-$G_{i\alpha}$ antibodies preincubated with the antigen peptide.
A. Proteins were separated by SDS-PAGE on a 4-20% polyacrylamide gradient gel and immunoblotted with anti-phosphotyrosine antibodies. The molecular weights of the identified proteins are indicated.
B. Proteins were separated by SDS-PAGE on a 10-20% polyacrylamide gradient gel and immoblotted with anti-$G_{i\alpha}$ antibodies. The position of $G_{i\alpha}$ is indicated. The bands at about 50 kDa are the heavy chains of the immunoglobulins.

it was clear that the same amount of $G_{i\alpha}$ was recovered in the immunoprecipitates from resting, thrombin- and epinephrine-stimulated platelets (Fig. 1B). Moreover, the specificity of the immunoprecipitations was confirmed by the absence of $G_{i\alpha}$ in the immunocomplexes obtained using neutralized antibodies (Fig. 1B)

Epinephrine increases the association of phosphotyrosine proteins to $G_{i\alpha}$

The increased reactivity of the 63 and 55 kDa proteins to anti-phosphotyrosine antibodies detected in the $G_{i\alpha}$ immunoprecipitates from epinephrine-stimulated platelets could be due to either increased association of these proteins or increased tyrosine phosphorylation. To distinguish between these two possibilities, platelet lysates from resting and epinephrine-stimulated cells were probed with anti-phosphotyrosine antibodies on western blotting. As shown in Fig. 2, epinephrine alone was not able to induce tyrosine phosphorylation in human platelets, or to increase the phosphotyrosine content of proteins already phosphorylated in resting cells. Therefore, the higher reactivity of the 63 and 55 kDa proteins to anti-phosphotyrosine antibodies in the $G_{i\alpha}$ immunocomplex from epinephrine-stimulated platelets was due to an epinephrine-induced increase in association of these proteins to $G_{i\alpha}$ rather than to epinephrine-induced tyrosine phosphorylation.

Epinephrine increases the association of tyrosine kinases to $G_{i\alpha}$

Most of the Src-related tyrosine kinases that are expressed in human platelets have molecular masses ranging from 55 to 62 kDa, and they are often tyrosine phosphorylated (14-17). Therefore, we

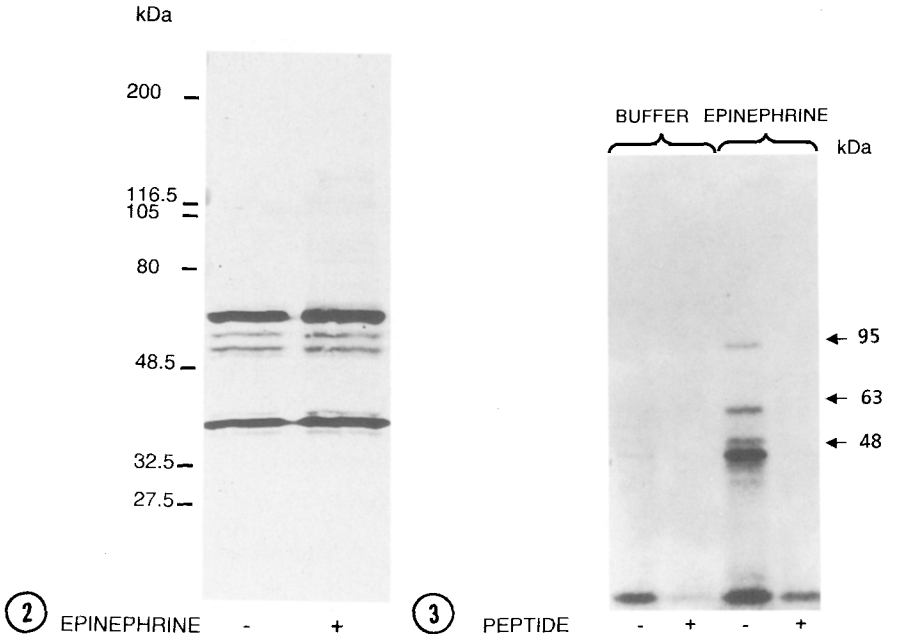

Figure 2. Epinephrine does not induce tyrosine phosphorylation. Platelet samples were incubated without or with 100 μM epinephrine at 37°C for 30 sec. Reactions were stopped by adding SDS-sample buffer. Proteins were separated by SDS-PAGE on a 4-20% polyacrylamide gradient gel, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibodies. The positions of the molecular weight markers are indicated on the left.

Figure 3. Epinephrine stimulates the association of protein kinase(s) with $G_{i\alpha}$. Resting and epinephrine-stimulated platelets (100 μM epinephrine, 30 sec at 37°C) were immunoprecipitated with anti-$G_{i\alpha}$ antibodies or anti-$G_{i\alpha}$ antibodies neutralized with the antigen peptide. Immunocomplexes were subjected to kinase assay as described under 'Materials and Methods', and then separated on a 4-20% polyacrylamide gradient gel. Proteins were transferred to nitrocellulose and subjected to autoradiography. The molecular weight of the principal phosphorylated proteins are indicated.

considered that one or more of the tyrosine phosphorylated proteins associated with $G_{i\alpha}$ might be a tyrosine kinase. To test this possibility, we assayed for the kinase activity in the $G_{i\alpha}$ immunoprecipitates. Resting and epinephrine-stimulated platelets were lysed and immunoprecipitated with the anti-$G_{i\alpha}$ antibodies. As a control for the specificity of the reaction, similar samples were immunoprecipitated with anti-$G_{i\alpha}$ antibodies preincubated with the immunizing peptide, a condition that specifically blocks the recovery of $G_{i\alpha}$ (Fig. 1B). The immunocomplexes on the protein A-Sepharose beads were incubated with [γ-^{32}P]ATP at 37°C for 15 min. The beads were then washed and the proteins separated by SDS-PAGE, transferred to nitrocellulose and analyzed by autoradiography. As shown in Fig. 3 several phosphorylated proteins were detected in the immunocomplex from epinephrine-stimulated platelets, but not from resting platelets. The molecular weight of the more evident proteins were 95, 63 and 48 kDa. When the $G_{i\alpha}$ immunoprecipitations were blocked by the immunizing peptide, none of these phosphorylated proteins was detected (Fig. 3). This confirmed that their phosphorylation was catalyzed by kinase(s) specifically associated with $G_{i\alpha}$ in epinephrine-stimulated but not in resting platelets. The identity of

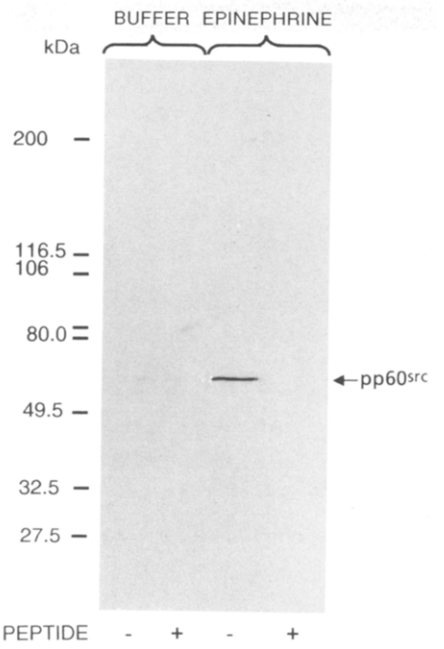

Figure 4. Epinephrine stimulates the association of pp60^{src} with $G_{i\alpha}$. The immunocomplexes obtained from resting and epinephrine-stimulated platelets (100 µM epinephrine, 30 sec) using anti-$G_{i\alpha}$ antibodies or neutralized anti-$G_{i\alpha}$ antibodies were separated on a 4-20% polyacrylamide gradient gel. Proteins were transferred to nitrocellulose and probed with anti-pp60^{src} antibodies. The position of molecular weight markers are indicated on the left and pp60^{src} is indicated on the right.

the phosphorylated proteins shown in Fig. 3 is unknown. However, it is known that a general feature of pp60^{src} and other Src-related kinases is the ability to catalyze autophosphorylation. Therefore, it is possible that the phosphorylated proteins shown in Fig. 3, or at least some of them, are not only the substrates for the $G_{i\alpha}$-associated kinases, but the kinases themselves. It is noteworthy, in this regard that the phosphorylated protein of 63 kDa in Fig. 3 has the same electrophoretic mobility of the 63 kDa tyrosine-phosphorylated protein in Fig. 1A. In previous experiments we also found the strongest tyrosine-phosphorylated protein detected in a whole lysate from resting platelets (Fig 2) had an apparent molecular mass of 63 kDa in our experimental conditions, and it was identified as pp60^{src} (not shown). Therefore, pp60^{src} was a good candidate for the $G_{i\alpha}$-associated kinase.

Epinephrine increases the association of pp60^{src} with $G_{i\alpha}$

We probed the $G_{i\alpha}$ immunocomplexes obtained from resting and epinephrine-stimulated platelets with anti-pp60^{src} monoclonal antibodies. As shown in Fig. 4, pp60^{src} was clearly detected in the $G_{i\alpha}$ immunoprecipitate from epinephrine-stimulated cells. The association of pp60^{src} to $G_{i\alpha}$ was specific, because it was not observed when the immunoprecipitation was blocked by the immunizing peptide (Fig. 4). In other experiments, we found that pp60^{src} was recognized by specific antibodies and the 63 kDa protein detected with anti-phosphotyrosine antibodies had the same electrophoretic mobility (not shown). Interestingly, pp60^{src} was not detected in the $G_{i\alpha}$ immunoprecipitate from resting platelets (Fig. 4), although a 63 kDa tyrosine phosphorylated protein was present (Fig. 1A). Therefore, we believe that another tyrosine phosphorylated protein of 63 kDa, distinct from pp60^{src},

is constitutively associated with $G_{i\alpha}$ in human platelets. The association of $pp60^{src}$ with $G_{i\alpha}$ is specifically induced by epinephrine, and this accounts for the increased reactivity to anti-phosphotyrosine antibodies of the 63 kDa band in the $G_{i\alpha}$ immunoprecipitate from epinephrine-stimulated platelets shown in Fig. 1A.

The anti-$G_{i\alpha}$ antibodies used in our experiments must be considered anti-$G_{i\alpha}$-common antibodies, because they do not discriminate among $G_{i\alpha-1}$, $G_{i\alpha-2}$ and $G_{i\alpha-3}$ (27). Therefore, on the basis of the presented results, it is impossible to say which one of the three $G_{i\alpha}$ isoforms is associated with $pp60^{src}$ in epinephrine-stimulated platelets. $G_{i\alpha-2}$ is the predominant isoform present in platelets and it is responsible for the inhibition of adenylate cyclase (1-3). $G_{i\alpha-2}$ is much more abundant than $G_{i\alpha-3}$, and very low amounts of $G_{i\alpha-1}$ have been detected (2). The fact that a relatively low percentage of the total platelet $pp60^{src}$ was associated with $G_{i\alpha}$ in epinephrine-stimulated cells suggested that $G_{i\alpha-1}$ or $G_{i\alpha-3}$, rather than $G_{i\alpha-2}$ might be responsible for this interaction. The identity of the 63 kDa protein distinct from $pp60^{src}$ and the 58 and 55 kDa proteins associated with $G_{i\alpha}$ is still unknown. These molecular weights are comparable with the molecular weights of some Src-related kinases. Therefore, in the absence of data with specific antibodies, the possibility that more tyrosine kinases, other than $pp60^{src}$, are associated with $G_{i\alpha}$ in human platelets cannot be ruled out. The association of more than one Src-related kinase with the same protein is not an unusual event, because it has already been demonstrated for glycoprotein IV, phosphatydylinositol 3-kinase and $p21^{ras}$ GTPase activating protein (17, 22, 24).

The functional relevance of the association of $pp60^{src}$ and $G_{i\alpha}$

The role of the interaction between $pp60^{src}$ and $G_{i\alpha}$ is still unclear. Epinephrine, induces this association but does not seem to activate tyrosine kinases, whereas thrombin activates tyrosine kinases and it does not induce the association of $G_{i\alpha}$ with $pp60^{src}$. Therefore, it seems unlikely that the association of $G_{i\alpha}$ with $pp60^{src}$ leads to the activation of the kinase. However, several aspects of epinephrine mechanism of action on platelets are still waiting for an explanation. Among these, the well known ability of epinephrine to potentiate the effects of other agonists and to reverse the desensitization to thrombin after prolonged stimulation, and the recently demonstrated suppression of the rap1B.GAP-activated GTPase activity (28). Therefore, it is possible that the epinephrine-induced association of $G_{i\alpha}$ with $pp60^{src}$ may play a role in one or more of these events

Of particular interest is the report describing translocation of $pp60^{src}$ to the platelet cytoskeleton during thrombin-stimulated aggregation (29). As epinephrine stimulates the association of $G_{i\alpha}$ with the cytoskeletal matrix (30), and induces the physical coupling of $G_{i\alpha}$ with $pp60^{src}$, the present work provides a functional link between these two events.

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