Structural Requirements for PAK Activation by Rac GTPases*

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The Rho family GTPases, Rac1 and Rac2, regulate a variety of cellular functions including cytoskeletal reorganization, the generation of reactive oxygen species, G1 cell cycle progression and, in concert with Ras, oncogenic transformation. Among the many putative protein targets identified for Rac (and/or Cdc42), the Ser/Thr kinase p21-activated kinase (PAK) is a prime candidate for mediating some of Rac's cellular effects. This report shows that Rac1 binds to and stimulates the kinase activity of PAK1 approximately 2- and 4–5-fold, respectively, better than Rac2. Mutational analysis was employed to determine the structural elements on Rac and PAK that are important for optimal binding and activation. The most notable difference between the highly homologous Rac isomers is the composition of their C-terminal polybasic domains. Mutation of these six basic residues in Rac1 to neutral amino acids dramatically decreased the ability of Rac1 to bind PAK1 and almost completely abolished its ability to stimulate PAK activity. Moreover, replacing the highly charged polybasic domain of Rac1 with the less charged domain of Rac2 (and vice versa) completely reversed the PAK binding/activation properties of the two Rac isomers. This polybasic domain differences account for the disparate abilities of Rac1 and Rac2 to activate PAK. PAK proteins also contain a basic region, consisting of three contiguous lysine residues (Lys66-Lys67-Lys68), which lies outside of the previously identified Cdc42/Rac-binding domain. Mutation of these Lys residues to neutral residues decreased PAK binding to activated Rac1 and Rac2 (but not Cdc42) and greatly reduced PAK1 activation by Rac1, Rac2, and Cdc42 proteins in vivo. In contrast, mutation of lysines 66–68 to basic Arg residues did not decrease (and in some cases enhanced) the ability of Rac1, Rac2, and Cdc42 to bind and activate PAK1. Our studies suggest that the polybasic domain of Rac is a novel effector domain that may allow the two Rac isomers to activate different effector proteins. In addition, our results indicate that a basic region in PAK is required for PAK activation and that binding of Rac/Cdc42 to PAK is not sufficient for kinase activation.

The Rho GTPases belong to the Ras superfamily of small GTP-binding proteins and consist, in mammalian cells, of several distinct Rho (RhoA, -B, and -C isoforms), Rac (Rac1, -2, and -3 isoforms), Cdc42 (Cdc42Hs and G25K isoforms), RhoG, RhoE, and TC10 subfamily members (1). By cycling between inactive GDP- and active GTP-bound forms, Rho GTPases act as molecular switches and thereby regulate signal transduction pathways that control a wide range of biological processes. For instance, Rho GTPases play a crucial role in actin cytoskeleton reorganization and have been implicated as key regulators of cell motility and adhesion in response to extracellular stimuli. Rho regulates focal adhesion assembly and actin stress fiber formation in lysophosphatidic acid- or bombesin-stimulated Swiss 3T3 cells (2); Rac induces membrane ruffling and lamellipodia at the plasma membrane upon growth factor stimulation (3); and Cdc42 triggers the formation of filopodia and focal complexes in the cell periphery when activated by bradykinin (4). In some cell types, cross-talk between Rho GTPases is believed to occur, such that Cdc42 activation leads to Rac activation, which subsequently leads to Rho activation (5). In addition to their effects on cytoskeletal remodeling, Rho GTPases (Rac and Cdc42) stimulate the activity of the c-Jun N-terminal/stress-activated mitogen-activated protein kinase (6–8), and the p38/HOG mitogen-activated protein kinase (9, 10); are required (Rho) for activation of the serum response transcription factor, SRF (11); and induce (Rho, Rac, and Cdc42) G1 cell cycle progression and DNA synthesis (8). Rho GTPases are also key components of signaling pathways leading to oncogenesis (12, 13). In addition to inducing the expression of growth-promoting genes, Rho GTPases can trigger an invasive phenotype (Rac1) and appear to be required (Rac and Rho) for Ras-induced malignant transformation (12, 13). In contrast to other Rho family members, Rac proteins have an additional distinct biological role. Rac regulates the activity of the NADPH oxidase enzyme complex in phagocytic cells (14, 15), and the superoxide anion (O2−) generated by this complex plays a key role in bactericidal host defense mechanisms.

Mammalian cells contain two Rac genes, Rac1 and Rac2. Although cells of myeloid lineage, such as neutrophils, express both Rac1 and Rac2 proteins, Rac2 expression in these cells is 20-fold higher than Rac1 expression. In distinct contrast, nonmyeloid cells exclusively express the Rac1 isoform. Rac1 and Rac2 have 92% sequence identity overall and exhibit their highest amino acid divergence at their C termini, particularly in a region that constitutes a polybasic domain. The polybasic domain in Rac1 is composed of six contiguous basic amino acids, whereas in Rac2 three of these six residues have been converted to neutral amino acids. It is currently unknown whether the two Rac isoforms are differentially activated by extracellular stimuli, stimulate different effectors and/or regulate distinct biological functions. In neutrophils, Rac1 and Rac2 are equally able to stimulate the respiratory burst in vitro in a semirecombinant system (16); however, their effectiveness in vivo may differ (17).

In order to regulate the various aforementioned biological processes, Rho GTPases interact with multiple downstream effectors to trigger complex, interconnected signaling pathways. One of these downstream targets is a family of serine/threonine protein kinases termed p21^c^c^d^d^2^2^/c^c^c^c^c^1^-activated ki-
nases (PAKs), which are stimulated by Rac and Cdc42, but not Rho, in a GTP-dependent manner (18). PAKs are related to Ste20p of Saccharomyces cerevisiae, a protein kinase required for pheromone-induced mitogen-activated protein kinase activation, and are believed to play a similar role in mammalian cells by regulating the c-Jun N-terminal/stress-activated mitogen-activated protein kinase and p38 mitogen-activated protein kinase pathways (9, 10, 19). Increasing evidence suggests that PAKs are involved in certain aspects of cytoskeletal remodeling, consistent with their role as potential effectors of Rac/Cdc42 GTPases. Recent studies have shown that PAK1 translocates to areas of active cytoskeletal rearrangement upon growth factor stimulation (20), and a PAK1 mutant, postulated to mimic the structural changes induced by GTPase binding, caused lamellipodia formation and membrane ruffling when microinjected or transfected into fibroblasts (21). Furthermore, PAK phosphorylates the heavy and light chains of myosins (22–24), thereby modulating actin-stimulated ATP hydrolysis. Studies from this laboratory also link PAKs to the stimulation of the neutrophil respiratory burst (25). For instance, one of the NADPH oxidase components, the cytosolic p47phox protein, is phosphorylated by activated PAK on several physiologically relevant sites. Multiple phosphorylations precede p47phox translocation to the membrane, assembly of the NADPH oxidase complex, and subsequent superoxide anion generation. In addition, extracellular stimuli capable of inducing O2- production in neutrophils, such as the chemotactic peptide fMet-Leu-OH, PAK1 activation. PAKs activate PAKs in a time-dependent manner that closely parallels the time courses of Rac and p47phox translocation and superoxide anion production (25).

In this report, we have demonstrated that Rac1 binds to and stimulates the kinase activity of PAK much more efficiently than Rac2, and we have identified the polybasic domain of Rac as a novel effector site for Rac/PAK interaction. This domain is critical for effective Rac/PAK binding and appears to dictate the differential potency of PAK stimulation by Rac1 versus Rac2. Furthermore, we identified a polybasic region on PAK that is important for Rac/PAK interaction. This domain is upstream of and distinct from the previously defined Cdc42/ Rac interactive binding (CRIB) domain. Mutational analysis of this PAK domain revealed that PAK activation by GTPases in vivo is dependent on the basic charge of this domain. Our results reveal previously unidentified structural regions on Rac and on PAK that have critical impact on GTPase-dependent PAK activation.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Constructs**—Wild type and activated Rac1 and Rac2 C-terminal mutants were generated by polymerase chain reaction-directed mutagenesis as described previously (26). Template DNA consisted of wild type or activated (G12V) human Rac1 or Rac2 cDNA containing a Glu-Glu (EEEEYMPME) epitope tag at their N termini, and forward and reverse primers consisted of oligonucleotides encoding the mutations. Table I summarizes the specific mutants generated. The Rac1(6Q) mutant contains 6 neutral amino acids of Rac1 or Rac2, respectively. Table I summarizes the specific mutants generated. The Rac1(6Q) mutant contains 6 neutral amino acids of Rac1 or Rac2, respectively. Table I summarizes the specific mutants generated. The Rac1(6Q) mutant contains 6 neutral amino acids of Rac1 or Rac2, respectively. Table I summarizes the specific mutants generated. The Rac1(6Q) mutant contains 6 neutral amino acids of Rac1 or Rac2, respectively.

| Rac 1 (WT)* | Rac 1 (6Q) | Rac 2 (WT) |
|-------------|------------|------------|
| pGEX-4T-3   | pGEX-4T-3  | pGEX-4T-3  |
| pCMV6       | pCMV6      | pCMV6      |
| pRK5        | pRK5       | pRK5       |

*The site and nature of amino acid substitutions are indicated. Hphens indicate sites where amino acid residues are identical to Rac1(wt). Both wild type and activated (G12V) versions of each protein were generated.

**pGEX2T, a baculovirus-expression vector. Escherichia coli- and baculovirus-expressed GTPases were purified as glutathione-S-transferase fusion proteins by affinity chromatography on glutathione-Sepharose beads (Amersham Pharmacia Biotech) and cleaved with thrombin (from human plasma; Sigma) (27). Processed GTPases were separated from unprocessed proteins by partitioning in Triton X-114. The purified Rac proteins were assayed for GTP·S binding activity (27), and equivalent amounts of GTP-bound Rac proteins were utilized.

Human cDNA encoding wild type and mutant PAK1 was subcloned into the Myc epitope tag-containing expression vectors pCMV6 and pRK5 as described (21). Polymerase chain reaction was carried out using internally derived oligonucleotides encoding the desired mutant PAK1 sequence, K66–68L, K68–68R, or K66–68Q. Two overlapping strands containing the mutated sequence were then used as polymerase chain reaction templates to allow synthesis of full-length PAK1 containing the mutations.

**Cell Culture**—Transient transfections in COS-7 cells (1 x 10 6 cells/100-mm dish) were performed as described by Zhang et al. (9) using Lipofectamine (Life Technologies, Inc.) or SuperFect (Qiagen) for 40 h. Co-transfections with N-terminal Myc-tagged wild type or mutant PAK1/pCMV6 or PAK1/pRK5 plasmid constructs and Myc-tagged wild type or mutant Rac1 or Rac2/pRK5 plasmid constructs were performed for 24 h in HeLa cells at a DNA ratio of 9:1 or 4:1.

**Preparation of Cell Lysates and Immunoprecipitation**—24–40 h post-transfection, COS-7 or HeLa cells were scraped from their 100-mm dish into 250 μl of ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl2, 1 mM dithiothreitol, 150 mM NaCl, 10% glycerol, 2% Nonidet P-40, 50 IU/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin). After 15 min on ice, the lysates were pelleted for 5 min at 14 K at 4 °C, and the supernatants were collected.

Aliquots of PAK-transfected cell lysates were diluted with detergent-free lysis buffer and incubated with anti-Myc monoclonal antibody (9E10) overnight at 4 °C, followed by a 60-min incubation with 50 μl of a 1:2 slurry of bovine serum albumin-coated protein G-Sepharose beads (Amersham Pharmacia Biotech). Beads were pelleted and washed three times with lysis buffer, three times with detergent-free lysis buffer, and twice with kinase buffer.

**Kinase Assays**—In vitro PAK kinase assays were performed essentially as described (25). Anti-Myc immunoprecipitates were incubated in 70 μl of kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 0.2 mM dithiothreitol, 20 μM ATP, and 5 μCi of [γ-32P]ATP (NEN Life Science Products) in the presence or absence of 0.25–3 μg of GTPγS-bound Rac proteins for 20 min at 30 °C. For analysis of PAK autophosphorylation, incubations were stopped by adding Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (7% gels), followed by autoradiography and PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA). Substrate phosphorylation by activated PAK1 was measured by adding 2 μg of a peptide derived from the NADPH oxidase component, p47phox (amino acids 324–331), to the kinase assay. After the 20-min incubation, an aliquot of the kinase reaction supernatant was spotted onto phosphocellulose filters (Pierce) and washed according to the manufacturer's instructions, and the amount of radiolabel incorporated into substrate was quantitated in a liquid scintillation counter. In vitro PAK kinase activation was evaluated after co-transfection as described above, except 5 μM okadaic acid and 1 μM microcystin LR (LC Laboratories) were added to the lysis buffer. Aliquots of cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (6.5% gels), and activation of PAK1 was determined by Western blot analysis of the mobility shift caused by PAK1 autophosphorylation.
solid domain of Rac1 accounts for its enhanced affinity for PAK, the Rac2 → 1 mutant exhibited increased binding, comparable with Rac1(wt) levels. These results demonstrate that differences in the polybasic domains of the two Rac isoforms completely account for their disparate affinities for the effector protein PAK1.

The Polybasic Domain of Rac Is Required for PAK Activation in Vitro and in Vivo—To investigate the relative ability of Rac1 versus Rac2 to activate PAK and determine the role of the polybasic domain in PAK activation, immunoprecipitates of PAK1(wt)-expressing cell lysates were incubated with bacterially expressed, GTPγS-activated wild type, mutant, or chimeric Rac1/Rac2 proteins in vitro, and autophosphorylation of the kinase was monitored by autoradiography as an index of PAK activation (Fig. 2). Optimal stimulation of PAK autophosphorylation was observed with Rac1(wt), leading to a high level of 32P incorporation into the molecule and the appearance of multiple, slower migrating autophosphorylated forms of PAK1.

In vitro activation by Rac2(wt) resulted in ~5-fold less PAK1 autophosphorylation than Rac1(wt), and the Rac1 polybasic mutant (Rac1(6Q)) had ~50-fold lower levels of PAK1 autophosphorylation in comparison with Rac1(wt). A similar pattern of PAK activation was also observed with post-translationally modified, baculovirus-expressed Rac1(wt), Rac2(wt), and Rac1(6Q) proteins (Fig. 2). A control mutation containing a Ser substituted for the Cys residue just upstream of the polybasic region (Rac1(C178S)), exhibited stimulatory activity toward PAK1 comparable with the Rac1(wt) protein. The ability of the Rac1/Rac2 chimeric proteins to stimulate PAK autophosphorylation corresponded well with their observed differences in binding affinity to the kinase. The replacement of the polybasic domain of Rac2 with the polybasic domain of Rac1 (Rac2 → 1) increased PAK1 autophosphorylation to Rac1(wt) levels and the reverse replacement (Rac1 → 2) diminished PAK1 autophosphorylation to Rac2(wt) levels. The importance of the polybasic domain probably depends on charge, rather than specific amino acid sequence, since a mutant protein, Rac1 → 1 (KR switch) (Fig. 2; see also Table I), containing different, but similarly charged amino acids substituted for the polybasic domain of Rac1, showed levels of PAK1 autophosphorylation comparable with Rac1(wt).

The effect of the various C-terminal Rac mutants on PAK autophosphorylation correlated well with their ability to stimulate PAK-dependent substrate phosphorylation. Fig. 3 confirms that Rac1(6Q) cannot stimulate PAK activity toward an exogenous substrate and that the ability of the Rac1 and Rac2

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**Legend for Fig. 1**: The polybasic domain of Rac1 conveys high binding affinity to PAK1. Decreasing amounts of lysate (15, 10, 5, 2.5, and 1.25 µl) from PAK1(wt)-overexpressing COS-7 cells were electrophoresed and blotted onto nitrocellulose. After renaturation, the blots were probed with 1 µg/ml [35S]GTPγS-labeled wild type and mutant Rac proteins as indicated. The autoradiograms of the blots were quantitated on a PhosphorImager. The depicted values are representative of three separate experiments. (Rac mutants are listed in Table I).

**Legend for Fig. 2**: The polybasic domain of Rac1 is required for efficient PAK1 autophosphorylation. Wild type PAK1 was expressed and immunoprecipitated from COS-7 cells and assayed for kinase activation in the presence of 1 µg (E. coli-derived proteins) or 2 µg (baculovirus-derived proteins) of the indicated GTPγS-labeled wild type and mutant Rac proteins (Rac mutants are listed in Table I and were prepared as described under “Experimental Procedures”). Rac proteins used in the left, middle, and right panels were E. coli-derived GTPγS-labeled wild type (wt), E. coli-derived GTPγS-labeled GTPase-deficient G12V mutant (V12), and baculovirus-derived, post-translationally modified GTPγS-labeled wild type proteins (BV), respectively. The stimulation of PAK1 autophosphorylation in one representative experiment, which was repeated eight times (E. coli-derived GTPases) or one time (BV-derived GTPases) with similar results is shown.
isoforms to stimulate PAK activity is largely dependent on the composition of their polybasic domains. The observed effects of wild type and mutant Rac proteins on PAK activity were reproducible over a wide range of Rac protein concentrations (0.25–3 μg/kinase reaction), which were calculated based upon the active, GTP-bound form of each protein.

The relative ability of Rac1, Rac2, and Rac1(6Q) to activate PAK was also assessed in vitro. Activated forms (G12V) of Rac1, Rac2, Rac1(6Q), and control vector plasmid were co-transfected with Myc-tagged PAK1(wt) plasmid into HeLa cells after 24 h. Cell lysates were blotted and probed with anti-Myc antibody, and PAK activation was evaluated by assessing the appearance of autophosphorylated bands, which migrate more slowly in SDS-polyacrylamide gels. Although Rac2 and Rac1(6Q) were expressed at significantly higher levels than Rac1 in co-transfected HeLa cells, Rac2 and Rac1(6Q) showed only a 29–34 and 10–12% ability, respectively, to stimulate PAK autophosphorylation, in comparison with Rac1 (Fig. 4).

Our combined results indicate that the polybasic domain of Rac plays a crucial role in PAK1 binding and activation and demonstrate that the presence of an intact versus a disrupted polybasic domain dictates the relative ability of Rac1 versus Rac 2 to bind to and activate PAK1.

The Polybasic Domain of PAK Is Required for PAK Activation—A core GTPase-binding domain on PAK1 (amino acids 75–88) was postulated (18) and further reduced to a minimal CRIB motif (amino acids 75–88 in PAK1) (28–30). To determine which PAK residues might be involved in the differential binding of Rac1 versus Rac2 to PAK1, we investigated the binding of Rac1, Rac2, Rac1(6Q), and Cdc42 proteins to PAK1 fragments in overlay assays (data not shown). These experiments suggested that a polybasic region upstream of the CRIB domain might affect the interaction between Rac proteins and PAK1. To elucidate the influence of this highly charged domain (Lys66–Lys68) on PAK binding and activation by GTPases, the three basic residues in PAK1 were mutated to neutral Gln, hydrophobic Leu, or basic Arg residues. The PAK mutants were expressed in COS-7 or HeLa cells and analyzed for in vitro GTPase binding. Comparable amounts of PAK1(wt) and the three PAK mutants were renatured on nitrocellulose and incubated with [35S]GTPγS-Rac1, – Rac2, and – Cdc42, and bound radiolabel was quantitated (Fig. 5A). Compared with the binding to PAK1(wt) (the binding of each GTPase to PAK1(wt) is set to 100%), Rac1 binding to PAK1(K66–68L) and PAK1(K66–68Q) was reduced to 27 and 32%, respectively; Rac2 binding was reduced to 71 and 68%, respectively; and Cdc42 binding was not affected. In contrast, Rac1 and Cdc42 binding to PAK1(K66–68R) was equivalent to PAK1(wt) binding; and Rac2 binding to PAK1(K66–68R) was ~50% increased in comparison with PAK1(wt) binding. These results indicate that the basic charge of Lys66–Lys68 plays an important role in Rac1/PAK1 binding but is not required for Cdc42/PAK1 binding. Moreover, although the basic charge of Lys66-Lys68 slightly facilitates Rac2/PAK1 binding, a more positive basic charge (such as that afforded by the PAK(K66–68R) mutant) is required for efficient Rac2/PAK2 binding.3

To evaluate the role of lysines 66–68 in GTPase-stimulated PAK activity, we assessed the ability of GTPases to activate wild type and mutant PAK1 proteins in vivo. We co-transfected HeLa cells with PAK1(wt) or the three PAK1 mutants and the active form of Rac1, Rac2, or Cdc42; prepared PAK1 immunoblots; and assessed the mobility of PAK1 as an index of PAK activation. All co-transfection experiments revealed high and comparable expression levels of PAK1, PAK1 mutants, and GTPases by PhosphorImager analysis. Fig. 5B compares activation of PAK1(wt) by Rac1, Rac2, and Cdc42 (normalized to 100% for each GTPase) with activation of PAK1(66–68) mutants by these GTPases. Mutation of the basic Lys residues of PAK1 to neutral Gln residues (K66–68Q) decreased PAK1 activation by Rac1(6Q) (see Table I) in HeLa cells (DNA ratio 9:1). Stimulation of PAK activity was visualized by anti-Myc immunoblots of cell lysates and quantitated by a PhosphorImager. Activation of PAK1 (68 kDa) results in autophosphorylated, slower migrating bands on 6.5% gels (panel 1). The expression levels of the activated Rac proteins in the same lysates were confirmed with anti-Myc immunoblots (panel 2) and subsequently quantitated. Similar results were obtained in two independent experiments.

Note that the data in Fig. 5A for PAK1(wt) and each of the GTPases is normalized to 100%, but, as shown in Fig. 1, Rac2(wt) protein has a 2-fold lower affinity for PAK1(wt) than Rac1(wt).
Rac-mediated PAK Stimulation

Fig. 5. The polybasic domain of PAK1 is required for efficient activation by GTPases. A. PAK1(wt) and mutant PAK1(K66–68L), PAK1(K66–68R), and PAK1(K66–68Q) were expressed in COS-7 cells, blotted in expression level-adjusted amounts, and compared for GTP-Rac1, -Rac2, and -Cdc42 binding with overlay assays. The mean values ± S.D. of three independent experiments are shown. The binding of each GTPase to PAK1(wt) is normalized to 100% for easier comparison within one group. B. Wild type PAK1 or the PAK1 mutants PAK1(K66–68L), PAK1(K66–68R), or PAK1(K66–68Q) were co-transfected with activated forms of Rac1, Rac2, and Cdc42 in HeLa cells, as described under "Experimental Procedures" (DNA ratio 4:1), and cell lysates were prepared after 24 h. PAK activity was assessed by Western blotting with anti-Myc antibody, detecting the faster migrating, nonactivated and the slower migrating, hyperphosphorylated active forms of PAK1. Rac1, Rac2, and Cdc42 expression levels were monitored in the same cell lysates with anti-Myc antibody. The PAK1(wt) activation by each GTPase was normalized to 100% as described for A. The bar graph shows one of two similar experiments.

Note that the data in Fig. 5B for PAK1(wt) and each of the GTPases is normalized to 100%, but, as shown in Fig. 4, Rac2 protein has an ~3.5-fold lower ability to activate PAK1(wt) than Rac1.

DISCUSSION

Previously, two Rac/Cdc42 structural domains were implicated in the ability of these GTPases to interact with their target protein kinase, PAK. Mutational analysis of residues corresponding to the N-terminal Ras effector domain (residues 26–45) revealed that these amino acids are critical for PAK activation in vitro and in vivo (25, 31, 33, 34). In addition, Rac/Rho chimeras identified residues 143–175 as another important site (35). In this report, we have investigated the importance of a C-terminal polybasic domain in Rac, which is located immediately upstream of the CAAx box, for PAK activation. This domain is present in several members of the Ras superfamily of small GTP-binding proteins (including Rac1, K-Ras4B, Rap1A, and Rap1B) and is believed to play a crucial role in their biological activity. Specifically, this domain has been shown to be important for Rac-mediated NADPH oxidase activation (37–39) and lamellipodia formation, K-Ras4B-induced malignant transformation (40), and interaction of Rap1A with its regulatory exchange protein smg p21 GDS (41). The biological activity of the polybasic domain has generally been attributed to its ability to facilitate plasma membrane targeting and thereby enable proper localization and function of GTPases (36).

This study now shows that the polybasic domain of Rac1 is a novel effector domain that enables Rac to optimally bind to and activate its target protein kinase, PAK. Substitution of these basic amino acids in Rac1 with neutral residues decreased binding of the GTPase to PAK ~4-fold and nearly abolished Rac1-stimulated PAK activation both in vitro and in vivo. This effect was dependent on the ionic charge of the polybasic domain rather than its specific amino acid composition. Our results also demonstrate that Rac1 binds to and stimulates the kinase activity of PAK1 ~2-fold and 5-fold, respectively, more efficiently than Rac2 in vitro. Similarly, intracellular activa-

\footnote{Note that the data in Fig. 5B for PAK1(wt) and each of the GTPases is normalized to 100%, but, as shown in Fig. 4, Rac2 protein has an ~3.5-fold lower ability to activate PAK1(wt) than Rac1.}

\footnote{J. H. Jackson, unpublished data.}
tion studies revealed that Rac1 activates PAK1 3-fold more efficiently than Rac2 in vivo.

Rac1 and Rac2 are 92% identical to each other (only 15 of their 192 amino acids are different) and are most disparate at their C termini. Importantly, while Rac1 contains a polybasic domain consisting of six contiguous basic amino acids at its C terminus, the comparable domain in wild type Rac2 protein is disrupted by three neutral amino acids. Swapping the intact polybasic domain of Rac1 for the disrupted polybasic domain of Rac2 (and vice versa) completely reversed the PK binding and PAK activation properties of the Rac isomers. Mutant Rac2 protein containing Rac1’s polybasic domain exhibited PK binding/activation properties identical to wild type Rac1 protein; and mutant Rac1 protein containing Rac2’s polybasic domain exhibited PK binding/activation properties identical to wild type Rac2 protein. It is apparent, therefore, that differences in their polybasic domains completely account for the differential ability of Rac1 versus Rac2 to bind/activate PAK. Importantly, substitution of neutral amino acids for the polybasic amino acids of Rac1 and swapping these domains between Rac1 and Rac2 did not alter their intrinsic GTP-binding properties or their ability to undergo post-translational modifications (data not shown).

It appears, therefore, that the Rac proteins contain at least three distinct effector domains in regard to PK activation. Interestingly, while there are marked differences between the polybasic domains of Rac1 versus Rac2, residues 26–45 and 143–175 of Rac1 and Rac2 are 100 and 92%, respectively, identical. Upon GTP binding, residues 26–45 of Rac undergo a conformational change, which is a prerequisite for binding and activation of target proteins such as PKA. Since the polybasic domains in Rac1 and Rac2 significantly differ, this effector domain could control specificity and enable the Rac isomers to activate different target proteins. Our data with Rac1- and Rac2-induced PK activation are consistent with this notion. In addition, a previous study, which demonstrated that Rac2 interacts with the NADPH oxidase component p67phox 6-fold better than with Rac1 (17) further supports this premise. It is possible therefore that in myeloid cells that express both Rac isoforms, Rac1 and Rac2 could have highly distinct and specific functions.

It has been established that association of the GTP-bound form of Rac or Cdc42 with the effector protein PAK will lead to autophosphorylation of the kinase and subsequent substrate phosphorylation. A minimal CRIB region present in a number of potential Rac/Cdc42 target proteins is believed to be necessary for these effector proteins to bind to and be activated by GTPases (28, 29). This study now clearly demonstrates that an additional region, the polybasic domain (lysines 66–68) upstream of the CRIB domain, is important for GTPase binding and stimulation of PAK activity. Moreover, our results indicate that the effects of this domain are mediated by its basic charge rather than a certain amino acid composition. Interestingly, the GTPases varied greatly in their requirement of this domain for efficient PAK binding. Rac1 was highly dependent on the domain for PK binding, Rac2 was intermediate, and Cdc42 did not require the domain at all for binding. In contrast, all of the GTPases required the PK polybasic domain for efficient PAK1 activation. Our studies suggest, therefore, that this domain plays a direct role in PAK activation independent of its effects on GTPase binding. In addition, our studies indicate that the binding properties of the different GTPases for the same effector protein can substantially differ. Moreover, our results demonstrate that binding of GTPases to PAK1 is not sufficient for PAK1 activation.

The basic domain in PAK1, identified in our study, is distinct from the previously identified CRIB domain, which has been reported to be necessary for binding of effectors to activated Rho GTPases. The two other cloned forms of PAK, PAK2 and PAK3, have 100% sequence identity in both the CRIB domain and the polybasic domain (amino acids 66–68 in human PAK1). The highly conserved homology suggests that this positively charged domain is likely to be important for the biological activity of all three PAK isoforms. Since this domain is not present in the related mammalian Cdc42-interacting kinase ACK (42) or the S. cerevisiae homolog Ste20p (43), it appears that this domain is a specialized feature for PAK activation by GTPases.

In conclusion, the data we have presented here establish novel regions on the GTPase Rac and the effector protein PAK that are crucial for interaction of these proteins. Two basic domains, the polybasic C-terminal amino acids 183–188 in Rac and the Lys residues 66–68 in PAK are required elements for Rac/PK interaction and Rac-induced stimulation of PAK kinase activation. The identification of the specific association sites required for Rac interaction with effector proteins such as PAK should facilitate dissection of the molecular mechanisms by which these GTPases regulate biological processes. In addition, our studies suggest that the C-terminal polybasic effector domain of Rac may allow the two Rac isoforms to activate different effector proteins and mediate different biological functions.

Acknowledgments—We thank Alan Hall for providing the pRK5 vector. We acknowledge the support of Lurayne Sanders, Pamela Hall, Kieran Moloney, and Gary Bokoch.

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