THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 29, Issue of July 21, pp. 17321–17326, 1995
Printed in U.S.A.

Integrin $\alpha_{IIb}\beta_3$-mediated Translocation of CDC42Hs to the Cytoskeleton in Stimulated Human Platelets*

(Received for publication, October 24, 1994, and in revised form, March 14, 1995)

Debabrata Dash‡, Martin Aepfelbacher, and Wolfgang Siess§

From the Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten bei der Universität München, Pettenkoferstrasse 9, 80336 München, Germany

To investigate the function of the human Ras-related CDC42 GTP-binding protein (CDC42Hs) we studied its subcellular redistribution in platelets stimulated by thrombin-receptor activating peptide (TRAP) or ADP. In resting platelets CDC42Hs was detected exclusively in the membrane skeleton (9.6 ± 1.5% of total) and the detergent soluble fraction (90 ± 4%). When platelets were aggregated with TRAP or ADP, CDC42Hs (10% of total) appeared in the cytoskeleton and decreased in the membrane skeleton, whereas RhoGDI (guanine-nucleotide dissociation inhibitor) and CDC42HsGAP (GTPase-activating protein) remained exclusively in the detergent-soluble fraction. Upon prolonged platelet stimulation CDC42Hs disappeared from the cytoskeleton and reappeared in the membrane skeleton. Rac translocated to the cytoskeleton with a similar time course as CDC42Hs. When platelets were stimulated under conditions that precluded the activation of the $\alpha_{IIb}\beta_3$ integrin and platelet aggregation, cytoskeletal association of CDC42Hs was abolished. Translocation of CDC42Hs to the cytoskeleton but not aggregation was also prevented by cytochalasins B or D or the protein tyrosine kinase inhibitor genistein. Platelet secretion and thromboxane formation were not required but facilitated the cytoskeletal association of CDC42Hs. The results indicate that in platelets stimulated by TRAP or ADP, a fraction of CDC42Hs translocates from the membrane skeleton to the cytoskeleton. This process is reversible and is mediated by activation of the $\alpha_{IIb}\beta_3$ integrin and subsequent actin polymerization and protein-tyrosine kinase stimulation. CDC42Hs might be a new component of a signaling complex containing specific cytoskeletal proteins and protein-tyrosine kinases that forms after activation of the $\alpha_{IIb}\beta_3$ integrin in platelets.

The cell division cycle proteins, CDC42, belong to the Rho family of Ras-related GTP-binding proteins, which also includes RhoA, -B, -C, and -G, Rac1 and -2, and TC10 (1). This group of proteins has been implicated in the control of cytoskeletal organization (2, 3). Like other Ras-related proteins that bind and hydrolyze GTP, CDC42 proteins are thought to cycle between GTP-bound (active) and GDP-bound (inactive) states and are regulated by GTPase-activating proteins and guanine nucleotide exchange factors (4). Furthermore, similar to several other small GTP-binding proteins of the Ras superfamily, CDC42 proteins contain a C-terminal, geranylgeranylated cysteine residue that is also carboxymethylated (5). These post-translational modifications are surmised to promote important protein-membrane and protein-protein interactions (6–8).

The human CDC42 proteins (CDC42Hs) are the homologs of the yeast cell division cycle protein, CDC42Sc, which has been implicated in bud site assembly during the yeast cell cycle (9–11). Mutations in the CDC42Sc gene result in changes of cell shape and disruption of actin filaments, suggesting a role in cytoskeletal organization (9). Two almost identical CDC42Hs cDNAs have been cloned from placental and fetal brain libraries (12, 13). The two cDNA-predicted CDC42 proteins are identical except for an 8-amino acid segment at the C terminus. The specific function of CDC42Hs proteins in mammalian cells remains to be defined. It has recently been reported that spreading of differentiating human monocytes is associated with a dramatic 30-fold increase in membrane-associated CDC42Hs (14).

CDC42Hs is expressed in high concentrations in platelets (15) that can be rapidly induced to change their shape, to aggregate, and to secrete the contents of their granules. This series of functional responses is associated with specific and profound changes of the cytoskeleton (16, 17). In a variety of cells cytoskeletal changes and cell motility are induced through integrin activation (18–21). In circulating discoid platelets the $\alpha_{IIb}\beta_3$ integrin, which serves as fibrinogen receptor, is not available for binding by fibrinogen. The $\alpha_{IIb}\beta_3$ integrin has to be activated before fibrinogen can bind to it. Activation of the $\alpha_{IIb}\beta_3$ integrin is regulated by specific signal transduction mechanisms and alterations of the cytoskeleton initiated during platelet shape change (reviewed in Ref. 22). Subsequently, fibrinogen binds to the $\alpha_{IIb}\beta_3$ integrin, leading to further cytoskeletal rearrangements and platelet aggregation (reviewed in Ref. 23). Thus, platelets may represent an ideal model system to study the function of CDC42Hs in mammalian cells. In this study we demonstrate that CDC42Hs translocates from the membrane skeletal and cytosolic fractions of the cell to the actin-rich cytoskeleton following platelet aggregation. The CDC42Hs translocation is mediated by activation of the $\alpha_{IIb}\beta_3$ integrin and is dependent on actin polymerization and protein-tyrosine kinase activation.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antibodies were raised against peptides corresponding to amino acids 167–183 and 17–28 of the sequences

* This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (Si 274) and the August-Lenz-Stiftung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a research fellowship from the Alexander von Humboldt Foundation (Bonn, Germany) and on leave of absence from the Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221 005, India.

§ To whom correspondence should be addressed: Prof. Wolfgang Siess, Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten bei der Universität München, Pettenkoferstr. 9, 80336 München, Germany. Tel.: 49-89-5160-4380; Fax: 49-89-5160-4382.

of CDC42Hs and RhoGDI,[1] respectively, and affinity-purified on columns containing the respective peptides coupled to SulfoLink gel (Pierce) (14). Chicken polyclonal antibodies were raised against a glutathione *S*-transferase fusion protein of CDC42HsGAP (24). The specificities of the antibodies against CDC42Hs and CDC42HsGAP were tested on Western blots of pure recombinant CDC42Hs, Rac1 or RhoA, or CDC42HsGAP. Pure recombinant proteins were prepared by expression as glutathione *S*-transferase fusion proteins in *Escherichia coli* and cleavage with thrombin (25). Antibodies against Rac1, Rac2, and Rac1/Rac2 were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies PY20 and Z027 against phosphotyrosine were purchased from Zymed Laboratories (San Francisco, CA). The horseradish peroxidase-conjugated secondary antibodies were from Amersham Corp. Apyrase, ADP, RGDS, acetylsalicylic acid, sodium orthovanadate, fetal bovine serum, Triton X-100, leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride, cytochalasin B, and cytochalasin D were from the Sigma. Tween 20 and the reagents for electrophoresis were obtained from Bio-Rad. Genistein and daidzein were obtained from Biomol (Hamburg, Germany). The chemiluminescence-based Western blot detection system ECL was from Amersham Corp. The thrombin receptor-activating peptide (TRAP) (SFLLRN) was custom synthesized by Dr. Arnold (Max Planck Institute, Martinsried, Germany).

Preparations of Washed Platelets—Washed human platelets were prepared as described previously (26). Platelet-rich plasma was obtained from 200 ml of freshly drawn human blood, anticoagulated with 0.1 volume of 3.8% trisodium citrate and centrifuged at 180 × *g* for 20 min. Unless otherwise stated, platelet-rich plasma was incubated with 1 mM acetylsalicylic acid for 15 min at 37 °C. Citric acid (9 mM) and EDTA (5 mM) were added, and platelets were pelleted by centrifugation at 800 × *g* for 15 min. They were resuspended in 3 ml of washing buffer (20 mM Hepes, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, and 1 mM EGTA, supplemented with 5 mM glucose and 3 ADPase units/ml of apyrase, pH 6.2) and diluted to 30 ml with the same buffer but containing 0.6 ADPase units of apyrase/ml. Platelets were pelleted and resuspended in 20 ml of the resuspension buffer (pH 7.4), which was the same as the washing buffer but without EGTA and apyrase. The final platelet concentration was adjusted to 1×10^9/ml.

Platelet Activation and Aggregation—Aliquots (1.5 ml) of washed platelets were incubated with stirring (1800 rpm) at 37 °C in a LABOR aggregometer (Fresenius, Bad Homburg, Germany) for 1 min prior to the addition of TRAP (10 μM). $CaCl_2$ (50 μM) was added 15 s before TRAP. Aggregation was measured by the percentage change of the light transmission. In some experiments, aggregation was prevented by (*a*) incubating the platelets at 37 °C in the absence of stirring, (*b*) preincubating platelets with EGTA (2 mM) for 20 min at 37 °C, or (*c*) preincubating platelets with RGDS (4 mM) for 1 min at 37 °C prior to stimulation with TRAP. In other experiments, platelets were pretreated with cytochalasin B (20 μM), cytochalasin D (2 μM), or Me_2SO (0.2%, control) at 37 °C for 1 min prior to exposure to TRAP. In further experiments, platelets were preincubated with genistein (100 μM and 150 μM), daidzein (100 μM and 150 μM), or Me_2SO (0.2%, control) for 2 min prior to the addition of TRAP.

Isolation and Analysis of Detergent Lysates of Platelets—Cytoskeletal fractions were prepared by using a modification of the method described by Phillips *et al.* (27). Briefly, platelet suspensions (1.5 ml) were lysed before or at various intervals after agonist addition by adding equal volumes of ice-cold 2 × Triton lysis buffer (pH 7.5) containing 2% Triton X-100, 100 mM Tris-HCl, 10 mM EGTA, 10 mM EDTA, 2 mM sodium orthovanadate, 21 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 μM pepstatin A, and 0.56 trypsin inhibitor unit/ml aprotinin. Samples (3 ml) were vortexed for 10 s, left on ice for 30 min, and spun at 15,600 × *g* for 15 min at 4 °C in a Kontron analytical centrifuge (type ZK 401). The pellets were washed once without resuspension in 1 × Triton X-100 (1%) lysis buffer. The pellets were resuspended in 300 μl of 1 × lysis buffer plus 75 μl of 5 × sample buffer (250 mM Tris-HCl, 8.2 g/100 ml SDS, 40% (v/v) glycerol, 0.05 g/100 ml bromphenol blue, 5% (v/v) 2-mercaptoethanol, and 5 mM sodium orthovanadate, pH 6.8) and heated at 95 °C for 10 min. For immunoblotting with anti-Rac antibodies the cytoskeletal pellet was resuspended in 200 μl of 1 × lysis buffer plus 50 μl of 5 × sample buffer, followed by boiling.

To obtain the membrane skeletal fraction (28), supernatants of detergent extracts were further centrifuged at 100,000 × *g* for 2.5 h at 4 °C in a Beckman ultracentrifuge (L5–50) with a 70.1 Ti rotor. The pellets were resuspended as above. The Triton-soluble supernatant fractions were mixed at a ratio of 4:1 with 5 × sample buffer and boiled. Thus, the final detergent-insoluble samples (both cytoskeleton and membrane skeleton preparations) were concentrated 10 times relative to the Triton-soluble fractions.

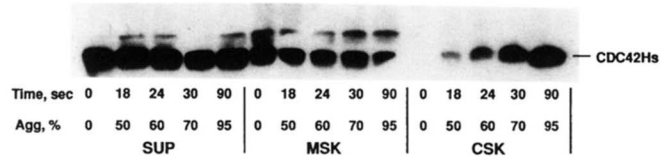

FIG. 1. **Translocation of CDC42Hs to the cytoskeleton during platelet aggregation.** Aliquots of washed human platelets (1.5 ml) were stimulated by TRAP (10 μM) for the times indicated. Platelet aggregation (*Agg %*) was measured. Platelets were lysed with the buffer containing Triton X-100, and the cytoskeletal (*CSK*), membrane skeletal (*MSK*), and soluble (*SUP*) fractions were isolated. The proteins in each fraction were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted using an anti-CDC42Hs antibody. The experiment is representative of six different experiments.

Triton X-100-insoluble cytoskeletons of control and activated platelet-rich plasma were prepared according to the method of Carrol *et al.* (29). Briefly, platelet-rich plasma (2 × 1.5 ml) was mixed before and at various times after agonist addition with equal volumes of 2 × Triton X-100 lysis buffer (see above), vortexed and centrifuged at 10,000 × *g* for 5 min. The supernatants were discarded. The pellets were washed twice with 1 × Triton X-100 lysis buffer. The final pellets were resuspended in 300 μl of 1 × Triton X-100 lysis buffer plus 75 μl of 5 × sample buffer and heated at 95 °C for 10 min.

Protein Gel Electrophoresis and Immunoblotting of CDC42Hs, Rac1/Rac2, CDC42HsGAP, RhoGDI, and Phosphotyrosine—Platelet proteins were separated by overnight vertical electrophoresis of samples on 1.5-mm-thick and 20-cm-long SDS-polyacrylamide (12%) slab gels (Protean II xi system, Bio-Rad) at a constant current of 13 mA/gel. Proteins were electrophoretically transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) by using the Nova Blot semidry system (Pharmacia Biotech. Inc., Bromma, Sweden) and the procedure recommended by the manufacturer. In some experiments proteins were separated and electroblotted using the Mini Protean II system (Bio-Rad). Blocking of residual sites on the membranes was effected by incubating the blots for 1 h at room temperature with 20% (v/v) fetal bovine serum in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.3% Tween 20. The blots were incubated for 1 h with one of the following primary antibodies: (*a*) polyclonal anti-CDC42Hs antibody (1:500), (*b*) monoclonal anti-phosphotyrosine antibody PY20 and Z027 (1:2000 dilution each), (*c*) polyclonal antibodies against Rac1 (1:150), Rac2 (1:150), or Rac1/Rac2 (1:500), (*d*) polyclonal anti-RhoGDI antibody (1:500), or (*e*) polyclonal anti-CDC42HsGAP antibody (1:2000). Following washing, the blots were incubated for 45 min with peroxidase-labeled donkey anti-rabbit IgG (1:2500) for anti-CDC42Hs and anti-GDI primary antibodies, sheep anti-mouse IgG (1:15,000) for the primary antibody against phosphotyrosine, or rabbit anti-chicken IgG (1:20,000) for anti-CDC42HsGAP primary antibody. After three washes of the blot, antibody binding was detected using the enhanced chemiluminescence (ECL) system (Amersham Corp.). Bands corresponding to CDC42Hs and RhoGDI were measured by laser densitometry (Ultroscan XL, Pharmacia Biotech Inc.).

To examine the proteolytic cleavage of actin-binding protein and talin, platelet proteins were separated on a 6% SDS-polyacrylamide gel electrophoresis slab gel, followed by staining of the gel with 0.2% Coomassie Brilliant Blue, destaining, and drying. Calpain activity was judged from the extent of degradation of actin-binding protein and talin. To quantify actin in platelet cytoskeletal preparations platelet proteins were separated by SDS-polyacrylamide gel electrophoresis (12%) and stained with Coomassie Blue, and the bands corresponding to actin were measured by laser densitometry.

RESULTS

Aggregation-dependent Reversible Translocation of CDC42Hs and Rac but not of RhoGDI and CDC42HsGAP to the Platelet Cytoskeleton—In unstimulated platelets CDC42Hs was present in both the membrane skeleton and the supernatant fraction but was absent from the cytoskeleton (Fig. 1). Laser densitometric evaluations of several ($n = 5$) immunoblots indicated that 90 ± 4% of total CDC42Hs was present in the

[1] The abbreviations used are: GDI, guanine nucleotide dissociation inhibitor; GAP, GTPase-activating protein; RGDS, Arg-Gly-Asp-Ser; TRAP, thrombin receptor-activating peptide.

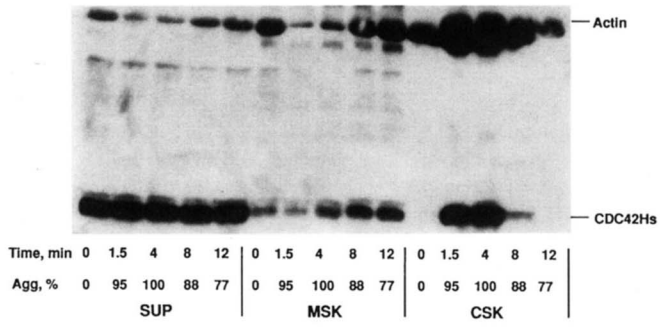

FIG. 2. **Disappearance of CDC42Hs from the cytoskeleton upon prolonged platelet aggregation.** Aliquots (1.5 ml) of washed human platelets were stimulated with TRAP (10 μM) for the times indicated. For further details and definitions of abbreviations see the legend to Fig. 1. The experiment is representative of six different experiments.

supernatant fraction whereas 9.6 ± 1.5% (mean ± S.D.) was in the membrane skeleton. Upon platelet stimulation with 10 μM TRAP the protein appeared in the cytoskeleton. The degree of cytoskeletal association of CDC42Hs correlated with the extent of platelet aggregation (Fig. 1); at 50% aggregation the CDC42Hs protein band was barely detectable, and at 95% aggregation the association was maximal. At maximal aggregation about 10% of total CDC42Hs translocated to the cytoskeleton, whereas its decrease in the membrane skeleton amounted to 6–8% of total CDC42Hs. No significant change of CDC42Hs during platelet aggregation was observed in the soluble fraction; the calculated decrease was 2–4% of total CDC42Hs.

The association of CDC42Hs with the platelet cytoskeleton was reversible; on prolonged activation with TRAP CDC42Hs gradually decreased and completely disappeared from the cytoskeleton after 12 min and reappeared in the membrane skeleton (Fig. 2) while platelet aggregation was reduced only by 35% (data not shown). These changes were paralleled by changes in the distribution of actin that decreased in the cytoskeleton and concomitantly increased in the membrane skeleton and the soluble fraction.

We observed in the membrane skeleton and the soluble fraction an additional protein band recognized by the anti-CDC42Hs antibody that showed a slightly lower electrophoretic mobility than the major CDC42Hs band. The major band most likely represents the prenylated form of CDC42Hs because it exactly comigrated with the CDC42Hs band of nucleated human U-937 cells, which are considered to contain almost exclusively the prenylated form of proteins. The additional upper band might represent the unprocessed CDC42Hs because it comigrated with the recombinant CDC42Hs expressed in *E. coli* that lacks post-translational modifications (Fig. 3). Interestingly, this putative unprocessed CDC42Hs did not translocate to the cytoskeleton upon platelet aggregation.

Rac GTPases are the closest homologs of CDC42Hs within the Ras family, and at least one Rac isoform, Rac1, seems to be present in platelets (30). Using antibodies specific for Rac1 and Rac2 both proteins were found in human platelets, but more Rac1 than Rac2 immunoreactivity was detected (data not shown). Interestingly, Rac (12% of total) translocated to the cytoskeleton in aggregating platelets with a time course similar to CDC42Hs (Fig. 4).

RhoGDI, which forms stable complexes with CDC42Hs, Rho, and Rac proteins in the cytosol of cells (31, 32), was exclusively confined to the soluble fraction. Upon platelet aggregation RhoGDI did not translocate to the cytoskeleton (Fig. 5). The GAP specific for CDC42Hs also did not associate with the cytoskeleton in the aggregated platelets. However, we observed a faint appearance of this protein in the membrane skeletal

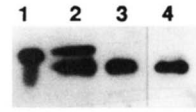

FIG. 3. **Comparison of the electrophoretic mobilities of the cytoskeletal and membrane skeletal CDC42Hs with the processed and unprocessed forms of the protein.** Proteins were electrophoretically separated and immunoblotted with an anti-CDC42Hs antibody. *Lane 1*, recombinant CDC42Hs protein expressed in *E. coli* (unprocessed); *lane 2*, membrane skeleton obtained from resting platelets; *lane 3*, cytoskeleton obtained from aggregated platelets; *lane 4*, lysate from human monocytic U-937 cells (processed).

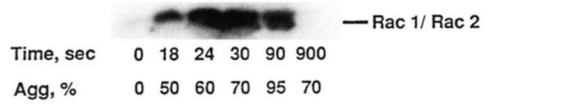

FIG. 4. **Translocation of Rac to the cytoskeleton during platelet aggregation.** Aliquots of washed human platelets (1.5 ml) were stimulated with TRAP (10 μM) for the times indicated. Platelets were lysed with the buffer containing Triton X-100, and the cytoskeletal fractions were isolated as described under "Experimental Procedures." Proteins in the cytoskeleton were immunoblotted using an anti-Rac1/Rac2 antibody. The experiment is representative of three different experiments. *Agg, %*, platelet aggregation.

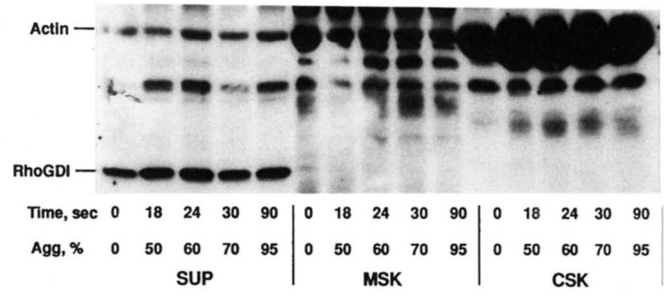

FIG. 5. **RhoGDI remains in the detergent-soluble fraction during platelet aggregation.** Platelets were stimulated by TRAP (10 μM) as described in the legend to Fig. 1. Immunoblotting was done using an antibody against RhoGDI. This figure is typical of three different experiments. Abbreviations are as defined in the legend to Fig. 1.

fraction of the platelets aggregated for 90 s (Fig. 6).

The Redistribution of CDC42Hs Is Mediated by the Activation of the $\alpha_{IIb}\beta_3$ Integrin and Is Dependent on Actin Polymerization and Protein-Tyrosine Kinase Activity—Platelet aggregation requires fibrinogen binding to the membrane glycoprotein IIb-IIIa complex ($\alpha_{IIb}\beta_3$ integrin), which is activated during platelet activation and shape change. To test the involvement of fibrinogen receptors in the cytoskeletal association of CDC42Hs we stimulated platelets (*a*) in the absence of stirring, which prevents platelet aggregation but allows fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin, (*b*) after preincubating platelets with EGTA at 37 °C to dissociate the $\alpha_{IIb}\beta_3$ integrin, or (*c*) in the presence of RGDS to inhibit fibrinogen binding. Fig. 7 shows that these conditions, which completely inhibited platelet aggregation (data not shown), prevented cytoskeletal association of CDC42Hs. We conclude from these data that the conformational change of the $\alpha_{IIb}\beta_3$ integrin evoked by fibrinogen binding is required but not sufficient for the translocation of CDC42Hs to the cytoskeleton. Subsequent signaling events of the activated fibrinogen receptor are necessary.

Platelet aggregation is associated with an increase in actin polymerization and translocation of the $\alpha_{IIb}\beta_3$ integrin as well as regulatory proteins, such as the small GTP-binding proteins Rap1B (33) and Rap2B (34), phosphatidylinositol 3-kinase (35, 36), and pp60$^{c\text{-}src}$ (35, 37, 38) to the actin-rich cytoskeleton. We asked next whether the increase of actin polymerization is required for $\alpha_{IIb}\beta_3$-mediated association of CDC42Hs with the

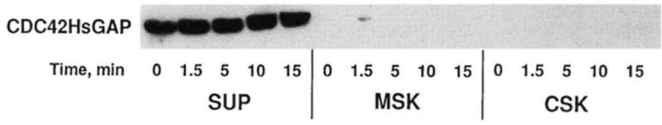

FIG. 6. **CDC42HsGAP does not associate with the cytoskeleton during platelet aggregation.** Platelets were stimulated by TRAP (10 μM) for the times indicated. Immunoblotting was done using chicken anti-CDC42HsGAP. For further details and definitions of abbreviations see the legend to Fig. 1. The experiment is representative of three different experiments.

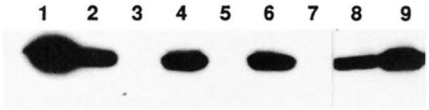

FIG. 7. **Translocation of CDC42Hs to the cytoskeleton in stimulated platelets is mediated by $\alpha_{IIb}\beta_3$ integrin activation.** Washed human platelets were exposed to TRAP (10 μM) for 90 s under the following conditions: without stirring (*lanes 2* and *3*), with stirring (*lanes 8* and *9*), after preincubation with EGTA (2 mM) for 20 min at 37 °C (*lanes 4* and *5*), and after preincubation with RGDS (4 mM) for 1 min at 37 °C (*lanes 6* and *7*). EGTA and RGDS pretreatment completely inhibited platelet aggregation (data not shown). Platelets were lysed with Triton lysis buffer. Cytoskeleton and soluble fractions were isolated and immunoblotted with an antibody against CDC42Hs as described. *Lane 1*, recombinant CDC42Hs protein; *lanes 2*, *4*, *6*, and *8*, soluble fractions; *lanes 3*, *5*, *7*, and *9*, cytoskeletal fractions. The experiment is representative of three different experiments.

cytoskeleton. When cells were pretreated with either cytochalasin B or cytochalasin D, both inhibitors of actin polymerization, before addition of TRAP, no suppression of platelet aggregation was seen (Fig. 8*A*), despite an effective reduction of actin in the cytoskeleton (Fig. 8*B*, *bottom*). The increase of actin in the cytoskeleton from 42.7% in the resting platelets to 81.0% in TRAP-aggregated platelets was reduced to 22.6 and 26.9% after treatment with cytochalasins B and D, respectively. Cytochalasin treatment completely abolished the cytoskeletal association of CDC42Hs with retention of the protein in the membrane skeletal fractions (Fig. 8*B*, *top*). These results indicate that stimulation of actin polymerization in aggregated platelets is required for the cytoskeletal association of CDC42Hs.

Because it has been reported that stimulation of protein tyrosine phosphorylation in platelets occurs subsequently to the activation of the $\alpha_{IIb}\beta_3$ integrin (39–41) and the major nonreceptor protein-tyrosine kinase, $pp60^{c\text{-}src}$, associates with the cytoskeleton under similar conditions as does the CDC42Hs protein (36, 37, 42, 43), we asked next whether inhibition of tyrosine kinase activity might affect the translocation of CDC42Hs. Pretreatment of platelets with the protein-tyrosine kinase inhibitor genistein (100 and 150 μM) inhibited the cytoskeletal association of CDC42Hs by about 95% compared with the Me_2SO-treated control platelets aggregated to the same extent (Table I). Pretreatment with genistein effectively reduced the tyrosine phosphorylation of several platelet proteins compared with Me_2SO- or daidzein-treated samples (data not shown), as observed previously (44–46). These observations indicate a role for protein-tyrosine kinase activity in aggregated platelets in inducing cytoskeletal association of CDC42Hs.

We further studied whether secretion is also required for the association of CDC42Hs with the cytoskeleton. Aggregation induced by a strong agonist like TRAP is accompanied by the secretion of the contents of platelet α granules and dense bodies. α granules contain various adhesion molecules (fibrinogen, fibronectin, and von Willebrand factor) that bind to the $\alpha_{IIb}\beta_3$ integrin (for review, see Ref. 22). Platelet-rich plasma, pretreated with the cyclooxygenase inhibitor aspirin, was stimulated with ADP. Under these conditions strong aggregation occurs without secretion from α granules and dense bodies. We observed the translocation of CDC42Hs to the cytoskeleton under these conditions, indicating that secretion was not necessary for that effect (Fig. 9). However, ADP caused a smaller percentage of CDC42Hs to translocate to the cytoskeleton than TRAP (compare *lanes 5* and *8* with *lanes 9* and *10* in Fig. 9). Also aspirin-treated platelets that were aggregated by ADP or TRAP contained less CDC42Hs in the cytoskeleton than nontreated cells at 95% aggregation. The results indicate that secretion and prostaglandin endoperoxides/thromboxane A_2 are not necessary, but enhance the association of CDC42Hs with the cytoskeleton.

DISCUSSION

We show in this study that a significant fraction of the Ras-related GTP-binding protein CDC42Hs translocates to the cytoskeleton in aggregating platelets. From comparisons with the electrophoretic mobility of the post-translationally processed and the unprocessed forms of CDC42Hs, we suggest that the prenylated form of the protein associates with the cytoskeleton. Mutational analysis in yeast suggested that the CDC42Sc (*Saccharomyces cerevisiae*) protein, which is 80% identical to the human CDC42Hs protein, is involved in cytoskeletal organization, but so far no direct interaction with the cytoskeleton could be demonstrated. The increase in cytoskeletal CDC42Hs during platelet aggregation was accompanied by a decrease in membrane skeletal CDC42Hs. The calculated decrease in soluble CDC42Hs (2–4%) was too small to be detectable, since 90% of CDC42Hs was in the cytosolic fraction. Thus, CDC42Hs might have translocated from the membrane skeleton and from the cytosol to the cytoskeleton. Interestingly, two proteins, RhoGDI and CDC42HsGAP, which interact with CDC42Hs were both not found in the cytoskeleton of aggregated platelets. These regulatory proteins will keep CDC42Hs in its inactive GDP-bound form; RhoGDI is known to form stable complexes with the GDP-bound form of Rho proteins (47, 48) and inhibits GDP dissociation, and CDC42HsGAP stimulates GTP hydrolysis (24, 49). The fact that both proteins were still present in the cytosol of aggregated platelets indicates that CDC42Hs (if translocating from the cytosol to the cytoskeleton) must have dissociated from RhoGDI and is also most likely not accessible to inactivation by CDC42HsGAP. Thus, cytoskeletal CDC42Hs might be present in its active, GTP-bound form. Recently, GTP-bound CDC42Hs has been reported to interact specifically with the protein-tyrosine kinase $p120^{ACK}$ and the serine/threonine protein kinase $p65^{PAK}$ (50, 51).

We observed no association of CDC42Hs with the cytoskeleton during platelet shape change. This initial platelet response is characterized by a specific reorganization of the cytoskeleton, stimulation of actin polymerization, and increased content of actin, myosin, and actin-binding protein in the cytoskeleton (17, 52). Hence it is unlikely that CDC42Hs plays a role in the rearrangement of the cytoskeleton during this initial platelet response, which is also associated with the activation of the integrin $\alpha_{IIb}\beta_3$. Platelet aggregation is associated with further specific changes in the cytoskeleton (27), most importantly the translocation of the integrin $\alpha_{IIb}\beta_3$ from the membrane skeleton to the cytoskeleton (27, 53–55). Activation of the integrin $\alpha_{IIb}\beta_3$ has been shown to cause other components of the membrane skeleton to undergo increased association with the cytoskeleton. To these components belong the cytoskeletal proteins talin and vinculin (36, 56) and the protein-tyrosine kinases $pp60^{c\text{-}src}$ (36, 37, 56) and $pp62^{c\text{-}yes}$ (56). The integrin $\alpha_{IIb}\beta_3$-dependent translocation of CDC42Hs from the membrane skeleton to the cytoskeleton shown in this study indicates that CDC42Hs belongs to this complex of specific signal-

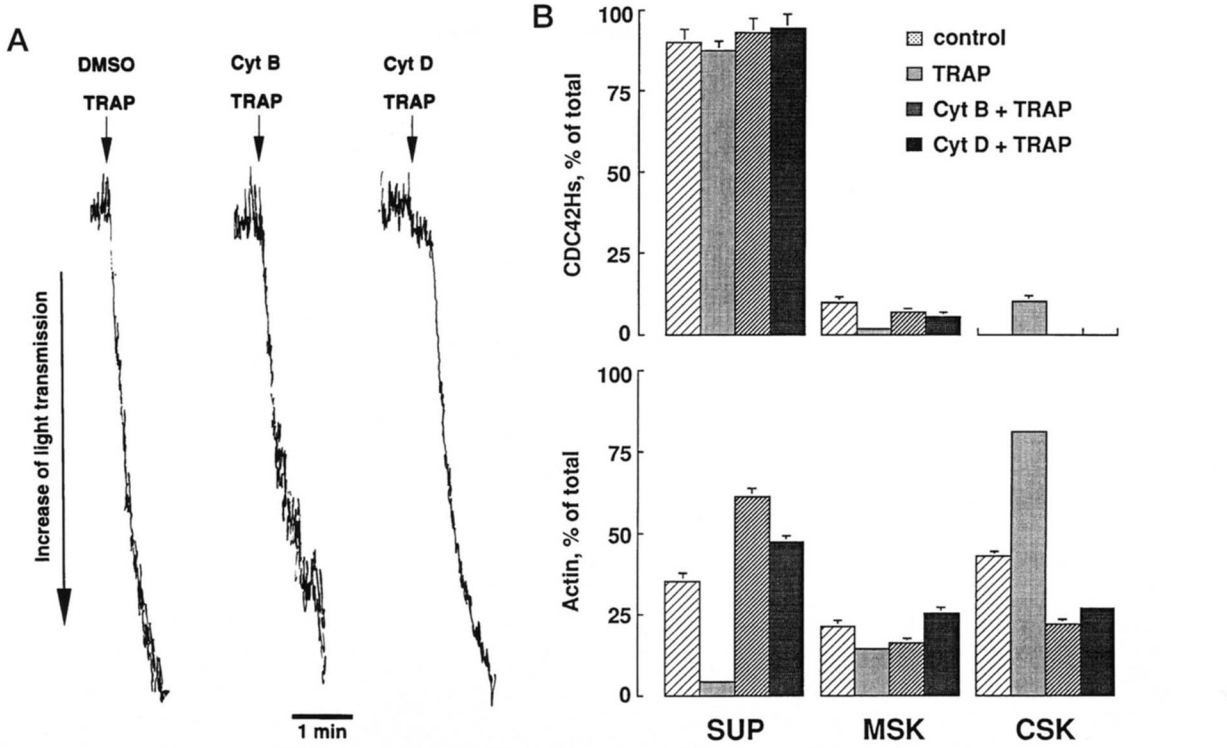

FIG. 8. **Effect of cytochalasins B and D on the cytoskeletal association of CDC42Hs in platelets aggregated by TRAP.** Washed platelets were incubated with either Me_2SO (*DMSO*) (0.2%, *control*), cytochalasin B (20 μM), or cytochalasin D (2 μM) at 37 °C for 1 min, followed by exposure to TRAP (10 μM). *A*, aggregation tracings of Me_2SO- and cytochalasin (*Cyt*)-pretreated platelets stimulated by TRAP (*arrow*). *B*, soluble (*SUP*), membrane skeletal (*MSK*), and cytoskeletal (*CSK*) fractions were subjected to electrophoresis and immunoblotting using an anti-CDC42Hs antibody. The *upper part* shows the laser densitometric quantitation of CDC42Hs in the different fractions. The *lower part* shows the laser densitometric measurement of actin in the Coomassie Blue-stained gel. Data are mean ± S.D. (n = 3).

TABLE I
Effect of genistein on cytoskeletal association of CDC42Hs

Suspensions of washed platelets were pretreated with Me_2SO (0.2%, control) or genistein (100 or 150 μM) for 2 min at 37 °C, and then exposed to TRAP (10 μM) for 35 s (Me_2SO), 45 s (genistein, 100 μM) or 60 s (genistein, 150 μM) to obtain the same level of aggregation (change of light transmission 70%) in all samples. Values are expressed as percentage (mean ± S.D., n = 3) of CDC42Hs or actin present in the Me_2SO-treated platelets.

	% of protein in cytoskeleton	
	CDC42Hs	Actin
Me_2SO + TRAP	100	100
Genistein, 100 μM + TRAP	5.0 ± 1.8	98.2 ± 8
Genistein, 150 μM + TRAP	0.7 ± 0.4	95.4 ± 10

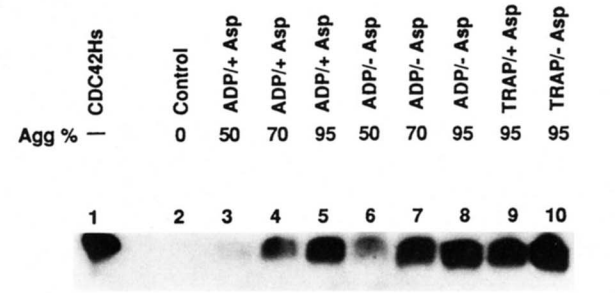

FIG. 9. **Effect of aspirin on the cytoskeletal association of CDC42Hs in the platelets aggregated by TRAP or ADP.** Platelet-rich plasma was incubated with either aspirin (1 mM) (*lanes 3–5* and *9*) or ethanol (0.2%) (*lanes 2*, *6–8*, and *10*) for 15 min at 37 °C and stimulated with either ADP (10 μM) (*lanes 3–8*) or TRAP (10 μM) (*lanes 9–10*). Platelet aggregation (*Agg %*) was measured. Cytoskeletal fractions were isolated as described, subjected to SDS-polyacrylamide gel electrophoresis, and analyzed for CDC42Hs by immunoblotting. *Lane 2*, control without agonist addition; *lane 1*, recombinant CDC42Hs.

ing molecules that might regulate integrin-actin interaction.

The finding that the GTPase Rac cotranslocated with CDC42Hs might indicate a common target protein in the cytoskeleton. Both CDC42Hs and Rac bind to and activate the serine/threonine kinase $p65^{PAK}$ (51) and the p85 subunit of phosphatidylinositol 3-kinase (57). The latter enzyme also translocates to the cytoskeleton of activated platelets (35, 36) and at this location might be activated by the CDC42Hs or Rac proteins.

It has been shown that the binding of fibrinogen to the integrin $\alpha_{IIb}\beta_3$ results in increased tyrosine phosphorylation of several proteins in stimulated platelets (39–41, 58–61). We observed that the cytoskeletal association of CDC42Hs in aggregated platelets could be prevented by inhibition of actin polymerization with cytochalasin B and D and inhibition of protein-tyrosine kinase activity. Cytochalasin D has been reported to inhibit translocation of $pp60^{c\text{-}src}$ to the cytoskeleton (42) and to reduce tyrosine phosphorylation of several platelet proteins (42, 61, 62). Therefore, protein-tyrosine phosphorylation appears to occur subsequently to actin polymerization and $pp60^{c\text{-}src}$ translocation in aggregated platelets. Our results showing that both cytochalasin and genistein blocked CDC42Hs association with the cytoskeleton indicate that (*a*) actin polymerization and (*b*) protein-tyrosine kinase activity are required for this process. It is possible that a protein-tyrosine kinase translocates to the actin cytoskeleton and phosphorylates itself or a cytoskeletal protein, thereby providing docking sites for CDC42Hs. A tyrosine kinase ($p120^{ACK}$) that binds to GTP-bound CDC42Hs and has some homology to the focal adhesion kinase was detected recently (50). The possibility that CDC42Hs could be tyrosine-phosphorylated was raised previously (63), but we did not find tyrosine-phosphorylated CDC42Hs in aggregated platelets (data not shown).

We observed that the cytoskeletal association of CDC42Hs

protein upon prolonged stimulation with TRAP was reversible. CDC42Hs completely disappeared from the cytoskeleton after 12 min of stimulation, whereas aggregation was decreased only by 35%. CDC42Hs disappearance from the cytoskeleton was accompanied by a corresponding increase in the membrane skeleton; hence, proteolysis of CDC42Hs as a cause for the complete disappearance from the cytoskeleton could be excluded. We also did not observe any stimulation of platelet calpain, which is known to degrade cytoskeletal proteins such as talin and actin-binding protein. We found that cytoskeletal actin content was decreased upon prolonged agonist stimulation, which might be related to the reversible association of CDC42Hs with the cytoskeleton.

In summary, we have shown that CDC42Hs incorporation into the cytoskeleton is a specific event that is mediated by $\alpha_{IIb}\beta_3$ integrin activation and is dependent on both actin polymerization and tyrosine kinase activity. Platelet secretion and thromboxane formation are not required but facilitate cytoskeletal CDC42Hs association. CDC42Hs might play a role in the reorganization of the platelet cytoskeleton observed during platelet aggregation.

Acknowledgments—We thank John A. Glomset for helpful discussions, Alan Hall for providing the CDC42HsGAP cDNA, Ulrike Korf for preparation of the chicken anti-CDC42HsGAP antibody, and F. Haag for the skillful photographic reproduction of the autoradiographs.

REFERENCES

1. Vincent, S., Jeanteur, P., and Fort, P. (1992) *Mol. Cell. Biol.* **12,** 3138–3148
2. Hall, A. (1992) *Mol. Biol. Cell* **3,** 475–479
3. Ridley, A. J., and Hall, A. (1994) *EMBO J.* **13,** 2600–2610
4. Boguski, M. S., and McCormick, F. (1993) *Nature* **366,** 643–654
5. Yamane, H. K., Farnsworth, C. C., Xie, H., Evans, T., Howald, W. N., Gelb, M. H., Glomset, J. A., Clarke, S., and Fung, B. K.-K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 286–290
6. Glomset, J. A., Gelb, M. H., and Farnsworth, C. C. (1990) *Trends Biochem. Sci.* **15,** 139–142
7. Cox, A. D., and Der, C. J. (1992) *Curr. Opin. Cell Biol.* **4,** 1008–1016
8. Marshall, C. J. (1993) *Science* **259,** 1865–1866
9. Adams, A. E. M., Johnson, D. I., Longnecker, R. M., Sloat, B. F., and Pringle, J. R. (1990) *J. Cell Biol.* **111,** 131–142
10. Johnson, D. I. (1993) in *The ras Superfamily of GTPases* (Lacal, J. C., and McCormick, F., eds) pp. 297–312, CRC Press, Boca Raton, FL
11. Ziman, M., Preuss, D., Mulholland, J., O'Brien, J. M., Botstein, D., and Johnson, D. I. (1993) *Mol. Biol. Cell* **4,** 1307–1316
12. Munemitsu, S., Innis, M. A., Clark, R., McCormick, F., Ullrich, A., and Polakis, P. (1990) *Mol. Cell. Biol.* **10,** 5977–5982
13. Shinjo, K., Koland, J. G., Hart, M. T., Narasimhan, V., Johnson, D. I., Evans, T., and Cerione, R. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9853–9857
14. Aepfelbacher, M., Vauti, F., Weber, P. C., and Glomset, J. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4263–4267
15. Polakis, P. G., Snyderman, R., and Evans, T. (1989) *Biochem. Biophys. Res. Commun.* **160,** 25–32
16. Jennings, L. K., Fox, J. E. B., Edwards, H. H., and Phillips, D. R. (1981) *J. Biol. Chem.* **256,** 6927–6932
17. Kometani, M., Sato, T., and Fujii, T. (1986) *Thromb. Res.* **41,** 801–809
18. Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988) *Annu. Rev. Cell Biol.* **4,** 487–525
19. Ruoslahti, E. (1991) *J. Clin. Invest.* **87,** 1–5
20. Hynes, R. O. (1992) *Cell* **69,** 11–25
21. Hynes, R. O., and Lander, A. D. (1992) *Cell* **68,** 303–322
22. Siess, W. (1989) *Physiol. Rev.* **69,** 58–178
23. Fox, J. E. B. (1993) *Adv. Exp. Med. Biol.* **344,** 175–185
24. Lancaster, C. A., Taylor-Harris, P. M., Self, A. J., Brill, S., van Erp, H. E., and Hall, A. (1994) *J. Biol. Chem.* **269,** 1137–1142
25. Smith, D. B., and Johnson, K. S. (1988) *Gene (Amst.)* **67,** 31–40
26. Siess, W., and Lapetina, E. G. (1990) *Biochem. J.* **271,** 815–819
27. Phillips, D. R., Jennings, K. K., and Edwards, H. H. (1980) *J. Cell Biol.* **86,** 77–86
28. Fox, J. E. B., Boyles, J. K., Berndt, M. C., Steffen, P. K., and Anderson, L. K. (1988) *J. Cell Biol.* **106,** 1525–1538
29. Carroll, R. C., Butler, R. G., Morris, P. A., and Gerrard, J. M. (1982) *Cell* **30,** 385–393
30. Polakis, P. G., Weber, R. F., Nevins, B., Didsbury, J. R., Evans, T., and Snyderman, R. (1989) *J. Biol. Chem.* **264,** 16383–16389
31. Regazzi, R., Kikuchi, A., Takai, Y., and Wollheim, C. B. (1992) *J. Biol. Chem.* **267,** 17512–17519
32. Hancock, J. F., and Hall, A. (1993) *EMBO J.* **12,** 1915–1921
33. Fischer, T. H., Gatling, M. N., McCormick, F., Duffy, C. M., and White, G. C., II (1994) *J. Biol. Chem.* **265,** 17257–17261
34. Torti, M., Ramaschi, G., Sinigaglia, F., Lapetina, E. G., and Balduini, C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7553–7557
35. Grondin, P., Plantavid, M., Sultan, C., Breton, M., Mauco, G., and Chap, H. (1991) *J. Biol. Chem.* **266,** 15705–15709
36. Zhang, J., Fry, M. J., Waterfield, M. D., Jaken, S., Liao, L., Fox, J. E. B., and Rittenhouse, S. E. (1992) *J. Biol. Chem.* **267,** 4686–4692
37. Horvath, A. R., Muszbek, L., and Kellie, S. (1992) *EMBO J.* **11,** 855–861
38. Clark, E. A., and Brugge, J. S. (1993) *Mol. Cell. Biol.* **13,** 1863–1871
39. Ferrell, J. E., Jr., and Martin, G. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2234–2238
40. Golden, A., Brugge, J. S., and Shattil, S. J. (1990) *J. Cell Biol.* **111,** 3117–3127
41. Bachelot, C., Cano, E., Grelac, F., Saleun, S., Druker, B. J., Levy-Toledano, S., Fischer, S., and Rendu, F. (1992) *Biochem. J.* **284,** 923–928
42. Oda, A., Druker, B. J., Smith, M., and Salzman, E. W. (1992) *J. Biol. Chem.* **267,** 20075–20081
43. Pumiglia, K. M., and Feinstein, M. B. (1993) *Biochem. J.* **294,** 253–260
44. Dhar, A., Paul, A. K., and Shukla, S. D. (1990) *Mol. Pharmacol.* **37,** 519–525
45. Shukla, S. D. (1990) *Lipids* **26,** 1028–1033
46. Murphy, C. T., Kellie, S., and Westwick, J. (1993) *Eur. J. Biochem.* **216,** 639–651
47. Ueda, T., Kikuchi, A., Ohga, N., Yamamoto, J., and Takai, Y. (1990) *J. Biol. Chem.* **265,** 9373–9380
48. Sasaki, T., Kato, M., and Takai, Y. (1993) *J. Biol. Chem.* **268,** 23959–23963
49. Barfod, E. T., Zheng, Y., Kuang, W.-J., Hart, M. J., Evans, T., Cerione, R. A., and Ashkenazi, A. (1993) *J. Biol. Chem.* **268,** 26059–26062
50. Manser, E., Leung, T., Salihuddin, H., Tan, L., and Lim, L. (1993) *Nature* **363,** 364–367
51. Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-S., and Lim, L. (1994) *Nature* **367,** 40–46
52. Heptinstall, S., Glenn, J., and Spangenberg, P. (1992) *Thromb. Haemost.* **68,** 727–730
53. Fox, J. E. B. (1985) *J. Biol. Chem.* **260,** 11970–11977
54. Cohen, I., Bark, D. L., and White, J. G. (1989) *Blood* **73,** 1880–1887
55. Kouns, W. C., Fox, C. F., Lamoreaux, W. J., Coons, L. B., and Jennings, L. K. (1991) *J. Biol. Chem.* **266,** 13891–13900
56. Fox, J. E. B., Lipfert, L., Clark, E. A., Reynolds, C. C., Austin, C. D., and Brugge, J. S. (1993) *J. Biol. Chem.* **268,** 25973–25984
57. Zheng, Y., Bagrodia, S., and Cerione, R. A. (1994) *J. Biol. Chem.* **269,** 18727–18730
58. Ferrell, J. E., Jr., and Martin, G. S. (1988) *Mol. Cell. Biol.* (1988) **8,** 3603–3610
59. Golden, A., and Brugge, J. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 901–905
60. Nakamura, S., and Yamamura, H. (1989) *J. Biol. Chem.* **264,** 7089–7091
61. Huang, M.-M., Lipfert, L., Cunningham, M., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (1993) *J. Cell Biol.* **122,** 473–483
62. Haimovich, B., Lipfert, L., Brugge, J. S., and Shattil, S. J. (1993) *J. Biol. Chem.* **268,** 15868–15877
63. Hart, M. J., Polakis, P. G., Evans, T., and Cerione, R. A. (1990) *J. Biol. Chem.* **265,** 5990–6001