RhöG is a new GTPase that has high sequence similarity with members of the Rac subfamily (Rac1, Rac2, and Rac3), including the regions involved in effector recognition and binding. To characterize its biological properties, we have compared the activity of RhöG and Rac1 in a number of experimental systems, including the study of their subcellular localization, oncogenic potential, activation of effectors, and effect on F-actin dynamics. Our study indicates that RhöG and Rac1 share overlapping, but not identical, signal transduction pathways. In contrast to previous results, we also provide evidence that RhöG works in parallel to Rac1 rather than as a Rac1 upstream activator. Using an extensive collection of Rhö/Rac1 chimeras and point mutants, we demonstrate that the different biological properties of RhöG and Rac1 can be traced to specific amino acid variations in their switch I, β2/β3 hairpin, α helix, and C-terminal polybasic regions. Taken collectively, our results highlight the complexity of the signal transduction pathways activated by Rhö/Rac GTPases and provide insight into the structural determinants that mediate the differential engagement of biological responses by GTPases of very similar structure.

GTP hydrolases of the Rhö/Rac family participate in the generation of coordinated cellular responses to extracellular stimuli (1, 2). This GTPase family is composed of numerous members that can be classified according to structural similarity in Rho (RhöA, RhöB, RhöC, RhöD, RhöE, and TTF), Rac (Rac1, Rac2, Rac3, and RhöG), and Cdc42 (Cdc42 and TC10) subfamilies (2). The majority of these proteins are characterized by a common mechanism of activation during signal transduction (2). In non-stimulated cells, Rhö/Rac proteins are maintained in an inactive state because of the presence of bound GDP molecules. After cell stimulation, they exchange GDP by GTP, an event inducing structural changes in their switch I and II regions that makes them competent for effector binding. At the end of the stimulation cycle, Rhö/Rac GTPases become inactive again because of the hydrolysis of GTP to yield GDP. This cycle is regulated by GEFs and GAPs. GEFs accelerate the activation of the GTPases during cell stimulation by catalyzing the release of the bound GDP. By contrast, GAP catalyze the hydrolysis of GTP into GDP, therefore allowing the rapid inactivation of the GTPases at the end of the stimulation cycle (2). In addition to these regulatory molecules common to all GTP-binding proteins, Rhö/Rac proteins utilize regulatory mechanisms that are different from other GTPase families. Thus, the expression of some Rhö/Rac family members is inducible during cell stimulation or differentiation (i.e. Rac2 and RhöG) (3, 4). Moreover, most Rhö/Rac proteins bind to negative regulatory molecules known as Rhö GDIs. These proteins regulate the subcellular localization of Rhö/Rac GTPases by extracting them from the plasma membrane and keeping them sequestered in the cytosol (2, 5, 6). In addition, recent data suggest that GDIs may inhibit Rhö/Rac signaling by removing effector molecules from the activated GTPase (7).

One key biological function of Rhö/Rac family members is the regulation of F-actin polymerization and cytoskeletal dynamics (1, 2). For instance, it has been shown that Rac1 promotes the formation of lamellipodia and membrane ruffling, that RhöA induces stress fibers and focal adhesions, and that Cdc42 is in charge of generating microspikes and filopodia in a number of cell types (1). These proteins have also been linked to other cellular processes such as apoptosis/cell survival, immune functions, vesicle trafficking, tissue contractility, cell cycle progression, and transcriptional changes (1, 2, 8). These activities are the result of the direct activation of different subsets of effector molecules, including adaptor proteins, actin polymerization/branching agents, and kinases of different substrate specificity (1, 2, 8). The function of Rhö/Rac-dependent pathways have also been associated with a number of human disorders such as cancer, hereditary immunodeficiencies, human immunodeficiency virus replication, X-linked mental retardation, deafness, and other developmental abnormalities (9). These observations have fueled the interest for the functional characterization of all members of this GTPase family.

RhöG is a new member of the Rhö/Rac family that is still poorly characterized. This GTPase was initially discovered as a ubiquitously expressed immediate early gene that was induced by a number of extracellular stimuli such as serum, thrombin, and fibroblast growth factor (4). Later on, the activity of this...
GTPase has been linked to cytoskeletal changes and the abrogation of cell contact inhibition in fibroblasts (10), neurite outgrowth in rat pheochromocytoma cells (11), and axogenesis in primary sympathetic postganglionic neurons (12). More recently, RhoG has been linked to the stimulation of the nuclear factor of activated T-cells (13), a transcriptional factor important for the activation of genes encoding interleukin 2 and other cytokines (14). One interesting property of RhoG is its close structural relationship with Rac1. These two GTPases share 72% overall sequence identity and, perhaps importantly, are very similar in the regions involved in effector binding, such as the switch I (92% sequence identity), the β/β3 hairpin (75% sequence identity), and the switch II domain (89.5% sequence identity). This structural likeness suggests that these two proteins must utilize similar signal transduction pathways. However, one unexpected observation is that RhoG appears to act not in parallel but upstream of Rac1, allowing the activation of this GTPase during specific cellular responses (10, 11). A similar relationship has also been reported for RhoG and Cdc42 (10, 11). For instance, it has been shown that the constitutively active version of RhoG can induce the formation of membrane ruffles and filopodia that can be blocked in turn by the expression of dominant negative mutants for Rac1 and Cdc42, respectively (10). Moreover, it has been reported that the RhoG-dependent neurite outgrowth can be eliminated by the expression of the same interfering mutants in rat pheochromocytoma cells (11). Although linear pathways of activation between different Rho/Rac GTPases have been previously described (1, 2), this is the first example of an activation of one GTPase by one closely related member of the same subfamily. The structural similarity of RhoG and Rac1 prompted us to investigate in detail their effector specificities and signaling relationships. To this end, we compared the biological properties of these two GTPases in a number of cellular responses, including subcellular localization, activation of signaling pathways, and oncogenesis. Moreover, we have used two independent techniques to verify the upstream position of RhoG over Rac1 in signal transduction. The results obtained indicate that RhoG and Rac1 act in non-linear, parallel pathways that share overlapping, but not identical, signaling elements. Specifically, we found that RhoG and Rac can promote JNK activation and induce similar changes in the F-actin cytoskeleton. However, they differ in subcellular localization, ability to bind PAK1, and transforming activity. To understand in detail the structural basis that allows the engagement of separate signaling elements by these two GTPases, we used an extensive collection of chimeric proteins in which specific fragments of Rac1 were swapped by the equivalent regions of RhoG. In addition, we utilized point mutants that targeted divergent residues between these two GTPase in the regions presumably involved in assuring substrate selectivity. All these mutant proteins were tested in PAK1 binding, cellular transformation, and subcellular localization. Our results indicate that amino acid residues within the switch I, β/β3 hairpin, α5, and hypervariable region of RhoG and Rac1 are involved in the differential engagement of those cellular responses.

EXPERIMENTAL PROCEDURES

Expression Vectors—All RhoG and Rac1 point mutants described in this work were generated using the QuikChange™ mutagenesis kit (Stratagene) according to the manufacturer’s instructions. RhoGβ61L/Rac1 chimeric cDNAs were generated by PCR using the Elongase polymerase (Invitrogen) following a previously described protocol (15). The oligonucleotide sequences and protocols for generating each specific chimera are available upon request. The pGEX vector containing the PAK1 CRIB domain was provided by Dr. R. Cerione (Cornell University, Ithaca, NY). The GST-PAK1 CRIB domain was expressed in Escherichia coli according to standard procedures (16).

RESULTS

RhoG and Rac1 Have Overlapping, but Not Identical, Signaling Pathways—To compare the signaling specificities of RhoG and Rac1, we studied their respective activities in four different biological assays. First, we analyzed their subcellular localization and their effects on the F-actin cytoskeleton in COS1 and NIH3T3 cells. As previously described (21), the constitutively active version of Rac1 (Q61L mutant) induces robust membrane ruffling in COS1 cells, showing a preferential subcellular localization in plasma membrane and membrane ruffles (Fig. 1A). A small fraction of Rac1Q61L was also

Mammalian expression vectors encoding different versions of RhoG, Rac1, H-Ras, Cdc42, Vav3 (41–66), RhoA, and H-Ras have been previously described (17, 18).

Tissue Culture Conditions—All cell types were cultured at 37 °C and an atmosphere of 5% CO2 in a Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 1% l-glutamine, and 1% penicillin-streptomycin. All tissue culture reagents were obtained from Invitrogen.

Immunofluorescence—Cells were grown on uncoated glass coverslips introduced into six-well plates (50,000 cells/plate) and transfected with 1 μg of the indicated plasmids for 30 h using liposomes (FuGENE 6, Roche Diagnostics), as described previously (18). Cells were then rinsed in phosphate-buffered saline and fixed with 3.7% formaldehyde (Sigma) for 15 min, permeabilized by incubation with phosphate-buffered saline containing 0.5% Triton X-100 for 10 min, and blocked for 10 min in 2% bovine serum albumin, 0.1% sodium azide, 0.1% Triton X-100 in 25 mM Tris-buffered solution. Cells were incubated with anti-AU5 antibodies (Babco) (1:1000 dilution) for 1 h and then incubated with a mouse secondary antibody coupled to Cy2 (Jackson Immunoresearch) (1:200 dilution) for 45 min. For staining the F-actin cytoskeleton, cells were incubated with rhodamine- phalloidin (Molecular Probes) for 20 min (18). After three washes in 25 mM Tris-buffered saline solution containing 0.1% Triton X-100, the coverslips were mounted onto microscope slides using a standard mounting medium (Silkslide, Molecular Probes). Antibody dilutions were made in blocking solution. All steps were conducted at room temperature. Fluorescence images were captured with a Zeiss LSM510 confocal microscope. Fluorochromes were excited using an 10Ar laser (488 nm excitation-wavelength) for Cy2 and a HeNe laser (543 nm excitation wavelength) for both Cy3 and rhodamine.

Focus Formation Assays—NIH2T3 cells (150,000 cells/10-cm plate) were transfected with 20 μg of cells molecular weight calf thymus DNA (Roche Diagnostics) and the indicated plasmids using the calcium-phosphate precipitation method (19). After 24 h, DNA/calcium phosphate precipitates were removed by two washes with Dulbecco’s modified Eagle’s medium containing 5% calf serum and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum for an additional 24 h. After this period, cells were fixed with formaldehyde and stained with Giemsa to count the foci of transformed cells. All transfections were done in duplicate.

GST Pull-down and Kinase Assays—COS1 cells growing in 10-cm dishes were transfected with 5 μg of the indicated constructs using the DEAE-dextran method (20). 48 h after transfection, cells were washed on ice-cold phosphate-buffered saline solution and then disrupted in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 0.5% Triton X-100, 5 mM β-glycerophosphate, 1 mM dithiothreitol, a protease-inhibitor mixture (Complete, Roche Molecular Biochemicals), and 10 μg of the GST-PAK1 CRIB domain fusion protein. Cell lysates were precleared by centrifugation at 14,000 rpm for 10 min at 4 °C and then incubated with glutathione-Sepharose beads (Amer- sham Biosciences) for 2 h at 4 °C. Beads were washed three times in lysis buffer without GST-PAK1 CRIB domain and boiled in SDS-PAGE sample buffer to release the bound proteins. Eluates were separated by electrophoresis, transferred to nitrocellulose filters, and subjected to immunoblot analysis using a monoclonal antibody to the AU5 epitope (Babco). Immunocomplexes and pulled down proteins were visualized by chemiluminescence techniques using a commercial kit (ECL, Amer- sham Biosciences). JNK immunocomplex assays were done as described (20), using a GST-ATF2 fusion protein as phosphate acceptor. Hemaglutinin antibodies used in the immunocomplex assays were from Babco.

Informatics—Confocal image analysis was carried out with the LSM 5 Image Browser program (version 2.8). Protein tertiary structures were elaborated using the Cn3D 4.0 program.
detected in the cytosol (Fig. 1A). Wild type Rac1 induced no detectable morphological change and displayed a uniform distribution in the cytosol (Fig. 1A), a result consistent with its high GDP content and its constitutive association with Rho GDIIs (see Supplementary Information, Fig. S1) (2). Wild type RhoG showed identical distribution as that of Rac1 in COS1 cells (Fig. 1A). However, RhoGQ61L localization was missed in intracellular vesicles and, unlike Rac1Q61L, was totally absent from the plasma membrane and ruffles (Fig. 1A). The differential localization of RhoG and Rac1Q61L correlated with their differential binding to Rho GDI (see Supplementary Information, Fig. S1), as previously described for other Rho/Rac GTPases (2). The RhoG-containing vesicles are from endocytic origins, because they co-localize with internalized epidermal growth factor receptor.2 In contrast to previous reports (22), no association of RhoGQ61L could be found with the endoplasmic reticulum or lysosomes. Despite the differential localization of the activated forms of RhoG and Rac1, both induced strong membrane ruffling when expressed in COS1 cells (Fig. 1A). When expressed in murine fibroblasts, Rac1Q61L and RhoGQ61L induced extensive lamellipodia in the cell periphery (Fig. 1B). In the case of Rac1, this phenotype was associated with the formation of a perinuclear actinomyosin ring (Fig. 1B). This morphological phenotype was similar to that induced by the Vav oncprotein (Fig. 1B), a well known Rac1/RhoG activator (23, 24). RhoGQ61L-expressing cells lacked this contractile structure and, instead, displayed a continuous membrane ruffle around the cell periphery. In contrast to previous results (10), no filopodia were found in RhoGQ61L-expressing cells (Fig. 1A and B). For the sake of comparison, we found that RhoAQ63L-induced cell rounding and Cdc42Q61L-promoted extensive filopodia formation when expressed in the same cell context (Fig. 1B). These results indicate that, despite their different subcellular localizations, RhoG and Rac1 induce similar morphological phenotypes.

Next, we analyzed the transforming potential of these GTPases when transfected into mouse fibroblasts (NIH3T3 cells). Rac1Q61L induces cellular transformation in this system (approximately 1.4–3.0 × 10^3 foci μg^-1 of transfected DNA) (Fig. 1C). This transformation is characterized by the generation of foci of transformed cells of very small size (Fig. 1C). In contrast, RhoGQ61L does not induce cell transformation under identical experimental conditions (Fig. 1C). The lack of transforming activity of RhoGQ61L is not because of lack of GDP/GTP cycling, because a fast cycling mutant of RhoG (F28L) also lacked transforming activity (see Supplementary Information, Fig. S2) (25, 26). The wild type versions of Rac1 and RhoG

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2 R. M. Prieto-Sánchez and X. R. Bustelo, manuscript in preparation.
that RhoG could activate Rac1 or Cdc42
situation of RhoG over Rac1. First, we explored the possibility
crepancy, we decided to further evaluate the possible upstream
experimental approach, we observed that RhoGQ61L could not
is already locked in the GTP-bound, activated state. Using this
constitutively active form of the GTPase, because this protein
GTPase should result in higher levels of transforming activity.
formation assays. As previously described for Ras GEFs (27),
RhoGQ61L. In this respect, a similar biological response was
ably a synergistic effect induced by the activation of parallel
pathways rather than the direct activation of Cdc42 by
previously described that RhoG appears to activate Rac1 and
structural similarity.
RhoG Is Not An Upstream Activator of Rac1—It has been
previously described that RhoG appears to activate Rac1 and
and the Vav oncoprotein promoted the activation of JNK in
these conditions (Fig. 1D, right panel). These results indicate that
RhoG and Rac1 induce overlapping, but not identical,
signaling pathways and biological responses despite their high
structural similarity.

found when RhoGQ61L was co-expressed in the same cell
system with an unrelated GTPase, the H-Ras oncoprotein (G12V
mutant) (Fig. 2A, left panel). These results indicate that
the activated version of RhoG does not seem to act as a 
bona fide
activator of Rac1 and/or Cdc42.

To further confirm these empirical observations, we investi-
gated whether RhoGQ61L could promote the incorporation of
GTP into wild type Rac1 in vivo. To this end, we expressed wild
type Rac1 in COS1 cells either alone or in the presence of
RhoGQ61L or its switch I mutants (F37L and Y40H). As positive
control, we included a well known activator of Rac1, the Vav
oncoprotein. After the transfection, the levels of GTP-Rac1
were determined using pull-down experiments with a GST-PAK1 CRIB
fusion protein (upper panel). Total cell lysates were processed in parallel
for anti-AU5 immunoblotting to determine the levels of expression of
each protein (lower panel). The migration of the GTPases is indicated by
arrows on the left side of each panel.

RhoG and Rac1. Instead, Rac1Q28L did show efficient binding to
PAA1 under the same experimental conditions (Supplemental Materials, Fig. S3).

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Structural Basis of RhoG and Rac1 Signaling Specificity

FIG. 2. A, left panel, cooperativity of RhoGQ61L with other GTPases in
cell transformation. NIH3T3 cells were transfected with plasmids en-
coding the indicated GTPases either in the absence (gray bars) or
presence (black bars) of a vector encoding RhoGQ61L. Foci were quanti-
fied after 15 days as indicated under "Experimental Procedures." Values
represent the means of two duplicates. Right panel, cooperativity of
Rac1 with other regulatory proteins in cell transformation. NIH3T3
cells were transfected with plasmids encoding the indicated proteins
and foci were quantified as described above. B, wild type Rac1 is
activated by the Vav oncoprotein but not by RhoGQ61L. AU5-Rac1 was
expressed in COS1 cells either alone or in the presence of the indicated
proteins. As control, AU5-Rac1Q61L was expressed in parallel in the
same cellular system. After 48 h, the GTP-bound levels of Rac1 were
determined using pull-down experiments with a GST-PAK1 CRIB
fusion protein (upper panel). Total cell lysates were processed in parallel
for anti-AU5 immunoblotting to determine the levels of expression of
each protein (lower panel). The migration of the GTPases is indicated by
arrows on the left side of each panel.

Structural Determinants for the Binding to PAK1: Role of the
β2 and α5 Regions—Because RhoG and Rac1 behave differ-
Structural Basis of RhoG and Rac1 Signaling Specificity

Fig. 3. A, schematic representation of the RhoGQ61L/Rac1 chimeras used in this study. The sequences corresponding to RhoGQ61L and Rac1 are indicated as black and white boxes, respectively. Some of the structural domains of the GTPases are shown at the bottom. B, binding of RhoGQ61L/Rac1 chimeras to PAK1. COS1 cells were transiently transfected with plasmids encoding the indicated proteins (AU5-tagged). After 24 h, cells were lysed and pull-down experiments were conducted using a GST-PAK1 CRIB domain fusion protein. After the experiment, aliquots of PAK1-CRIB bound proteins (upper panel) and of total cellular lysates (lower panel) were subjected to immunoblot analysis with AU5 antibodies. Arrows indicate the mobility of the GTPases. C, primary sequences of the switch I, β2, and β3 regions of selected Rho/Rac family proteins. Amino acids conserved in all proteins are shown on the white background, those conserved in three GTPases are shaded in gray, and those conserved in less than three GTPases are shaded in black. D, binding of Rac1Q61L and RhoGQ61L mutants to PAK1. Cell extracts were obtained from COS1 cells expressing ectopically the indicated proteins and subjected to pull-down experiments with a GST-PAK1 CRIB domain fusion protein. After the experiment, aliquots of PAK1-CRIB bound proteins (upper panels) and of total cellular lysates (lower panels) were analyzed by Western blot analysis using AU5 antibodies. Arrows indicate the mobility of the GTPases.

ently in terms of PAK1 activation, transforming activity, and subcellular localization, we decided to investigate the molecular basis of such signaling specificity. We first focused our attention on the differential binding of the serine/threonine kinase PAK1. To that end, we made two chimeras that combined in the same molecule N-terminal residues of Rac1Q61L (residues 1–85 or 1–120) with the C-terminal domains of the GTPases. Amino acids conserved in all proteins are shown on the white background, those conserved in three GTPases are shaded in gray, and those conserved in less than three GTPases are shaded in black. D, binding of Rac1Q61L and RhoGQ61L mutants to PAK1. Cell extracts were obtained from COS1 cells expressing ectopically the indicated proteins and subjected to pull-down experiments with a GST-PAK1 CRIB domain fusion protein. After the experiment, aliquots of PAK1-CRIB bound proteins (upper panels) and of total cellular lysates (lower panels) were analyzed by Western blot analysis using AU5 antibodies. Arrows indicate the mobility of the GTPases.

To verify whether this position was sufficient for PAK1 binding, we decided to generate the reciprocal mutation (S44V/A45M) in RhoGQ61L. We assumed that if this position was the only important for PAK1 binding, then this RhoG mutant would be able to associate with PAK1. Contrary to this hypothesis, we found that the S44V/A45M mutant of RhoGQ61L could not bind to the PAK1 CRIB domain (Fig. 3D). As positive control, Rac1Q61L and Rac1M45A/Q61L showed optimal association with the PAK1 CRIB domain (Fig. 3D), confirming the binding of Rac1 to PAK1. We next sought to identify the amino acids within this region of the GTPases that are used by PAK1 to discriminate Rac1 from RhoG. To aid in the identification of these residues, we used two different criteria: (i) the residue had to be different between RhoG and other PAK1-binding GTPases such as Rac1 and Cdc42; (ii) the candidate amino acid had to be at least partially exposed to the surface of the GTPases, a property indicative of accessibility for the interaction with effectors. Based on these criteria, we found one possible candidate in the Rac1 switch I region (Gly-30), three candidates in the Rac1 β2 region (Asn-43, Val-44, and Met-45), and one in the Rac1 β3 region (Pro-50) (Fig. 3C, arrows). The residues mentioned above are replaced in RhoG by amino acids Lys, Gln, Ser, Ala, and Thr, respectively (Fig. 3C, arrows). No residue fulfilling these two criteria was found in the switch II region of that GTPase. To check whether these residues were contributing to the binding specificity of PAK1, we generated a Rac1Q61L mutant in which the aforementioned residues were replaced by those found in the equivalent positions of RhoG. Given the proximity of the residues Val-44 and Met-45, we initially made a Rac1Q61L double mutant in those positions. Using GST-PAK1 CRIB pull-down experiments, we found that the Rac1Q61L mutants of the switch I region (G30K mutant) and the β3 region (P50T mutant) did not alter the binding of the constitutively active mutant of Rac1 toward PAK1 (Fig. 3D, upper panel on the left). Likewise, one of the Rac1Q61L β2 mutants (N43Q) showed no major effects on such an interaction (Fig. 3D, upper panel on the left). By contrast, the other Rac1Q61L β2 mutant (V44S/M45A) was severely impaired in its binding toward the PAK1 CRIB domain (Fig. 3D, upper panel on the left). We next generated single Rac1Q61L mutant proteins containing single replacements in either position 44 (V44S mutant) or 45 (M45A mutant) to determine which one of these two residues of Rac1Q61L was contributing to the specific binding of PAK1. As shown in Fig. 3D (upper panel on the right), only the V44S mutant of the Rac1Q61L protein disrupted the binding of PAK1. These results indicate that the β2 region and, specifically, the valine residue located at position 44, is a major structural determinant for the discrimination of Rac1 over RhoG by the serine/threonine kinase PAK1.
the Rac1<sup>V44S/Q61L</sup> and RhoG<sup>S44V/Q61L</sup> mutant proteins were incapable of associating with PAK1 because of spurious structural effects induced by the mutations, we also tested all our Rac1 and RhoG mutants in a different biological read-out, the ability to induce F-actin polymerization and membrane ruffling. To this end, COS1 cells expressing transiently these proteins were fixed, stained with both phalloidin-rhodamine (to detect F-actin) and anti-AU5 antibodies (to detect the GTPases), and analyzed by immunofluorescence. All Rac1 and RhoG mutants were similarly active in these two cellular responses (Fig. 4). Moreover, all mutants showed the expected subcellular localization for Rac1<sup>Q61L</sup> and RhoG<sup>Q61L</sup> in membrane areas and intracellular vesicles, respectively (Fig. 4). These results confirmed that the mutations of residues 44 and 45 of Rac1 and RhoG were not deleterious for the overall function of these proteins.

To identify the other region(s) outside the β2 region responsible for specific binding of PAK1 toward Rac1, we generated a new series of RhoG/Rac1 chimeric proteins (Fig. 5A). In these constructions, our aim was to replace progressively the C-terminal region of Rac1<sup>Q61L</sup> with the same regions present in RhoG (Fig. 5A). In addition, a subset of these chimeric proteins included the S44V/A45M mutation in the RhoG β2 region, because these residues were demonstrated to be essential for the binding of PAK1 to Rac1<sup>Q61L</sup> (see above, Fig. 3). Pull-down experiments indicated that all RhoG<sup>Q61L</sup>/Rac1 chimeric proteins containing the S44V/A45M mutation were capable of binding to PAK1, but only when the α5 region of Rac1 was present in the chimeric protein (Fig. 5, B and C, upper panels; compare the negative binding of chimera G with the positive binding of chimeras C and F). In good agreement with our previous results (Fig. 3), this binding was strictly dependent on the presence of the RhoG to Rac1 (S44VM45SM)mutation in the β2 region of RhoG, because chimeras lacking these amino acid changes could not bind PAK1 even when the Rac1 α5 helix was present (Fig. 5, B and C, upper panels, see chimeras B and E). All these RhoG/Rac1 chimeras were expressed at similar levels in vivo, as determined by Western blot analysis using anti-AU5 antibodies (Fig. 5, B and C, lower panels). These chimeras were also active in other biological assays, such as morphological change (see below, Fig. 8A). Taken together, these results indicate that PAK1 utilizes residues present in both the β2 and α5 regions of the GTPases to distinguish Rac1 from RhoG.

The examination of the α5 region of Rho/Rac GTPases under the same criteria used in Fig. 3 suggested the presence of two possible residues (Thr-167 and Asp-170) that could contribute to the specificity of the Rac1/PAK1 physical interaction (Fig. 6A). These two residues are replaced in RhoG by Glu and Ala, respectively (Fig. 6A, arrows). To investigate whether any of these residues was the second structural signal required for the specific interaction of PAK1 with Rac1, we tested whether the Rac1 to RhoG mutants (T167E, D170A, and T167E/D170A) partially recovered the binding to PAK1, although it was still significantly less active than the normal protein (Fig. 6B, upper panels). All these mutant proteins were expressed normally in cells, as determined by anti-AU5 immunoblotting experiments (Fig. 6B, lower left panel). In addition, we also found that the Rac1<sup>Q61L/T167E</sup> and Rac1<sup>Q61L/D170A</sup> mutants displayed a 20-fold lower binding affinity toward PAK1 than both Rac1<sup>Q61L</sup> and Rac1<sup>Q61L/T167E</sup> mutants (Fig. 6B, upper panel on the left). Interestingly, a Rac1<sup>Q61L</sup> mutant containing a double mutation in the α5 helix (T167E/D170A) partially recovered the binding to PAK1, although it was still significantly less active than the normal protein (Fig. 6C). Interestingly, residue Asp-170 of Rac1 is located from a spatial point of view close to the Val-44 residue, the other position important for PAK1 binding (Fig. 6D). This result indicates that the groove existing between the β2/β3 and α5 regions of Rac1 and RhoG is important for their discrimination by PAK1.

To verify whether the amino acids located at positions 44 and 170 were the only ones important for such binding, we generated a final RhoG<sup>Q61L</sup> mutant in which the serine and
two alanine residues located at positions 44, 45 (β2 region), and 170 (α5 region) were replaced by the amino acids present in Rac1 in those positions (Val, Met, and Asp, respectively). Although this mutant recovered a small amount of PAK1 binding with respect to RhoG44V/A45M/Q61L, its affinity to PAK1 was still very low compared with Rac1Q61L or even the Rac1Q61L/D170A mutant (Fig. 6B, upper panel on the right). All these versions of Rac1 and RhoG were similarly expressed at the protein level (Fig. 6B, lower panel on the right). This result suggests that despite the importance of Val-44 and Asp-170 for PAK1 binding, there have to be additional residues located within (or downstream) the α5 helix that ensure optimal PAK1 binding.

Structural Determinants for the Differential Transforming Activity of Rac1 and RhoG—To achieve this end, we utilized a similar strategy than that used above for the identification of the structural cues that determine the differential binding of PAK1. First, we used focus formation assays in NIH3T3 cells to evaluate the transforming activity of the seven RhoG/Rac1 chimeric proteins used before in our PAK1 binding assays (see Figs. 3A and 5A). As shown in Fig. 7A, no chimeric protein showed transforming activity in this assay under conditions in which Rac1Q61L induced elevated levels of cell transformation. This indicated that the N-terminal region of Rac1 also had important structural determinants for engaging the signaling pathways that contribute to cell proliferation. To identify them, we then tested the transforming activity of Rac1Q61L mutants with six Rac1 to RhoG mutations distributed in the switch I, β2, and β3 regions (G30K, N43Q, V44S/M45A, V44S, M45A, and P50T). In contrast to the results with PAK1 (Fig. 3), we found that the V44S/M45A and the V44S Rac1 mutants did not abolish Rac1Q61L transforming activity (Fig. 7B). Rac1N43Q/Q61L and Rac1Q61L/P50T mutants also showed similar transforming activities as that of Rac1Q61L (Fig. 7B). In contrast, the transforming activity of the Rac1G30K/Q61L mutant was 15-fold lower than Rac1Q61L (Fig. 7B). This result indicates that position Gly-30 of the switch I region of Rac1 represents a key residue for the transforming activity of this protein. Moreover, these observations suggest that the structural elements used to discriminate binding of PAK1 and cellular transforma-
Structural Basis of RhoG and Rac1 Signaling Specificity

One feature of Rho/Rac proteins is the versatility with which they exploit a similar molecular scaffold to induce specific and, in some instances, quite diverse biological functions. Thus, despite a similar three-dimensional structure, members of this family show distinct subcellular localizations, differential binding to upstream and downstream effectors, distinct affinities toward GAPs, and even different dynamics of their GDP/GTP exchange cycles (1, 2). As a consequence, the biological responses induced by these proteins are almost as diverse as the number of members of the Rho/Rac family. This structural flexibility is well exemplified by the results presented here, because we have observed that two highly related GTPases such as RhoG and Rac1 are still capable of inducing different cellular effects despite their high sequence similarity. Thus, we have shown that these GTPases share some common properties (cytoskeletal change and JNK activation), but differ in other important biological features such as the binding to PAK1, the ability to induce cell transformation, or their specific subcellular localization. This functional disparity is remarkable if we take into consideration that RhoG and Rac1 share almost identical switch I and II regions, the domains involved in the recognition and binding of effector molecules.

One consequence of the functional specialization of Rho/Rac proteins is that, to assemble a coordinated and coherent cellular response, the stimulated cell has to make sure that several GTPases are stimulated simultaneously. For instance, cell motility can only be achieved efficiently if Cdc42, Rac1, and RhoA are activated in a specific time frame to promote the advancement of the leading edge and the retraction of the posterior end of the migrating cell (1). This can be achieved by different means. On one hand, the cell can activate a GEF that can stimulate several members of the Rho/Rac family, thus allowing the simultaneous engagement of their signaling pathways (31). On the other hand, cells have developed signal transduction cascades in which the activation of one particular GTPase translates into the stimulation of other GTPases further downstream. Thus, it has been shown that fibroblasts have a linear pathway in which the activation of Cdc42 leads to the stimulation of Rac1 that, in turn, promotes the activation of RhoA (2). Interestingly, several publications have indicated that a pathway of this type is assembled by RhoG, because the activated version of this protein appears to activate the GTPases Rac1 and Cdc42 (10, 11). This model is primarily based on the indirect observation that dominant negative mutants of Rac1 and Cdc42 can block the effects induced by RhoGQ61L in cell morphology and neurite extension (10, 11). Although this model is congruent with previous observations with other GTPases, there are several observations that speak against it. At the empirical level, our results showing the lack of transforming activity of RhoG argue against the upstream position of this GTPase over Rac1, because the latter displays high oncopgenic potential. If a linear pathway existed, one would expect that RhoG were at least as transforming as its downstream element. This is not the case in our system. Moreover, we have demonstrated that the expression of RhoGQ61L does not have any effect in the activation of Rac1 either in vivo (using focus formation assays) or in vitro (by detecting the GTP levels of Rac1). Our results showing that RhoG is preferentially localized in cytoplasmic vesicles far away from the membrane-localized Rac1 indicate also that a close connection to allow the activation of Rac1 by this GTPase is difficult to achieve in vitro. Taken together, all this evidence is consistent with the idea that these two GTPases are functionally autonomous, regulat-

![Diagram](https://example.com/diagram.png)

**Fig. 8.** A, subcellular localization of RhoGQ61L/Rac1 chimera and their effect in the cellular cytoskeleton. COS1 cells were transfected with the indicated proteins and, after 30 h of culture, fixed and stained with mouse anti-A5 antibodies followed by Cy2-labeled antibodies to mouse IgGs, stained with rhodamine-phalloidin, and analyzed by immunofluorescence. Images show in green (right panels) and red (left panels) the localization of the indicated GTPases and F-actin, respectively. The structure of the chimeras can be found in Figs. 3A and 5A. B, summary of the amino acid residues important for the discrimination of the biological activities that are different in RhoG and Rac1. Amino acid residues are shown on black boxes. Biological activities are depicted on black boxes. The thickness of the arrow indicates the importance of a particular residue in assembly of the described biological response.
ing parallel pathways that share some, but not all, signaling elements. This is probably not an exclusive property for the RhoG and Rac1 relationship, because preliminary evidence indicates that RhoG and Cdc42 work also through parallel pathways. The lack of a linear relationship between RhoG and Rac1 is understandable from a mechanistic point of view, because RhoG and Rac1 share GEFs such as Trio, Vav1, Vav2, and Vav3 (24, 32). So, the stimulation of one of these GEFs should suffice to activate the simultaneous activation of both RhoG and Rac1 without the need of signaling intermediates. Although we have not attempted a careful experimental analysis to explain the basis of our discrepancies with previous publications, it is worth noting that similar conclusions have been reached by Wennerberg et al. (33) during the final elaboration of our work. In this case, they have explained the disagreement with previous reports by demonstrating that the inhibitory effect of the dominant negative mutant of Rac1 (Rac1N17) on RhoGQ61L-mediated morphological change is probably because of either nonspecific or cytoxic effects derived from the overexpression of Rac1N17 for extended periods of time (33). In agreement with this hypothesis, no inhibitory effects of Rac1N17 on RhoG signals are observed when the former protein is expressed during short periods of time (2–3 h instead of the usual 24–48 h) (33). These observations highlight the importance of taking into consideration the multiple caveats associated with the use of this type of dominant negative mutants (34).

The different biological activities of RhoG and Rac1 suggest that, despite their high sequence similarity, they must diverge in specific residues that determine the pattern of effector binding. We have used an extensive mutagenesis approach to identify some of those molecular cues. Our data indicate that the proper connection of RhoG and Rac1 GTPases with specific cellular responses involves at least four different structures (switch I, the β2/β3 hairpin, the α5 helix, and the C-terminal polybasic region), each of them accomplishing specific signaling tasks (Fig. S8). In the case of PAK1 binding, we have shown that the binding specificity is mediated by residues located in the β2 (Val-44) and α5 regions (Asp-170) (Fig. S8B). Mutation of each of these two residues of Rac1 into the equivalent amino acids present in RhoG leads to either the total (Val-44) or partial (Asp-170) loss of PAK1 association by Rac1. This is not because of deleterious effects of the mutations in the structure or activity of Rac1, because the mutations of those areas do not affect the subcellular localization or the morphological changes induced by Rac1N17L. Conversely, the S44V substitution in RhoGQ61L along with the inclusion of the α5 region of Rac1 (RhoGQ61L/Rac1 chimera F) allows the binding of PAK1, a result that demonstrates the importance of these two regions for the proper docking of PAK1 to the upstream GTPases. However, it should be noted that the RhoGQ44VQ61L/D170L mutant does not recover PAK1 binding as efficiently as the RhoGQ61L/Rac1 chimera F, indicating that, in addition to residue Asp-170 of the α5 helix, the overall conformation of this area or the presence of additional residues in the polybasic region must also contribute to the overall binding affinity of PAK1 toward the GTPases. Interestingly, other divergent residues between Rac1 and RhoG located in the switch I (position 30, Gly and Lys in Rac1 and RhoG, respectively), β2 (position 45, Met and Ala in Rac1 and RhoG, respectively), β3 (position 50, Pro and Thr in Rac1 and RhoG, respectively), and α5 regions (position 167, Thr and Glu in Rac1 and RhoG, respectively) do not contribute to the differential binding of PAK1 to these GTPases. Our results are consistent with the type of interaction that PAK1 establishes with Cdc42 and Rac1. As described earlier by Morreale and collaborators (35), PAK1 wraps around half of the Cdc42 molecule, making contacts in the switch I (Pro-34 and Val-36 residues), switch II (Leu-70), the β2 regions (residues 40–46), and α5 helix (Phe-169, Asp-170, and Ile-173). In agreement with such structure, the amino acid residues identified in our analysis match some of those areas. However, it was proposed that the α5 of Rac1 and Cdc42 worked as a passive contact site for PAK1 because, unlike the case of other effectors such as activated Cdc42-associated kinase and WASP, its replacement by the α5 region of RhoA (a non-CRIB domain binding protein) did not affect the overall affinity of the PAK1 association (29, 35). Our results suggest that the role of α5 is not entirely passive in this process, because the Rac1Q61L/D170A binds PAK1 poorly. One possible explanation for this discrepancy is that RhoA, Rac1, and Cdc42 share the same aspartic residue in position 170 (position 172 in RhoA) that is not conserved in RhoG. Accordingly, our prediction is that the α5 region of RhoA with the D172A mutation would not be compatible with PAK1 binding when transplanted into the Rac1 backbone.

We have obtained a different cartography of the residues responsible for the different behavior of RhoG and Rac1 in cell transformation. In this case, the important regions for this biological response are the switch I (position 30, amino acids Gly and Lys in Rac1 and RhoG, respectively) and the helix α5 (positions 167 and 170, Thr-Glu and Glu-Ala in Rac1 and RhoG, respectively) (Fig. 8B). Substitution of any of these three residues in Rac1 by the equivalent amino acids present in RhoG severely affects Rac1Q61L transforming activity. Conversely, the substitution of Lys-30 in RhoG by the glycine residue present in Rac1 induces a low, but significant (approximately 1 × 105 foci/μg of transfected DNA), transforming activity in the mutant RhoGQ61L protein. In contrast to the results with PAK1, the β2/β3 region was found not relevant for determining the specificity of this biological property. The common implication of residues of the α5 helix in the assembly of PAK1 binding and transforming activities is not entirely unexpected given the available evidence derived from other signaling and structural studies. For example, it has been shown that a C-terminal residue of the α5 region of Cdc42 (Leu-174) is the key specificity determinant for the binding to WASP and activated Cdc42-associated kinase (35). Likewise, the C-terminal region of the Rab3A α5 helix determines the specific binding to rabphilin 3A (36). N-terminal residues of the RhoA α5 helix also contribute to the binding of the serine/threonine kinase protein kinase N (37). Finally, residue Asp-170 of yeast Cdc42 appears to be very important for the assembly of specific signaling responses through the interaction with a hitherto unknown effector (38). These observations indicate that the contribution of GTPase α5 regions to signaling specificity is not restricted to RhoG but, rather, it is a usual event in the signal transduction of different GTPase subfamilies.

Our studies on the subcellular localization of RhoG and Rac1 indicate that these proteins also diverge in this biological property. As expected, the wild type versions of RhoG and Rac1 show a cytoplasmic distribution, without detectable localization in membrane-enriched areas. This is in agreement with the arrest in the cytoplasm of the GDP-bound forms of most Rho/Rac family GTPases through the binding of Rho GDIs (2). However, when the constitutively active versions of RhoG and Rac1 were analyzed, RhoGQ61L was detected mainly in intracellular vesicles that, in contrast to previous observations (22), did not match with lysosomal or endoplasmic reticulum markers. Instead, they seem to belong to the endocytic compartment, because they co-localize with typical markers for this pathway such as the epidermal growth factor receptor and its ligand. A percentage of these vesicles also colocalize with caveolin, a
marker for caveolae. No RhoGQ61L was found associated with the plasma membrane or membrane ruffles, indicating that the effect of this GTPase on F-actin polymerization is probably mediated by a second messenger intermediate. In contrast to these observations, Rac1Q61L was enriched at the plasma membrane and areas of membrane ruffling, in good agreement with previous observations (21). Using RhoGQ61L/Rac1 chimeras, we have observed that this differential behavior of RhoG and Rac1 can be mapped to a totally distinct area than the previously described for PAK1 binding and oncogenesis (Fig. 8B). Indeed, RhoG protein can be relocated to the plasma membrane just by replacing its hypervariable, C-terminal region by the equivalent area of Rac1.

In this report, we have given a new example of exquisite effector selectivity of GTPases. The case of RhoG and Rac1 is even more remarkable than other cases of signaling divergence in this family, because these two GTPases are highly homologous. Through the use of numerous mutations, we have been able to associate the functional differences between these GTPases to specific residues located in different areas of the molecule. One question that remains to be addressed is why these proteins need to be so selective. For instance, it is not obvious at first sight the biological reason for the binding of PAK1 to Rac1 and not to RhoG when this serine/threonine kinase binds with very high affinity to Cdc42. This question cannot be answered until we have genetic models for Rac1 and RhoG with which to dissect the actual contribution of each GTPase to cell signaling in vivo. Until that time, the reagents and information generated here provide a broad molecular foundation for continuing the analysis of the functional specificity of these two highly related GTPases. In addition, the elucidation of the mechanisms underlying these intermolecular interactions may serve as clues for the design of specific pharmacological agents that may inhibit in a specific fashion the signal transduction pathways connecting extracellular stimuli with cytoskeletal and mitogenic events.

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REFERENCES
1. Etienne-Manneville, S., and Hall, A. (2002) Nature 420, 629–635
2. Van Aelst, L., and D’Souza-Schorey, C. (1997) Genes Dev. 11, 2295–2322
3. Li, B., Yu, H., Zheng, W., Voll, R., Na, S., Roberts, A. W., Williams, D. A., Davia, R. J., Gosh, S., and Flavell, R. A. (2000) Science 288, 2219–2222
4. Vincent, S., Jeanteur, P., and Fort, P. (1992) Mol. Cell. Biol. 12, 3138–3148
5. Kaibuchi, K., Kuruda, S., and Amano, M. (1999) Annu. Rev. Biochem. 68, 709–749
6. Mackay, D. J., and Hall, A. (1998) J. Biol. Chem. 273, 20685–20688
7. Del Pozo, M. A., Kassows, W. B., Alderson, N. B., Meller, N., Hahn, K. M., and Schwartz, M. A. (2002) Nat. Cell Biol. 4, 232–239
8. Bustelo, X. R. (2002) Bioessays 24, 602–612
9. Boettner, B., and Van Aelst, L. (2002) Gene (Amst.) 286, 155–174
10. Gauthier-Rouviere, C., Vignal, E., Merian, M., Roux, P., Montcourrier, P., and Fort, P. (1998) Mol. Biol. Cell 9, 1379–1394
11. Katoh, H., Yasui, H., Yamaguchi, Y., Aoki, J., Fujita, H., Mori, K., and Negishi, M. (2000) Mol. Cell. Biol. 20, 7378–7387
12. May, V., Schiller, M. R., Epper, B. A., and Mains, R. E. (2002) J. Neurosci. 22, 6980–6990
13. Vigorito, E., Berberich-Siebelt, F., Chrypilo, S., Jankevsic, E., Klein-Hessling, S., Twardzik, T., and Avots, A. (2000) Biochim. Biophys. Acta 1498, 1–18
14. Li, R., and Zheng, Y. (1997) J. Biol. Chem. 272, 4671–4679
15. Sander, E. E., van Delft, S., ten Klooster, J. P., Reid, T., van der Kammen, R. A., Michiele, P., and Collard, J. G. (1998) J. Cell Biol. 143, 1385–1398
16. Schuobel, K. E., Movilla, N., Rosa, J. L., and Bustelo, X. R. (1998) EMBO J. 17, 6608–6621
17. Movilla, N., and Bustelo, X. R. (1999) Mol. Cell. Biol. 19, 7870–7885
18. van der Eb, A. J., and Graham, F. L. (1980) Methods Enzymol. 65, 826–839
19. Crespo, P., Bustelo, X. R., Aaronson, D. S., Coso, O. A., Lopez-Barabash, M., Barbacid, M., and Gutkind, J. S. (1996) Oncogene 13, 455–460
20. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
21. Vignal, E., Blangy, A., Martin, M., Gauthier-Rouviere, C., and Fort, P. (2001) Mol. Cell. Biol. 21, 8022–8034
22. Crespo, P., Schuobel, K. E., Ostrom, A. A., Gutkind, J. S., and Bustelo, X. R. (1997) Nature 385, 169–172
23. Bustelo, X. R. (2000) Mol. Cell. Biol. 20, 1461–1477
24. van der Eb, A. J., and Graham, F. L. (1980) Methods Enzymol. 65, 826–839
25. Lin, R., Bagrodia, S., Ceronie, R., and Manor, D. (1997) J. Biol. Chem. 272, 23633–23641
26. Lin, R., Bagrodia, S., Ceronie, R., and Manor, D. (1997) J. Cell Biol. 7, 794–797
27. Bustelo, X. R., Suen, K. L., LeFtheris, K., Meyers, C. A., and Barbacid, M. (1994) Oncogene 9, 2405–2413
28. Movilla, N., Dossu, M., Zheng, Y., and Bustelo, X. R. (2001) Oncogene 20, 8057–8065
29. Li, R., Debreceni, B., Jia, B., Gan, Y., Tsigay, G., and Zheng, Y. (1999) J. Biol. Chem. 274, 29648–29654
30. Zhang, B., Wang, Z. X., and Zheng, Y. (1997) J. Biol. Chem. 272, 21999–22007
31. Zheng, Y. (2001) Trends Biochem. Sci. 26, 724–732
32. Blangy, A., Vignal, E., Schmidt, S., Debant, A., Gauthier-Rouviere, C., and Fort, P. (2000) J. Cell Sci. 113, 729–739
33. Wennerberg, K., Ellerbroek, S. M., Liu, R. Y., Karnoub, A. E., Burrage, K., and Der, C. J. (2002) J. Biol. Chem. 277, 47810–47817
34. Feig, L. A. (1999) Nat. Cell Biol. 1, E25–E27
35. Morreale, A., Venkatesan, M., Mott, H. R., Owen, D., Nielitsch, D., Lowe, P. N., and Laue, E. D. (2000) Nat. Struct. Biol. 7, 384–388
36. Ostermeier, C., and Brunger, A. T. (1999) Cell 96, 363–374
37. Maesaki, R., Ihara, K., Shimizu, T., Kuroda, S., Kaibuchi, K., and Hakoshima, T. (1999) Mol. Cell. Biol. 4, 793–803
38. Kasinski, K. G., Chen, A. J., Rodal, A. A., and Drubin, D. G. (2000) Mol. Biol. Cell 11, 339–354