Tyrosine Phosphorylation of the Novel Protein-tyrosine Kinase RAFTK during an Early Phase of Platelet Activation by an Integrin Glycoprotein IIb-IIIa-independent Mechanism*

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A key regulatory event controlling platelet activation is mediated through the phosphorylation of several cellular proteins by protein-tyrosine kinases. The related adhesion focal tyrosine kinase (RAFTK) is a novel cytoplasmic tyrosine kinase and a member of the focal adhesion kinase (FAK) gene family. FAK phosphorylation in platelets is integrin-dependent, occurs in a late stage of platelet activation, and is dependent on platelet aggregation. In this study, we have investigated the involvement of RAFTK phosphorylation during different stages of platelet activation. Treatment of platelets with thrombin induced, in as early as 10 s, a rapid tyrosine phosphorylation of RAFTK in a time- and concentration-dependent manner. Treatment of platelets with thrombin in the absence of stirring or pretreatment of platelets with RGDS peptide prevented platelet aggregation, but not RAFTK phosphorylation. Furthermore, phosphorylation of RAFTK did not require integrin engagement since platelets treated with the 7E3 inhibitory antibodies that block fibrinogen binding to glycoprotein IIb-IIIa did not inhibit RAFTK phosphorylation. Similarly, platelets treated with LIBS6 antibodies, which specifically activate glycoprotein IIb-IIIa, did not induce RAFTK phosphorylation. Stimulation of platelets by several agonists such as collagen, ADP, epinephrine, and calcium ionophore A23187 induced RAFTK phosphorylation. Tyrosine phosphorylation of RAFTK in platelets is regulated by calcium and is mediated through the protein kinase C pathway. Phosphorylation of RAFTK is dependent upon the formation of actin cytoskeleton as disruption of actin polymerization by cytochalasin D significantly inhibited this phosphorylation. The RAFTK protein appears to be proteolytically cleaved by calpain in an aggregation-dependent manner upon thrombin stimulation. These results demonstrate that RAFTK is tyrosine-phosphorylated during an early phase of platelet activation by an integrin-independent mechanism and is not dependent on platelet aggregation, suggesting different mechanisms of regulation for FAK and RAFTK phosphorylation during platelet activation.

Bone marrow megakaryocytes produce platelets, cells critical for the maintenance of normal hemostasis (1). Upon blood vessel injury, platelets activate various intracellular signaling pathways involved in thrombus formation (1). A signal transduction cascade is initiated that causes platelets to change shape by extension of the filopodia, to secrete the contents of intracellular granules, and to aggregate by promoting the binding of the major integrin GPIIb-IIIa1 to its adhesive ligand, fibrinogen (2). Platelet activation also triggers polyphosphoinositol turnover, calcium mobilization and influx, and changes in protein phosphorylation (2).

Platelet activation is followed by a significant increase in the tyrosine phosphorylation of several cellular proteins by protein-tyrosine kinases (PTKs) (2). Many of these agonist-induced phosphorylation events in platelets have been shown to be regulated by integrins (2). Agonist-induced platelet tyrosine phosphorylation has been divided into three temporal waves based upon their dependence on integrin (GPIIb-IIIa) binding to fibrinogen or on subsequent platelet aggregation (2). PTKs such as src family kinases, mitogen-activated protein (MAP) kinase, cortactin and GAP are phosphorylated during an early phase of platelet activation by an integrin-independent mechanism. Syk is activated by fibrinogen binding to and dimerization of GPIIb-IIIa, while FAK is phosphorylated during a late stage of platelet activation that is dependent on platelet aggregation (2). Furthermore, PTKs such as Src family kinases and Syk have been shown to alter their cellular localization to the cytoskeleton upon platelet activation. It is believed that integrins and the cytoskeleton may serve to anchor and compartmentalize kinases to form signaling complexes that regulate many cellular functions (3, 4). An important research area of platelet activation is to identify the proteins involved in these signaling pathways and to dissect their mechanisms of regulation.

We have recently cloned and characterized a novel human cytoplasmic tyrosine kinase termed RAFTK (for related adhesion focal tyrosine kinase) (5), also known as Pyk2 or CAK-β (6, 7). RAFTK is related to FAK (48% identity, 65% similarity), which is known to play an important role in cell adhesion (4). Analysis of their deduced amino acid sequences also indicates that RAFTK, like FAK, lacks a transmembrane region, myristylation sites, and SH2 and SH3 domains. RAFTK also contains a kinase domain flanked by large N-terminal and C-terminal domains, and the C-terminal region contains a proline-rich stretch of residues (indicating its capability to interact with actin-binding proteins).

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This work is dedicated to the memory of Dananagoud Hiregowdara.

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* The abbreviations used are: GP, glycoprotein; FAK, focal adhesion kinase; PKC, protein kinase C; PTK, protein-tyrosine kinase; MAP, mitogen-activated protein; RAFTK, related adhesion focal tyrosine kinase; CMK, megakaryocytic cell line; RIPA, radioimmuno precipitation buffer; BAPTA-AM, [1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetoxyethyl]ester.
RAFTK Tyrosine Phosphorylation in Platelets

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RAFTK is rapidly tyrosine-phosphorylated in thrombin-stimulated platelets—To eludicate the role of RAFTK phosphorylation in platelets, the effect of thrombin on RAFTK tyrosine phosphorylation was investigated. Thrombin induced a dose- and time-dependent phosphorylation of RAFTK in platelets (Fig. 1). There appeared to be a clear threshold of response between 0.05 and 0.2 unit/ml thrombin. A time course of thrombin stimulation in platelets showed a rapid induction of RAFTK phosphorylation (Fig. 1B). Activation was observed as early as 10 s post-thrombin stimulation, reaching a maximum at 2 min and tapering off by 10 min (Fig. 1B). These results show that thrombin stimulation of platelets induces RAFTK phosphorylation in a time- and concentration-dependent manner.

RAFTK is an Endogenous Substrate for Calpain—We consistently observed an apparent decrease in RAFTK protein levels after thrombin stimulation (Fig. 1, A and B, bottom panels), despite using an equal number of platelets for the immunoprecipitations (×10^9/ml) and loading equal amounts of total proteins. This observation was consistent in repeated experiments and was not due to technical variabilities such as the number of platelets used or type of procedures followed for solubilization and preparation of platelets.

Calpain constitutes most of the calcium-dependent protease activity in platelets (12–14). Agonist-induced activation of calpain (12–15) and limited proteolysis of some specific substrates (16–18) have been reported during the course of platelet acti-
RESULTS

RAFTK Phosphorylation Is Regulated by Actin Polymerization—Thrombin stimulation in platelets leads to actin polym-
raftification and causes dramatic rearrangements of the cytoskeleton, thereby inducing the formation of focal contact-like areas (24). We examined whether the phosphorylation of RAFTK was affected by agents that disrupt the actin cytoskeleton. Pretreatment of platelets with cytochalasin D blocks agonist-induced actin polymerization, but does not inhibit platelet aggregation. We observed that pretreatment with cytochalasin D significantly inhibited the tyrosine phosphorylation of RAFTK in thrombin-stimulated platelets (Fig. 4, top panel). Furthermore, the proteolytic processing of RAFTK is not inhibited in cytochalasin D-treated platelets (Fig. 4, bottom panel). This finding is consistent with our previous observations that RAFTK processing is dependent on platelet aggregation, because cytochalasin D treatment inhibits actin polymerization, but not platelet aggregation.

**Phosphorylation of RAFTK Is Induced by Several Platelet Agonists**—To examine whether RAFTK is phosphorylated by agonists other than thrombin, platelets were activated by a strong agonist such as collagen and weak agonists such as ADP and epinephrine. RAFTK phosphorylation was studied in response to collagen, and under conditions of stirring, collagen caused platelet aggregation and the tyrosine phosphorylation of RAFTK (Fig. 5A).

Platelets were also activated by ADP and epinephrine in the absence of stirring. Under these conditions, GPIIb-IIIa was activated and was capable of binding to fibrinogen, and no aggregation took place. We monitored platelet aggregation in these experiments by aggregometry. ADP alone in the presence of stirring induced RAFTK phosphorylation after 2 min, while epinephrine did not have any effect on RAFTK phosphorylation (Fig. 5B). Furthermore, the combination of ADP and epinephrine in the presence of stirring induced a rapid RAFTK phosphorylation within 1 min (Fig. 5B).

**Phosphorylation of RAFTK by the Calcium Ionophore A23187**—RAFTK phosphorylation in platelets was studied in response to the calcium ionophore A23187 (1 μM). A23187 induced within 1 min a rapid tyrosine phosphorylation of RAFTK which reached its highest level within 2 min. These results indicate that RAFTK is rapidly phosphorylated in response to this calcium ionophore treatment, resulting in enhanced levels of phosphorylation and causing dramatic rearrangements of the cytoskeleton.
Fig. 5A, time course of RAFTK phosphorylation in collagen-stimulated platelets. Platelets were stirred in the absence or presence of collagen for the times indicated and then lysed in RIPA. Lysates were immunoprecipitated (IP) with RAFTK antibodies (R-4250) or normal rabbit serum (NRS) and analyzed on immunoblots probed with phosphotyrosine antibodies PY20 or anti-RAFTK. B, time course of the tyrosine phosphorylation of RAFTK in response to ADP and epinephrine in platelets. Platelets were unstimulated or stimulated with thrombin or a combination of ADP (10 μM) and epinephrine (10 μM), unstirred (−) or stirred (+) at the times indicated. Lysates were immunoprecipitated (IP) with RAFTK antibodies (R-4250) or normal rabbit serum (NRS) and analyzed on immunoblots probed with phosphotyrosine antibodies PY20 or anti-RAFTK. Results are representative of three independent experiments.

Tyrosine Phosphorylation of RAFTK Is Mediated by PKC in Platelets—The possible involvement of PKC in RAFTK stimulation in platelets was investigated. In response to thrombin stimulation in the presence of the PKC inhibitors calphostin C and bisindolylmaleimide, the tyrosine phosphorylation of RAFTK was completely blocked by both PKC inhibitors (Fig. 7).

DISCUSSION

In this report, we have examined the signaling mechanisms involved in the tyrosine phosphorylation of the newly identified protein-tyrosine kinase, RAFTK, a member of the tyrosine kinase FAK family. We have demonstrated that thrombin induced a rapid tyrosine phosphorylation of RAFTK in a time- and concentration-dependent manner. RAFTK phosphorylation, unlike FAK phosphorylation, occurred in the early phase of platelet activation, was not dependent on platelet aggregation, and did not require integrin engagement. In addition, stimulation of platelets by collagen, calcium ionophore A23187, ADP, and epinephrine induced RAFTK tyrosine phosphorylation. We also observed that RAFTK phosphorylation was regulated by calcium and mediated through the PKC pathway and that phosphorylation of RAFTK is dependent upon the formation of active cytoskeleton.

Agonist-induced platelet aggregation and secretion parallels a rapid and dramatic increase in the tyrosine phosphorylation of multiple proteins (3). The phosphorylation of these proteins occurs in three temporal phases that can be experimentally distinguished: the early tyrosine phosphorylation of proteins such as p21ras GAP (25), cortactin (20, 26), and p60src (3) occurs by an integrin-independent mechanism. Fibrinogen binding to the integrin GPIIb-IIIa initiates a second wave of tyrosine phosphorylation (23, 27), which is followed by a third wave of platelet aggregation-dependent tyrosine phosphorylation of several proteins such as FAK (21). Our study indicates that activation of the tyrosine phosphorylation of RAFTK in platelets is rapid (as early as 10 s) and is dependent on events induced by fibrinogen binding to GPIIb-IIIa (Fig. 3). Tyrosine phosphorylation of RAFTK does not require platelet aggregation (Fig. 3). This suggests that phosphorylation of RAFTK occurs in the early phase of platelet activation by an integrin GPIIb-IIIa-independent mechanism and that phosphorylation of RAFTK occurs upstream of the platelet aggregation stage, which is not mediated by fibrinogen binding to GPIIb-IIIa. Interestingly, these results are different from studies on FAK (21). FAK phosphorylation in platelets is dependent on coordinated signaling through occupied integrins and agonist receptors, and occurs in the late phase of platelet activation (2, 21, 23). We have previously shown (8) integrin-dependent phosphorylation of RAFTK upon stimulation with fibronectin in CMK megakaryocytic cells and in COS cells transfected with a FLAG-RAFTK pcDNA3 neo construct. Also, RAFTK phosphorylation was recently observed to be dependent on the β1 integrin in B cells (26). In this report, RAFTK phosphorylation was found to be independent of the integrin GPIIb-IIIa activation. These apparently discrepant results could be due to differences in the cell systems as well as the type of integrins utilized when cells are plated on fibronectin versus fibrinogen. However, other platelet integrins, such as the fibronectin integrin α5β1, might be involved in RAFTK tyrosine phosphorylation.

In this study, we examined tyrosine phosphorylation of RAFTK downstream from a G-protein-coupled thrombin receptor. Thrombin interacts with a membrane-bound receptor on platelets, which is coupled through the G protein. This interaction leads to activation of several forms of phospholipase C...
and inhibition of adenylate cyclase via the G proteins Gα and Gβ, respectively (29). Upon thrombin activation of platelets, phosphoinositide turnover, a rise in intracellular calcium, and protein kinase C activation are accompanied by a change in GPIIb-IIIa conformation and mobilization of arachidonic acid release via a calcium-sensitive cytosolic phospholipase A2 (30). RAFTK is phosphorylated rapidly and transiently upon the thrombin treatment of platelets, with its phosphorylation returning to base line levels within 10 min (Fig. 1B). Therefore, RAFTK could be added to the group of proteins (such as Src (3), GAP (25), cortactin (20, 26), and MAP kinase (3, 27)) that are phosphorylated on tyrosine during the early phase of platelet activation. Studies with PYK2/RAFTK in PC-12 cells have linked intracellular Ca2+ signals and both Gα or Gβ protein-coupled receptors with the activation of the MAP kinase signaling pathway (9, 10). In agreement with the results from PC-12 cells (9, 10), our studies in platelets demonstrated involvement of a similar G protein-linked pathway (thrombin) and involvement of Ca2+ and PKC in RAFTK phosphorylation. Furthermore, it has been shown that activation of the MAP kinase pathway in thrombin or collagen activated platelets is blocked by a PKC inhibitor indicating that it is either independent of Ras or that the Ras-MAP kinase pathway requires coactivation of PKC (31). Since thrombin was shown to stimulate the activity of the MAP kinases ERK1, ERK2, and p38 in platelets (32), it is possible that RAFTK might be linked with the MAP kinase pathway in platelets.

We observed an apparent dose- and time-dependent decrease in RAFTK protein levels after thrombin, collagen, or calcium ionophore treatments (Figs. 1, 5, and 6). Although the amount of RAFTK protein present was reduced, the anti-phosphotyrosine signal was strong, indicating that the level of tyrosine phosphorylation of RAFTK was greatly increased. The apparent decrease in RAFTK protein levels could be due to protein degradation (33) by calpain or other proteases (12, 13, 20, 34–36) or due to its redistribution to the actin-rich cytoskeletal complexes upon RAFTK phosphorylation (27). Calpain constitutes most of the calcium-dependent protease activity in platelets (12, 13). Activation of the calpain enzyme and cleavage of specific protein substrates, actin-hinding protein, P235, and spectrin were reported in platelets (17). Some of the known substrates of calpain are cytoskeletal proteins and also kinases such as Src (17). Thrombin is one of the agonists that activates calpain (12, 14, 15), and limited proteolysis of some specific substrates (16–18) was observed during the course of platelet activation. In this study, we demonstrate that the decrease in immunoreactive RAFTK protein levels is mediated by calpain in an aggregation-dependent manner (Fig. 2). Calpeptin, a membrane-permeable inhibitor of calpain (19), at a concentration of 20 μM did not inhibit platelet aggregation or secretion induced by thrombin, but inhibited the decrease in RAFTK protein levels (Fig. 2). We consistently observed two lower molecular mass RAFTK fragments of ~80 and ~75 kDa in thrombin-treated platelets, but not in untreated platelets or calpeptin-treated platelets (Fig. 2). Immunoprecipitations were performed with RAFTK antisera raised against a glutathione S-transferase-fusion protein containing the C terminus (681–1009 amino acid residues) of human RAFTK cdNA (8). Therefore, this antibody does not recognize fragments in the N terminus of RAFTK. Current studies are aimed at generating monoclonal and polyclonal antibodies for the various domains of RAFTK, which will be useful to further analyze RAFTK proteolytic cleavage products. Interestingly, FAK protein levels after thrombin stimulation were also decreased in our studies (Fig. 3B). In support of our observations, it has recently been reported that FAK undergoes sequential proteolytic modification by calpain in thrombin-, collagen-, and calcium ionophore A23187-stimulated platelets (34).

PKC plays a central role in the transduction of signals downstream for the receptor GPIIIa-IIIa which regulates cell adhesion through the formation of focal contacts (37). Studies in platelets adherent to fibrinogen and in other systems have shown that PKC regulates platelet spreading and the tyrosine phosphorylation of FAK and a few unknown proteins (38). Our results indicate that PKC is involved in RAFTK phosphorylation. Therefore, RAFTK tyrosine phosphorylation was completely blocked by the PKC inhibitors calphostin C and bisindoylmaleimide (Fig. 7), indicating that the activation of RAFTK in platelets is mediated by PKC.

The cytoskeleton is essential for many cellular functions including the regulation of cell shape, flexibility, and adhesive properties (39). Part of the cytoskeleton and plasma membrane form a region known as focal adhesion sites (40), which form adherent contacts with the extracellular matrix (40). The cytoskeleton of platelets plays an important part in the initial response to exogenous mediators, the release of these mediators, and the formation of stable aggregates (33, 37, 41–44). The cytoskeleton also plays a key role in integrin-dependent tyrosine phosphorylation in platelets (2, 45), and might serve to anchor and compartmentalize kinases and other signaling molecules as part of a signal transduction complex. Our results in this study indicate that phosphorylation of RAFTK is dependent upon the formation of actin cytoskeleton, since disruption of the actin cytoskeleton by cytochalasin D significantly inhibited RAFTK phosphorylation. Although we have not shown an association of RAFTK with actin cytoskeleton, our results in other cells such as CMK megakaryocytes cells demonstrate that RAFTK is colocalized with vinculin and talin in “focal adhesion-like structures” (8).

In conclusion, RAFTK may play an important role in a complex signaling cascade in platelets involving agonist receptors, G-coupled proteins, and the cytoskeleton. Our comparative analysis revealed that RAFTK phosphorylation occurs in the early phase of platelet activation, is not dependent on platelet aggregation, and is involved in an integrin-independent pathway that is mediated by PKC. Further analysis of the upstream and downstream signaling molecules associated with RAFTK will provide new insights into the early signaling pathways in platelets that may modulate platelet activation.

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