The Small GTPase Rac3 Interacts with the Integrin-binding Protein CIB and Promotes Integrin αIIbβ3-mediated Adhesion and Spreading*

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There are only three human isoforms of the small GTPase Rac, which together regulate a variety of cellular processes, including those related to actin cytoskeletal reorganization. A role for Rac3 in integrin-mediated adhesion and spreading has not been defined. We here report that CIB, a protein that binds to the αIIbβ3 fibrinogen receptor, interacts exclusively with activated (V12) Rac3 but not Rac1 or Rac2. Binding of V12Rac3 to CIB was mediated by the C-terminal end of Rac3 and by Rac3 membrane localization. Adhesion of cells on fibrinogen was accompanied by a specific increase in the levels of Rac3 but not Rac1 or Rac2 in the Triton-insoluble fraction of the cell. Also, CIB co-localized with active Rac3 to the periphery of cells adhering to fibrinogen. Expression of V12Rac3 and CIB stimulated αIIbβ3-mediated adhesion and spreading on fibrinogen. Moreover, adhesion through αIIbβ3 caused a marked increase in the levels of endogenous GTP-bound Rac3 but not Rac1. These combined results strongly implicate Rac3 and CIB in integrin-associated cytoskeletal reorganization during αIIbβ3-mediated adhesion.

The small GTPase Rac1 participates in the regulation of a large number of different cellular functions, including ones that involve the reorganization of the actin cytoskeleton such as happens during the adhesion and spreading of cells on extracellular matrix (1, 2). Spreading and adhesion are mediated by the integrin class of transmembrane receptors. The affinity of integrins for ligands can be regulated intracellularly by agonists through so-called inside-out signaling. Ligand-bound integrins transmit signals to the cell interior, resulting in post-receptor occupancy events, including actin cytoskeletal rearrangement, leading to the spreading of cells (3).

Integrin-mediated adhesion and spreading have been linked to the activity of Rac1. Experiments in T-cells demonstrated that V12Rac1 is not involved in the first steps leading to activation of the integrin receptor but, rather, that it plays a role in these post-receptor occupancy events (4). Within minutes of integrin engagement on normal human natural killer cells, the level of endogenous active GTP-bound Rac1 is increased (5).

Adhesion of NIH3T3 cells to fibronectin also caused a transient increase in the levels of activated Rac (6). The introduction of constitutively active V12Rac1, which is locked in its GTP-bound state, into cultured cells results in a dramatic spreading of these cells, accompanied by actin cytoskeletal rearrangements, which are integrin-dependent (2, 7, 8). Moreover, integrin engagement leads to downstream activation of previously identified Rac effectors (8). In combination, these data demonstrate that the cytoskeletal rearrangements associated with integrin activation are linked to Rac1, but the exact mechanism by which this takes place is not clear.

There are only three human Racs, which together are responsible for mediating the large variety of downstream cellular effects associated with activated Racs. They share between 89 and 93% identity in their amino acid sequence (9). Because the sequence of their so-called effector loop, the region to which most Rac effectors bind, is virtually identical, it is unclear how specificity is generated. Indeed, no proteins binding to the Rac effector loop have been reported that interact with only one single Rac family member.

The only region that differs substantially among Rac1, 2, and 3 is their C-terminal end (9), which also contains subcellular localization determinants. We reasoned, therefore, that this region is most likely to specify differential functions between the three Rac isoforms. In an attempt to isolate proteins interacting only with Rac3, we used the isolated C-terminal 48 amino acid residues of Rac3 to screen a yeast two-hybrid cDNA library. Here we report that the calcium- and integrin-binding protein CIB binds exclusively to Rac3 and that these proteins stimulate adhesion and spreading of cells in an αIIbβ3 integrin-dependent manner. This is the only protein identified to date that interacts exclusively with a single Rac isoform and links Rac3 with adhesion and spreading of cells through the fibrinogen receptor.

EXPERIMENTAL PROCEDURES

Plasmids—The C-terminal fragments of RAC3 and RAC1 encoding amino acids 145–192 were subcloned into the yeast bait vector pAS2-1 (Fig. 1A, CLONTECH). pAS2-1/V12S189Rac3 was used to isolate and subclone the C-terminal fragment containing the S189 mutation (10). In addition, that plasmid was used to generate GST/V12S189Rac3 in pLEF. The plasmids pAS2-1/S189Rac3, pLEF/GST-wtRac3, and pLEF/GST-V12Rac3 have been described previously (10). pcDNA3.1/Xpress-N17Rac3 was generated by PCR mutagenesis using RAC3

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§ The abbreviations used are: CIB, calcium- and integrin-binding protein; GST, glutathione S-transferase; HA, hemagglutinin; IB, immuno blot; PDZ, p121-binding domain; wt, wild type; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; TRITC, tetramethyl rhodamine isothiocyanate; EGFP, enhanced green fluorescent protein.
cDNA as a template (9). To express GST-V12Rac1 and GST-V12Rac2 in mammalian cells, a G12V mutation was generated by PCR mutagenesis using Rac1 and Rac2 cDNAs as templates (9). Mutations were verified by sequencing. Candidate positive cDNAs from the yeast two-hybrid screen were subcloned into pSG5 (Stratagene) as described previously (10). One each of NoA/H2A bait clones was designated. Soybean trypsin inhibitor was added, and 2× cells were washed twice with DMEM/0.1% BSA, trypsinized, and kept in suspension for 1 h. Cells were plated on Petri dishes coated with 20 μg/ml fibrinogen (A5 CHO) or with 10 μg/ml fibronectin (CHO-K1) or kept in suspension at 37 °C for 30 min. Cells were washed with cold PBS and lysed in Triton-lysine buffer. An aliquot of the lysate was solubilized in 2× Laemml sample buffer (total lysate) and boiled. The lysates were clarified by centrifugation at 19,000 × g for 20 min. The supernatant (Triton-soluble fraction) was removed, and the pellet was washed once with Triton-lysine buffer and finally solubilized in 2× Laemmli sample buffer (Triton-insoluble fraction). An equal amount of protein (10 μg) was loaded in each lane. Proteins were assayed using anti-Rac3, anti-Xpress, or anti-GST antibodies.

**Immunocytochemistry**—Transfected and serum-starved A5 CHO cells were plated on fibrinogen-coated Lab-Tek chamber slides (Nunc) for 15 or 30 min at 37 °C. Nonadherent cells were washed away, and adherent cells were fixed with 2% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked in PBS plus 3% normal goat serum (Zymed Laboratories Inc.) for 30 min. Cells were incubated for 1 h with primary antibodies including polyclonal anti-HA (Santa Cruz Biotechnologies, 1:4000), polyclonal anti-GST (1:50), monoclonal anti-Xpress (1:4000), or monoclonal anti-Myc (USC Core Facility, 1:200) antibodies, followed by incubation for 30 min with fluorescein isothiocyanate (1:50)- and TRITC (1:100)-conjugated secondary antibodies (Zymed Laboratories Inc.). Actin filaments were stained with TRITC-phalloidin (Sigma, 1:1000). Slides were then washed and incubated for 1 h with 300 μg/ml 6-diamidino-2-phenylindole (Molecular Probes). Slides were mounted in Vectashield (Vector Laboratories), and cells were analyzed using a Zeiss LSM 510 or a Leica TCS SP confocal microscope.

**Identification of a Specific Rac3-binding Protein CIB**—We screened a human placental cDNA library with C-terminal residues 145–192 of Rac3 as bait (C-term. Rac3, Fig. 1A) and isolated one clone, 107-14, independently six times. In a separate yeast two-hybrid screen using V12S189Rac3 as bait, we identified several other positives, including a CRIB domain-containing target as well as a protein with a serine/threonine kinase homology domain (10). The strength of interaction of clone 107-14 with C-term. Rac3 was comparable to that of the

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**RESULTS**

Identification of a Specific Rac3-binding Protein CIB—We screened a human placental cDNA library with C-terminal residues 145–192 of Rac3 as bait (C-term. Rac3, Fig. 1A) and isolated one clone, 107-14, independently six times. In a separate yeast two-hybrid screen using V12S189Rac3 as bait, we identified several other positives, including a CRIB domain-containing target as well as a protein with a serine/threonine kinase homology domain (10). The strength of interaction of clone 107-14 with C-term. Rac3 was comparable to that of the

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CIB failed to bind to the Rac1 C terminus in yeast. Therefore we co-transfected CIB with the V12 mutants of Rac1, Rac2, and Rac3 into CHO-K1 cells and compared the binding of CIB to these closely related small GTPases. As shown in Fig. 2B (Lysate), all three GST-tagged small GTPases and Xpress-tagged CIB were clearly expressed in these cells. However, remarkably, CIB was only able to complex with full-length V12Rac3, and failed to detectably bind to full-length V12Rac1 or V12Rac2 (Fig. 2B, GST-pull-down).

As discussed above, in small GTPases such as Rac, the prenylation of the carboxyl-proximal cysteine residue (Cys-189) is a major determinant for membrane association (22), and prenylation can be blocked by the C189S mutation. Because Cys-189 does not appear to be essential for the binding of Rac3 to CIB but will prevent its association with membranes, we tested the binding of CIB to V12S189Rac3 in eukaryotic cells. CHO-K1 cells were co-transfected with Xpress-tagged CIB plus GST-tagged V12Rac3 or V12S189Rac3 or V12S189Rac3. Xpress-tagged CIB protein bound to GST-Rac3 was analyzed using anti-Xpress antibodies. The constructs were efficiently expressed (Fig. 2C, Lysate). As shown in Fig. 2C (GST-pull-down), in comparison with V12Rac3, there was significantly less CIB complexed with the V12S189Rac3 mutant. These experiments indicate that membrane localization of Rac3 is important for the efficient interaction between CIB and Rac3.

We considered the possibility that CIB is involved in the activation of Rac3. As shown in Fig. 2A (GST-pull-down), there as a detectable interaction of wild type Rac3 with CIB, although the level of detected complex formation was much lower than that with constitutively active GTP-bound Rac3. Wild type Rac is mainly in the GDP-bound form. To examine if CIB is involved in the activation of Rac3, cells were transfected with CIB and wtRac3, and affinity precipitation with GST-PAK-PBD was performed as described (11), followed by immunoblotting with anti-Xpress antibodies. The expression level of HA-CIB was equal in each lane (Fig. 2D, Lysate). As controls, we transfected cells with CIB plus empty vector or plus V12Rac3. As shown in Fig. 2D, although GTP-bound V12Rac3 was clearly detectable using this method, there was no evidence that any of the wtRac3 was in the GTP-bound state in the presence of CIB. These data show that CIB and Rac3 specifically interact in mammalian cells but that this does not result in the activation of Rac3.

Levels of Active Rac3 in the Triton-insoluble Fraction Increase upon Fibronogen-mediated Adhesion—Both CIB and Rac3 are ubiquitously expressed. Although the normal cellular functions of Rac3 have not yet been defined, Rac1 was shown to be involved in integrin-mediated adhesion and spreading. CIB was originally identified in a yeast two-hybrid screen as a protein binding specifically to the C-terminal cytoplasmic domain of the α1β1 integrin. Therefore, we examined a possible role for Rac3 in α1β3 integrin-mediated spreading and adhesion.

The function and downstream signaling of the α1β3 integrin
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Lysates were also probed for HA-CIB (Fig. 3, upper panel). Lysates of CHO-K1 cells co-transfected with Xpress-tagged CIB plus Xpress-tagged wtRac3, or GST-tagged V12Rac3 or GST-tagged V12S189Rac3. Cell lysates were incubated with glutathione-agarose beads, and the presence of CIB was detected by immunoblotting with anti-Xpress antibodies. In each pull-down reaction equal amounts of Xpress-CIB proteins were present. The bottom panel shows the protein levels of Xpress-CIB (top) and GST-Rac3 (bottom) in total cellular lysates. D, lysates of CHO-K1 cells co-transfected with HA-CIB plus Xpress-tagged wtRac3, V12Rac3, or vector were incubated with GST-PAK-PBD immobilized on glutathione-agarose, and bound, active GTP-Rac3 was analyzed by Western blotting. Cell lysates probed for total Xpress-Rac3 (Lysate, upper panel) are shown as a control. Lysates were also probed for HA-CIB (Lysate, lower panel).

have been extensively investigated in the A5 CHO model system, in which αIIbβ3 is stably expressed. These cells can adhere to fibrinogen because of the presence of the αIIbβ3 integrin, which is lacking in the parental cell line; parental CHO-K1 cells are unable to adhere to fibrinogen-coated supports (12). Engagement of the αIIbβ3 integrin leads to association of specific proteins involved in signaling through this integrin with the cytoskeleton, where they are recovered in the Triton-insoluble fraction of the cell (23). To investigate whether CIB and V12Rac3 become incorporated into the Triton-insoluble fraction during fibrinogen-mediated adhesion, A5 CHO cells were transiently co-transfected with Xpress-CIB plus GST-V12Rac3. Cells were either allowed to adhere on fibrinogen or kept in suspension. The total amount of Rac3 or CIB was not different between adherent and non-adherent cells (Fig. 3A, lanes T). In suspended cells, a relatively low level of CIB was associated with the Triton-insoluble cytoskeletal fraction (Fig. 3A, right panel, lane IS). When cells were plated on fibrinogen, a 2- to 3-fold increase of CIB as well as V12Rac3 protein levels was detected in this fraction (Fig. 3A, left panel, lanes IS), indicating that both V12Rac3 and CIB become associated with the cytoskeleton, and αIIbβ3 integrin-mediated adhesion.

To compare the localization of CIB in the absence and in the presence of Rac3, we transfected A5 CHO cells with CIB or with CIB plus GST-tagged V12Rac3. Cells were adhered on fibrinogen for 30 min, and Triton-soluble and Triton-insoluble fractions were prepared. As shown in Fig. 3B, the amount of CIB in the Triton-insoluble fraction was increased in the presence of V12Rac3 (compare lanes IS), whereas the level of association with the V12Rac3 in Triton-insoluble fraction, which was already high, did not clearly change in the presence of CIB (Fig. 3C).

To be able to compare the localization of Rac3 with that of Rac2 and Rac1, we co-transfected Xpress-CIB with V12Rac1, 2, or 3, which were all GST-tagged, and repeated the experiment. GST was recovered only in the soluble fraction, indicating that there is no significant contamination of the insoluble fraction with soluble proteins. V12Rac3 levels increased in the insoluble fraction when the A5 CHO cells were allowed to adhere on fibrinogen, in concordance with the previous experiment (Fig. 3D). V12Rac1 and V12Rac2 clearly behaved differently: Their levels did not increase in the insoluble fraction of the fibrinogen-adherent cells (Fig. 3D). In addition, the overall amount of V12Rac2 associated with the insoluble fraction was less than that of V12Rac1 or V12Rac3 and is concordant with the difference in subcellular locations found for Rac1 and Rac2 in living cells (24).
Increased association of CIB and V12Rac3 with the Triton-insoluble fraction upon α1β1-mediated adhesion of A5 CHO cells to fibrinogen. A, A5 CHO cells were co-transfected with plasmids encoding Xpress-tagged CIB and GST-V12Rac3 and plated on fibrinogen (Adh.; adherent) or kept in suspension (Susp.). The Triton-soluble (S), Triton-insoluble (IS), and total (T) lysates were analyzed by immunoblotting using anti-Xpress (top) or anti-Rac3 (bottom) antibodies. B and C, A5 CHO cells were transfected with Xpress-CIB (B), GST-V12Rac3 (C), or both (B, C), and plated on fibrinogen. The Triton-soluble (S) and Triton-insoluble (IS) lysates were analyzed using anti-Xpress and anti-GST antibodies. D, A5 CHO cells were transfected with Xpress-CIB plus the V12Rac constructs indicated to the right and either kept in suspension or allowed to adhere to fibrinogen. As control, CHO-K1 cells transfected with Xpress-CIB plus GST were adhered to fibronectin or kept in suspension (bottom panel). The Western blots were reacted with anti-GST antibodies. The results of one of two independently performed experiments are shown.

To visualize CIB, transfected cells were stained for the Xpress tag. As shown in Fig. 4B (top), some cells showed a prominent CIB signal in a nuclear location, as confirmed by 4',6-diamidino-2-phenylindole labeling and optical sectioning (not shown). Other cells exhibited only cytoplasmic staining (Fig. 4B, bottom). Because myristoylation of overexpressed CIB was shown to be abolished by addition of an N-terminal tag (17), we also generated a C-terminal tagged CIB. Although the quantitative location of N-terminal (Xpress- or HA-) and C-terminal (myc-) tagged CIB differed, both proteins localized in the cytoplasm and in the nucleus (Figs. 4B and 5D, left panel). CIB contains two EF-hand motifs (13). These also constitute potential determinants of subcellular location for CIB (17); therefore, we examined the effect of deletion of the C-terminal EF-hand domain. In comparison with HA-CIB, HA-CIB del EF showed greatly decreased nuclear localization, suggesting that this domain indeed functions as a determinant for subcellular localization for CIB (Fig. 4C, compare left upper and lower panels).

Cells co-expressing CIB and V12Rac3 were very well-spread (Fig. 5A). V12Rac3 and CIB co-localized in the perinuclear area. Interestingly, both were also prominently localized at the cell periphery (Fig. 5A). The co-expression of CIB with V12Rac3 caused the increased translocation of CIB to that location, because in cells transfected with CIB alone, CIB was not concentrated at the edges of the cell (compare Fig. 4B to 5A).
the prominent signal for V12Rac3 at the cell periphery and the images shown were taken at the level of cell-substratum contact. Note the high spread morphology and the co-localization of V12Rac3 and CIB in the nucleus, respectively. Image points to membrane protrusions containing both V12Rac3 and V12Rac2 transfected cells, which are generally less round. Also note the differences in localization of the two Racs, with most of the V12Rac3 distributed between locations at the cell periphery, in the cytoplasm and juxtanuclear, whereas most of the V12Rac2 signal is juxtanuclear. Scale bar, 20 μm.

Cells transfected with V12Rac2 and CIB and plated on fibrinogen were morphologically distinct from those transfected with V12Rac3 and CIB and did not show the typical rounded morphology (compare Figs. 5D and 5E). Cells also lacked the prominent localization and co-localization at the cell periphery as seen with V12Rac3 and CIB. We also repeated the experiment with CIB and V12Rac1 (not shown). The morphology of many of these transfected cells was similar to that of cells transfected with V12Rac3 plus CIB, and both CIB and V12Rac1 were present at the cell periphery (not shown). However, our experiments described in Fig. 2B demonstrated that, in contrast to Rac3 and CIB, there is no physical interaction between Rac1 and CIB. Our combined data provide evidence that CIB is directed to the cell periphery by endogenous Rac3 in A5 CHO cells.

**Fig. 5. Comparison of A5 CHO cells adhering to fibrinogen and expressing CIB plus either V12Rac3, N17Rac3 or V12Rac2.** A, co-transfection of HA-CIB with Xpress-tagged V12Rac3. Note the highly spread morphology and the co-localization of V12Rac3 and CIB at the cell periphery and in the perinuclear area. Scale bar, 20 μm. B, co-transfection of HA-CIB with Xpress-tagged N17Rac3. Cells were stained for the HA-epitope (green), Xpress-epitope (red), and merged. C, lysates of A5 CHO cells transfected with the DNAs indicated above the lanes were Western-blotted and reacted with anti-GST (upper panel) or anti-Myc (lower panel) antibodies. Rac3 and Rac2 are indicated as 3 and 2, respectively. D, confocal microscopy images of fibrinogen-adherent A5 CHO cells co-transfected with GST-V12Rac3 and CIB-Myc and reacted with anti-Myc (left), anti-GST (middle), or merged (right). The images shown were taken at the level of cell-substratum contact. Note the prominent signal for V12Rac3 at the cell periphery and the circle-shaped, very spread morphology of the cells. An arrow in the merged image points to membrane protrusions containing both V12Rac3 and CIB. Large arrowheads and a small arrowhead in the left panel point to cells that have localization of CIB at the membrane/in the cytoplasm and in the nucleus, respectively. E, fibrinogen-adherent A5 CHO cells transfected with GST-V12Rac2 and CIB-Myc were imaged using confocal microscopy for Myc (left), GST (middle), or merged (right). The images were taken at the level of cell-substratum contact. Note the morphological differences between fibrinogen-adherent V12Rac3 and V12Rac2 transfected cells, which are generally less round. Also note the differences in localization of the two Racs, with most of the V12Rac3 distributed between locations at the cell periphery, in the cytoplasm and juxtanuclear, whereas most of the V12Rac2 signal is juxtanuclear. Scale bar, D, E, 20 μm.

Cells transfected with CIB and N17Rac3 behaved very differently from those transfected with CIB and V12Rac3. It was difficult to detect any transfected cells that adhered to the fibrinogen-coated plates. Those that did adhere exhibited a rounded shape and were not spread (Fig. 5B).

**DISCUSSION**

Mammalian cells express three different Rac proteins, which share a very high degree of amino acid identity. To date it has not been possible to assign specific functions to each. For example, although Rac2 was proposed to be the Rac responsible for the regulation of reactive oxygen species production in hematopoietic cells, active Rac1 can also stimulate this process (25, 26). Most Rac effectors bind to one or more of the so-called effector domains in Rac, which are almost identical in the three Rac proteins. Therefore, specificity must be conferred by spe-
CHO cells to immobilized fibrinogen. The result shown for Rac1 is the mean of three independently performed experiments using a colorimetric detection. The extent of adhesion is expressed as the percentage of maximal adherence of control transfected cells. Data from one experiment representative of two with similar results are shown. **,** statistically significant difference with control cells at $p < 0.001$. A, CHO cells expressing $\alpha_{1B}\beta_3$, were co-transfected with plasmids encoding $\beta$-galactosidase (control), Xpress-tagged CIB, V12Rac3, or CIB plus V12Rac3. Cells were adhered to fibrinogen for 30 min, and adherent cells were quantitated using a colorimetric detection. The extent of adhesion is expressed as the percentage of maximal adherence of control transfected cells. Data from two experiments are shown. **,** statistically significant difference with control cells at $p < 0.05$. B, cells in suspension; FG, fibrinogen-adherent. The gray and open bars represent Rac3 and Rac1, respectively. Levels of endogenous active and total Rac1 were measured using Rac1-specific antibodies (Transduction Laboratories). The result shown for Rac1 is the mean of three independently performed experiments.

Specific tempo-spatial expression or subcellular localization of Rac. In the current study we have identified a novel and unique type of specificity, which is conferred by the primary sequence of Rac.

Residues 143–175 of Rac contain the C-terminal effector domain (Fig. 1A), and this region is needed for activation of the NADPH oxidase and the c-Jun N-terminal kinase pathway (27–30). However, only two (conserved) amino acid substitutions are different in this area of Rac1 and Rac3, and it is thus unlikely to account for the differential binding of CIB to Rac1 and Rac3. The very C-terminal end, including residues 180–192 encompasses the most diverged region between Racs, consisting of a polybasic region and an immediately adjacent region that is necessary for post-translational modification of Rac. Both regions are involved in Rac membrane targeting. Interestingly, it has been shown that differences between the polybasic regions of Rac1 and Rac2 are responsible for the different affinities of these two proteins for activating the effector PAK (31). These studies, in combination with our finding that CIB binds to residues 145–192 of Rac3, and not Rac1, support the concept that the primary sequence of this domain may confer specificity to different Rac family members.

Numerous proteins have been identified that interact with Racs, and some bind to the related Cdc42 as well, but none have been reported to interact exclusively with a single Rac family member. Our two-hybrid screen differed fundamentally from those performed previously because we used the isolated C-terminal end of Rac3 as bait. Apparently, in the yeast nucleus, in the context of the whole Rac3 protein, this domain is improperly folded, has an abnormal conformation, or is not accessible, because CIB failed to interact with full-length S189Rac3 or V12S189Rac3 in yeast, although it readily bound to the isolated C-terminal end with or without the Cys-189 mutation. However, we found that when Rac3, and especially activated Rac3, was produced in mammalian cells, its C terminus apparently has the correct conformation, or is exposed, and is able to bind CIB. These results suggest that other important Rac-interacting proteins may not have been identified in the yeast two-hybrid system for similar reasons.

CIB appears to have at least two regions that are important for subcellular location. Stabler et al. (17) reported that overexpressed CIB becomes myristoylated and that addition of an N-terminal tag interferes with this modification. However, this group and others (14, 15) showed that N-terminally tagged CIB is nuclear and cytoplasmic, which is also where endogenous CIB is located (17). In fact, all forms of CIB (N-terminal, C-terminal, and untagged) showed a nuclear and cytoplasmic distribution, although detectable quantitative shifts in location were seen. Stabler et al. (17) noted that the location of CIB could also potentially be regulated by a calcium-myristoyl switch, in which the myristoyl group would be either exposed or sequestered, depending upon the state of calcium binding. Our finding that a CIB mutant lacking residues 130–191 becomes mainly cytoplasmic supports the concept that the EF-hand motif additionally determines its location and suggests that a C-terminal tag may also influence the distribution of CIB among the different subcellular locations. Therefore, we have used both N- and C-terminal-tagged CIB in our studies, but mostly the N-terminal-tagged form, because this was most frequently used in other CIB localization studies (14, 15, 17).

CIB was originally identified in the yeast two-hybrid system, because it interacts with the cytoplasmic domain of $\alpha_{1B}$. Vallar et al. (18) showed that CIB is not involved in activation of $\alpha_{1B}$ and, in fact, interacts preferentially with the high affinity state of $\alpha_{1B}\beta_3$. This places the function of CIB in the category of post-receptor occupancy events. Interestingly, studies of Rac1 in T-cell lines have also implicated Rac1 in the integrin clustering that happens subsequent to integrin activation (4). Very recently, the function of Rac1 in the formation of new integrin-mediated adhesions was further defined using an engineered monoclonal antibody antigen binding fragment: Rac1 was shown to be required for the recruitment of already activated $\alpha_\beta_3$ integrin at lamellipodia. The authors speculated that the mechanism by which these high affinity integrins are localized to cell edges may be through a more tight association with cytoskeletal elements (32).

Our experiments showed that CIB requires membrane localization of Rac3 for efficient interaction. Shock et al. (16) showed that in aggregated platelets, CIB and $\alpha_{1B}\beta_3$ become incorporated in the Triton X-100-insoluble cytoskeleton in a parallel manner. We have shown that Rac3 levels also increase in the cytoskeletal fraction upon $\alpha_{1B}\beta_3$-mediated adhesion and that, moreover, active Rac3 was located at the cell periphery to-
gether with CIB in cells adhering to fibrinogen. Finally, the binding of αIIbβ3 to fibrinogen sends a signal to within the cell, resulting in activation of endogenous Rac3. Vallar et al. (18) showed that CIB binds preferentially to the activated αIIbβ3. Taken together, the combined data suggest a model in which the engagement of αIIbβ3 intracellularly results in Rac3 activation, CIB binding, and their combined recruitment to the cell periphery at the site of cell-substratum contact. We speculate that an activity stimulated by activated Rac3 could then increase actin polymerization in the vicinity of activated αIIbβ3 and explain the increased incorporation of CIB, Rac3, and αIIbβ3 into the Triton X-100-insoluble cytoskeletal fraction. In this model, the increased adherence on fibrinogen that we measured of A5 CHO cells transfected with V12Rac3 and/or CIB is due to increasing incorporation of the integrin into the cytoskeleton and thus a strengthening of already established adhesive contacts, rather than the stimulation of formation of such contacts.

There is increasing evidence that an important function of Rac family GTPases is to direct Rac-binding proteins to certain subcellular locations (6, 33, 34). Our data are in agreement with this and, moreover, suggest that the C-terminal ends of the three Rac proteins can provide specificity to potential interacting partners.

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