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Rac Regulation of Transformation, Gene Expression, and Actin Organization by Multiple, PAK-Independent Pathways

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Rac1 and RhoA are members of the Rho family of Ras-related proteins and function as regulators of actin cytoskeletal organization, gene expression, and cell cycle progression. Constitutive activation of Rac1 and RhoA causes tumorigenic transformation of NIH 3T3 cells, and their functions may be required for full Ras transformation. The effectors by which Rac1 and RhoA mediate these diverse activities, as well as the interrelationship between these events, remain poorly understood. Rac1 is distinct from RhoA in its ability to bind and activate the p65 PAK serine/threonine kinase, to induce lamellipodia and membrane ruffling, and to activate the c-Jun NH_2-terminal kinase (JNK). To assess the role of PAK in Rac1 function, we identified effector domain mutants of Rac1 and Rac1-RhoA chimeric proteins that no longer bound PAK. Surprisingly, PAK binding was dispensable for Rac1-induced transformation and lamellipodium formation, as well as activation of JNK, p38, and serum response factor (SRF). However, the ability of Rac1 to bind to and activate PAK correlated with its ability to stimulate transcription from the cyclin D1 promoter. Furthermore, Rac1 activation of JNK or SRF, or induction of lamellipodia, was neither necessary nor sufficient for Rac1 transforming activity. Finally, the signaling pathways that mediate Rac1 activation of SRF or JNK were distinct from those that mediate Rac1 induction of lamellipodia. Taken together, these observations suggest that Rac1 regulates at least four distinct effector-mediated functions and that multiple pathways may contribute to Rac1-induced cellular transformation.

Rho family proteins constitute a major branch of the Ras superfamily of GDP-GTP-regulated switches (7). While both Ras and Rho family proteins are regulators of cell morphology and growth, they regulate distinct cellular processes. Two functions of Rho family proteins have been well characterized. First, these proteins regulate actin cytoskeleton organization (8). Microinjection studies have established that Rho proteins can act in concert and function in a cascade where CDC42Hs stimulates filopodium formation and also causes activation of Rac-mediated lamellipodium induction and Rac1 in turn causes activation of Rho-mediated stress fiber and focal adhesion formation (34, 43, 44).

Second, Rho proteins are regulators of gene expression (53). Unlike Ras, Rho family proteins do not activate the p42/p44 mitogen-activated protein kinases (MAPKs). Instead, Rac1 and CDC42Hs potently stimulate activity of kinases in the Jun N-terminal kinase (JNK; also referred to as stress-activated protein kinase [SAPK]) and p38 families (11, 33, 36). Interestingly, RhoA does not directly stimulate these pathways. Rac1, RhoA, and CDC42Hs also stimulate the activity of the serum response factor (SRF) which complexes with ternary complex factor (TCF)/Elk proteins to stimulate transcription of genes with serum response elements (SREs) in their promoter-enhancer regions (e.g., the c-*fos* promoter) (19). Importantly, the activation of SRF by Rac and CDC42Hs is not RhoA dependent; hence, these signals converge at an as-yet-uncharacterized point downstream.

More recent evidence points to an involvement of Rho family proteins in events beyond cytoskeletal remodelling. Similarly to the prototype Ras, Rho family proteins induce tumorigenic transformation of rodent fibroblasts (2, 24, 37, 39–41, 52). Furthermore, Rac1 and RhoA appear to be required for Ras transformation and presumably are components of a cascade downstream of Ras, although the linear cascade does not appear to be as well defined as in the control of cytoskeletal rearrangements (24, 39, 41). Rac1 can also confer an invasive phenotype and possibly metastatic potential to lymphocytes and may therefore be involved in multiple aspects of tumorigenesis (32). However, despite evidence for their involvement in transformation, the mechanism whereby Rho family proteins mediate transformation is presently unresolved.

Recently, two clues to the nature of Rac involvement in cellular transformation have been reported. One possible mechanism is based on the fact that Rac and Rho proteins are regulators of the expression of growth-promoting genes. The activation of the JNK and p38 kinase cascades by Rac stimulates the transcriptional activity of c-Jun and ATF-2 (15, 33). c-Jun, and to a lesser extent other AP-1 proteins, is necessary for cell cycle progression, and JNK and Jun function are crucial for cellular transformation by Ras (10, 21, 26). A specific role for SRF in Rac1 or RhoA transformation has not been defined, but SRF is a crucial mediator of c-*fos* transcription, and c-Fos is required for cell cycle progression and Ras transformation in some fibroblast models as well as being implicated in human tumorigenesis (20, 26, 45).

A second line of evidence linking Rac to cellular transfor-

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mation is provided by recent studies demonstrating a role for Rac, Rho, and CDC42Hs in cell cycle progression through the G_1 phase of the cell cycle (36). Constitutively activated mutants of these three Rho family proteins, as well as of Ras, caused G_1 progression and stimulation of DNA synthesis. Thus, it is possible that cyclin-dependent kinase activation by Rac and cell cycle progression are mechanistically linked.

Although morphological changes elicited by Ras and Rho family proteins have been postulated to be an important component of the transformed phenotype, the precise contribution of actin cytoskeletal reorganization to transformation is poorly understood. Furthermore, the interrelationship between Rac-induced gene expression and actin reorganization is not known. It has been suggested that the stimulation of signal transduction cascades composed of immediate effectors, such as PAK, as well as distal signaling kinases (JNK/SAPK and p38) and resultant transcriptional responses might be responsible for Rac-induced morphological changes. In support of this, studies of *Drosophila melanogaster* morphogenesis have suggested a role for the *Drosophila* homolog, DPAK, in cytoskeletal reorganization, a process which may require c-Jun (16). Alternatively, it is also possible that actin reorganization promotes the events that lead to changes in gene expression.

It is clear that Ras interacts with multiple downstream effectors to trigger a complex array of signaling pathways, and the case with Rho family proteins may be similar. The effector(s) that mediates Rac1 induction of lamellipodia, activation of JNK, p38, and SRF, cell cycle regulation, and transformation is unclear. The best-characterized effectors are the ubiquitously expressed family of serine/threonine kinases known as PAKs, but it is not known which, if any, Rac function they mediate. At least three mammalian PAK isoforms have been isolated: rat p65PAK/h-PAK-1, h-PAK-2, and mPAK-3 (4, 29, 31). Binding of PAKs to Rac and CDC42Hs stimulates their serine/threonine kinase activity (4, 29).

By virtue of its striking homology with the *Saccharomyces cerevisiae* protein Ste20, which is implicated in G protein-associated pheromone signaling to a MAPK cascade, PAK has been considered a likely candidate for a link from Rac and CDC42Hs to mammalian MAPKs (46, 57). Published reports have implicated p65PAK as the effector responsible in mammalian cells for activation of JNK/SAPK and p38 MAPK cascades (3, 38, 56). A linear pathway has thus been proposed, leading from Rac through the effector PAK to MEKK, MKK4 (SEK), and MKK3 and, eventually, JNK and p38. However, the direct target(s) of PAK is unknown, and overexpression of wild-type or activated PAKs activates JNK and p38 activity significantly less than Rac or CDC42Hs in some studies and not at all in others (3, 33, 56).

Recent studies have demonstrated that the use of Ras effector domain mutants, which are differentially impaired in downstream signaling activities, is a powerful approach for deciphering the specific signaling pathways critical for Ras transformation (23, 25, 55). For example, mutants of Ras which no longer bind to Raf but maintain signaling through other pathways and maintain transforming activity have been isolated (25). We have utilized an analogous approach to identify the critical sequelae of Rac activation required for Rac1-mediated actin cytoskeleton remodelling, signaling, transcriptional activation, and transformation. Our observations reveal that Rac1 binding to PAK is dispensable for Rac induction of lamellipodia, activation of JNK and SRF, and transformation of NIH 3T3 cells. However, PAK binding correlated with the ability of Rac1 to stimulate transcription from the cyclin D1 promoter. Additionally, Rac causes changes in the actin cytoskeleton by activation of pathways distinct from those that mediate Rac regulation of gene expression. Finally, since no one Rac action directly correlated with Rac1 transforming activity, we suggest that Rac regulation of cell growth is mediated by multiple Rac functions.

MATERIALS AND METHODS

Site-directed mutagenesis of Rac1. The human *rac*1(61L) cDNA (24) was subcloned into the *Bam*HI site of pBluescriptSK$^+$ and mutagenized with the Chameleon kit (Stratagene). Oligonucleotides were produced on an Applied Biosystems synthesizer with 5′ phosphate added. The selection primer was designed to replace the polylinker *Kpn*I site of pBluescriptSK$^+$ with an *Nde*I site: 5′GGG TAC CGG GCC CCC CCA TAT GGT CGA CGG TAT CGA TAA GC3′. Mutagenic oligonucleotides used were as follows: N26D, 5′GGA TAT ATT CTC CAG GAA ATG CAT CGG TTG TGT AAC TGA TCA GTA GG3′; E31V, 5′GTC AAA GAC AGT AGG GAT ATA CAC TCC AGG AAA TGC ATT GG3′; Y40C, 5′AAC ATT GGC AGA GCA ATT GTC AAA GAC3′; N43D, 5′CCA TCT ACC ATA ACG TCG GCA GAA TAA TTG TCA AAG ACA GTA GG3′. Mutagenesis was verified by dideoxy sequencing. The use of a random mutagenesis protocol to identify mutants of Rac12V that are impaired in effector binding has been described elsewhere (22).

cDNA sequences encoding Rac-Rho chimeras were generated by utilizing the unique conserved *Pvu*II site at amino acid 59 of Rac1 and that at amino acid 61 of RhoA. The amino acid sequences of Rac and Rho are identical from this point through amino acid 73; hence, these chimeras are labeled Rac^{73}Rho and Rho^{73}Rac to allow comparison with previous analyses (13). In each case, the carboxy-terminal sequence bears an activating mutation in the switch II region (61L in Rac1 and 63L in the RhoA sequence). Chimeric constructs were verified by sequencing.

Expression vectors and reporter plasmids. *rac*1(61L) sequences were subcloned into the expression vector pCGN-hyg (gift of M. Ostrowski), which is derived from the vector pCGN, for expression in COS-7 cells and NIH 3T3 cells (49). pCGN-hyg constructs encode the hemagglutinin (HA) epitope tag downstream of the cytomegalovirus promoter, in frame with the amino terminus of Rac1 sequences. *rho*A(63L) and wild-type and mutant *rac*1(61L) cDNA sequences were also subcloned into pGEX4T (Pharmacia) for bacterial expression as glutathione *S*-transferase (GST) fusion proteins. Sequences encoding Rac61L, RhoA(63L), Rac^{73}Rho, and Rho^{73}Rac were also subcloned into pcDNA3 (Invitrogen) for expression in mammalian cells. *rac*1(12V) sequences were subcloned into pcDNA3 (Rac12V/33N, Rac12V/35S, and Rac12V/40H) or pCGT (Rac12V/33N, Rac12V/37L, and Rac12V/40H) (50). The sequence encoding Rho^{73}Rac was cloned into the LexA vector pBTM116, and all other *rac* sequences were cloned into the LexA vector pLEVVJ10 for yeast two-hybrid analysis. Sequences encoding the putative Rac1 effectors PAK-3, ROK, and POR-3 as well as Raf-1 were subcloned into GAL4 activation domain expression vectors for yeast two-hybrid analysis as previously described (50). pJ3HPAK-1 and pCMV6M-PAK-1 encode amino-terminal HA and Myc epitope-tagged PAK-1, respectively (gifts of J. Chernoff). pZIP-*raf*/CAAX consists of c-Raf-1 coding sequences with the 18-amino-acid K-Ras4B carboxy-terminal plasma membrane targeting sequence linked in frame to the carboxy terminus of full-length Raf-1 (35).

A reporter for analysis of SRF activity was generated with a mutant SRE sequence which no longer binds TCF (Elk/SAP) proteins (19). Oligonucleotides representing the mutant SRE were synthesized with *Sal*I linkers, phosphorylated with T4 polynucleotide kinase (BRL), annealed, and ligated, and multimers of this sequence were isolated from nondenaturing polyacrylamide gels. A dimer of the mutant SRE was cloned into the unique *Sal*I site of the minimal reporter construct Δ56Fos-dE-Luciferase (14). The sequences of the mutant SRE oligonucleotides used were as follows: 5′TCG ACT GTA CTG TAT GTC CAT ATT AGG ACA TCT G3′ (top) and 5′TCG ACA GAT GTC CTA ATA TGG ACA TAC AGT ACA G3′ (bottom). Cyclin D1-luciferase (CD1-Luc) consists of sequences from −963 of human cyclin linked to luciferase (1). 5XGal-luciferase and Gal-Jun(1-223) have been previously described (48). Flag epitope-tagged expression vectors for JNK1 and p38 and the bacterial expression vector GST-Jun(1-79) were provided by M. Karin.

Yeast two-hybrid binding assay. The yeast strains and media for performance of the yeast two-hybrid binding analyses have been described previously (50). Rac1 sequences (61L, 61L/26D, 61L/31V, 61L/43D, Rac^{73}Rho, and Rho^{73}Rac) were expressed as fusions between the Rac1 protein and the DNA-binding domain of LexA (LBD). Rac1 binding partners were expressed as GAL4 activation domain fusions containing the Rac1 binding sequences from PAK-3, ROK, POR-3, and the Ras binding sequence from Raf-1 (50, 51). POR-3 was identified by yeast two-hybrid screening for Rac1-interacting proteins that exhibit properties of a Rac1 effector target by procedures that we have described previously (50). Briefly, LBD Rac12V was used as bait to screen a Jurkat cDNA library fused to the GAL4 activation domain of pGADGH. Sequence analysis of a partial clone of approximately 600 bp revealed that POR-3 did not show any homology with any known proteins. As described previously, ROKα is a Rac1- and RhoA-interacting protein (5, 28). Interactions between Rac-LexA DNA-binding domain fusions and the GAL4 activation domain of pGADGH. Sequence analysis of a partial clone of approximately 600 bp revealed that POR-3 did not show any homology with any known proteins. As described previously, ROKα is a Rac1- and RhoA-interacting protein (5, 28). Interactions between Rac-LexA DNA-binding domain fusions and the GAL4 activation domain fusions were assessed in the yeast reporter strain L40 and quantitated in a liquid β-galactosidase assay

TABLE 1. Ras and Rho family effector domain: correlation with biological and biochemical phenotype

Protein	Phenotype for Ras residue no. or property:																								
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	N[a]	T[b]	P[c]	J[d]	S[e]
RasH, K, and N	Q	N	H	F	V	D	E	Y	D	P	T	I	E	D	S	Y	R	K	Q	V	V	++	ND[g]	−	+	+
RhoA, B, and C	K	D	Q/E	*[f]	P	E	V	*	V	*	*	V	F	E	N	*	I/V	A	D	I	E	+	−	−	−	++
Rac1 and 2	T	*	A	*	P	G	*	*	I	*	*	V	F	*	N	*	S	A	N	*	M	+	+	+	++	++
CDC42Hs	T	*	K	*	P	S	*	*	V	*	*	V	F	*	N	*	A	V	T	*	M	−	ND	+	++	++

[a] NIH 3T3 transformation (references 24 and 39 to 41 and unpublished observation). ++, >1,000 foci per μg of DNA; +, <100 foci per μg of DNA; −, no focus-forming activity in NIH 3T3 assays.
[b] BW5147 T-cell invasiveness (32). +, induction of invasion; −, no induction of invasion.
[c] p65 PAK binding (29).
[d] JNK activation (11, 33, 36). ++, strong activation; +, weak activation; −, no activation.
[e] SRF activation (19). ++, strong activation; +, weak activation.
[f] *, identity with Ras.
[g] ND, not determined.

as previously described (50). Values (mean ± standard deviation of triplicate determinations) are given in Miller units in Fig. 1 (18).

Cell culture, transfection, and transformation assays. COS-7 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM; high glucose) supplemented with 10% fetal bovine serum or newborn calf serum, respectively. Transfection of COS-7 cells was achieved with the Lipofectamine reagent (GIBCO/BRL) as described by the manufacturer. Thirty hours after transfection, the medium was changed to DMEM containing 0.5% fetal bovine serum, and after 14 h of incubation, lysates were prepared as previously described (42). NIH 3T3 cells were transfected by calcium phosphate coprecipitation as previously described, allowed to recover for 30 h, and starved in DMEM with 0.5% newborn calf serum for 14 h before lysate preparation (9, 17, 54). Focus formation assays were performed with NIH 3T3 cells exactly as described previously (9). For cooperation with Raf/CAAX, cells seeded into 60-mm-diameter tissue culture dishes were cotransfected with 10 ng of pZIP-*raf*CAAX and 500 ng of *rac*1 or *rho*A expression vector. Cognate empty vectors of each plasmid were employed as controls. After 12 to 14 days of growth, the plates were stained with 0.4% crystal violet and photographed. NIH 3T3 cells were also stably transfected with all pCGN-hyg and chimeric pcDNA3 constructs and were utilized in soft agar assays as previously described (9). Cells used in these assays represent pooled populations of >50 individual colonies selected in hygromycin (200 mg/ml) or G418 (400 mg/ml). Expression of the mutant proteins was confirmed by Western blotting (data not shown). Cells (5×10^3/cm^2) were seeded into 60-mm-diameter plates in growth medium containing 0.3% soft agar, and colonies were photographed after 18 days on a Nikon phase-contrast microscope at ×40 magnification.

Transient-expression reporter gene assays. Analysis of luciferase expression in transiently transfected NIH 3T3 cells was performed as described with enhanced chemiluminescence reagents (Amersham) and a Monolight 2010 luminometer (Analytical Luminescence, San Diego, Calif.) (17). Two separate plasmid preparations of each mutant were analyzed to ensure reproducibility of data. All assays were performed in duplicate, and results shown represent the mean (± standard error of the mean [SEM]) of at least three independent assays for each reporter gene with each mutant. To allow comparison of the mutants in different expression vectors, results are expressed as a percentage of maximal stimulation, where the activated Rac1 (Rac12V or Rac61L) equals 100%.

Immunoprecipitation and in vitro kinase assays. PAK-1 activity was analyzed in COS-7 cells following transfection of HA or Myc epitope-tagged PAK-1 and the various Rac expression vectors. Cells were transfected in 100-mm-diameter plates, and after starving for 14 h, lysates were collected in 1 ml of lysis buffer containing protease and phosphatase inhibitors (42). PAK was immunoprecipitated with anti-HA (BAbCO) or anti-myc (9E10; Santa Cruz Biotechnology) antibody as indicated, and the kinase activity was measured by using 4 μg of myelin basic protein (MBP) as the substrate. In vitro kinase reactions were carried out for 20 min at 30°C and stopped with 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins were fractionated on SDS–12.5% PAGE and blotted to Immobilon filters (Millipore). The blots were dried and exposed to film for 1 to 3 h and then subjected to quantitation on a PhosphorImager (Molecular Dynamics). The blots were subsequently probed with antibodies to the appropriate epitope to visualize expression of the PAK construct. A tenth volume of the lysate used in the immunoprecipitations was fractionated on SDS–15% PAGE and probed with the appropriate antibody (anti-T7 [Novagen] or anti-HA [BAbCO]) to visualize Rac1 levels in the lysates. Following incubation with horseradish peroxidase-labelled anti-mouse secondary antibodies, Western blots were developed with enhanced chemiluminescence reagents (Amersham). These methods were also employed to analyze JNK-1 and p38 activity with the anti-Flag antibody (Kodak/IBI) and 2 μg of GST-Jun(1-79) or GST-ATF2(1-254), respectively, as the substrate.

Analysis of lamellipodium formation. Methods for analysis of lamellipodium formation were similar to those described in reference 18. Porcine aortic endothelial (PAE) cells (gift of Lena Claesson-Welsh) were injected with pCGN constructs at 1 μg/ml and with pCGT or pcDNA3 constructs at 2 μg/ml. Concentrations were chosen in order to obtain approximately 50% lamellipodium induction in the control (Rac61L or Rac12V) cells. After injection, cells were starved in serum-free medium and fixed after 13 to 15 h. Rac-injected cells were identified by coexpressed green fluorescent protein (GFP) (Green Lantern; BRL) injected at 25 μg/ml. The actin cytoskeleton was visualized by incubation with rhodamine-phalloidin (1 μg/ml) for 10 min. Cells expressing GFP which exhibited obvious lamellipodia were counted as positive. The standard error of these measurements was determined by dividing the coverslips into three areas and determining the mean of the three counts.

RESULTS

Effector domain mutants of Rac1 exhibit differential binding and activation of PAK. Rac1 and CDC42Hs, but not RhoA, bind and cause activation of the serine/threonine kinase PAK (4, 29). To address the specific contribution of PAK to the diverse signaling and biological activities of Rac1, we mutated specific residues in the Rac61L effector domain (spanning residues 25 to 45) that are identical to CDC42Hs but distinct from those seen in the RhoA, RhoB, and RhoC proteins (26D, 31V, and 43D) (Table 1). We also mutated a tyrosine residue that is conserved in all Ras superfamily proteins and has been shown to be important for Ras function (40C) (23, 25, 55). Another panel of mutants was derived by random mutagenesis of Rac12V to identify effector domain mutants that show differential binding to a panel of Rac1 effectors (PAK-3, POR-3, and ROK) in yeast two-hybrid analyses (33N, 35S, 37L, and 40H) (Table 2) (22). The ability of each Rac1 mutant to interact with PAK was determined in yeast two-hybrid binding analyses and in in vitro binding assays with bacterially expressed mutant proteins.

Several mutants showed impaired PAK interaction (Table 2). In particular, mutation of activated Rac1 at amino acid 43 to the cognate RhoA amino acid (Rac61L/43D) completely blocked the ability of Rac1 to interact with PAK in the yeast two-hybrid assay (Fig. 1A). Interestingly, this mutant was not compromised in its ability to interact with two other candidate Rac effectors, POR-3 and ROKα (Fig. 1A). This mutant was also severely impaired in PAK binding as determined by in vitro binding assays utilizing bacterially expressed Rac1 and wild-type PAK expressed in COS-7 cells (data not shown). Finally, neither wild-type nor mutant Rac1 proteins showed any ability to bind to the Ras effector, Raf-1 (Fig. 1A).

We also determined the ability of each mutant to activate PAK in vivo in transient-overexpression assays using COS-7 cells (Fig. 1B and C; Table 2). These analyses showed that all

TABLE 2. Properties of Rac1 effector domain mutants and Rac1-RhoA chimeras

Protein[a]	Value							
	Focus formation[b]	Lamellipodia[c]	Gal-Jun[d]	JNK[e]	PAK		SRF[h]	Cyclin D1[i]
					In vivo kinase[f]	Two hybrid[g]		
Rac12V	+++	+++	+++	+++	+	+	+++	+++
Rac61L	+++	+++	+++	+++	+	+	+++	+++
Rac61L/26D	+	+++	+++	++	+	+	++++	++
Rac61L/31V	++	+	++	+++	+	+	++	+++
Rac12V/33N	+++	ND	+++	+++	++	++	+	++++
Rac12V/35S	±	ND	+	±	ND	−	±	±
Rac12V/37L	++	−	±	+	±	+	−	±
Rac61L/40C	+++	+++	±	−	−	−	±	±
Rac12V/40H	+++	+++	±	−	−	−	±	±
Rac61L/43D	+	+++	+++	+++	±	−	+++	+
RhoA(63L)	++	SFs	±	−	−	−	+++	++++
Rac[73]Rho	+	SFs	±	−	−	−	+++	±
Rho[73]Rac	++++	++	+++	++	−	−	+++	+++++

[a] Oligonucleotide-directed or random mutagenesis of *rac*1(61L) or *rac*1(12V) cDNA sequences, respectively, was employed to generate mutant sequences encoding effector domain mutants of constitutively activated Rac1. Chimeric proteins are composed of the N-terminal 73 residues from Rac61L or RhoA(63L) and terminate with the reciprocal C-terminal amino acids.

[b] Appearance of transformed foci when cotransfected with pZIP-*raf*/CAAX in NIH 3T3 (UNC) focus formation assays. ±, <5 foci per dish (activity seen with Raf/CAAX alone); +, 5 to 10; ++, 10 to 20; +++, 20 to 30; ++++, >30. Note that the Rac12V/37L mutant did not cause transformation in the NIH 3T3 (CSHL) strain (22).

[c] Induction of lamellipodia in PAE cells. −, <5% injected cells with lamellipodia; +, 5 to 19%; ++, 20 to 35%; +++, >35%; SFs, induction of stress fibers.

[d] in vivo activation of Gal-Jun transcriptional activity. ±, 1 to 30% of maximal stimulation, where value for Rac61L = 100%; +, 30 to 60%; ++, 60 to 80%; +++, 80 to 120%.

[e] Rac1 activation of JNK1 in transiently transfected COS-7 cells. −, <2-fold activation relative to vector-only control; ±, 2- to 4-fold; +, 5- to 8-fold; ++, 8- to 12-fold; +++, >12-fold (activity seen with nonmutated Rac12V or Rac61L).

[f] Rac1 activation of p65 PAK1 in transiently transfected COS-7 cells. −, <2-fold activation relative to vector-only control; ±, 2- to 6-fold; +, 6- to 18-fold; ++, >18-fold.

[g] Induction of β-galactosidase activity in yeast two-hybrid analysis. −, <2 units of activity; +, 50 to 70 units; ++, >70 units. Data for Rac12V mutant proteins are derived from the method of Joneson et al. (22).

[h] In vivo activation of transcription from an SRF-dependent promoter element upstream of the luciferase gene. −, <5% of maximal stimulation, where value for Rac61L = 100%; ±, 5 to 25%; +, 26 to 50%; ++, 51 to 90%; +++, 91 to 120%; ++++, >120%.

[i] in vivo activation of transcription from the cyclin D1 promoter upstream of the luciferase gene. ±, 6 to 30% of maximal stimulation, where value for Rac61L = 100%; +, 31 to 50%; ++, 51 to 90%; +++, 91 to 120%; ++++, 121 to 150%; +++++, >150%.

mutants were fully capable of activating PAK catalytic activity except Rac61L/40C and Rac12V/40H, which were severely impaired, and Rac61L/43D and Rac12V/37L, which were substantially impaired. Since other single amino acid substitutions (e.g., 31V) failed to impair PAK binding and activation, it suggests that multiple residue differences between Rac1/CDC42Hs and RhoA effector domains determine recognition by PAK (Table 1).

Interaction with PAK-1 is neither necessary nor sufficient for Rac1 transforming activity. PAK-1 is the best-characterized candidate effector for Rac1, and several studies have implicated PAK in signaling downstream from Rac (3, 4, 29, 38, 46, 56, 57). To determine if Rac1 interaction with PAK was important for Rac1 transforming activity, we evaluated the transforming potential of each Rac1 mutant with two assays. First, previous studies have shown that coexpression of Rac1 with activated Raf-1 (e.g., Raf/CAAX or RafY340D) causes a synergistic enhancement of focus-forming activity in NIH 3T3 cells (24, 41). Utilizing the mutants described above, we found that the Rac61L/40C and Rac12V/40H mutants, which were severely impaired in PAK interaction and activation, were still capable of cooperative focus-forming activity when coexpressed with Raf/CAAX in NIH 3T3 cells (Fig. 2A and Table 2). In contrast, although Rac61L/26D bound and activated PAK as well as nonmutated Rac1 (Rac12V or Rac61L) in two-hybrid analysis and in vitro binding studies, it showed significantly impaired transforming activity (Fig. 2A and data not shown).

We also used the ability of activated Rac1 alone to promote anchorage-independent growth (24) to determine the consequences of each mutation to Rac1 transforming potential. For these analyses, we utilized pooled populations of multiple drug-resistant colonies of NIH 3T3 cells stably expressing each Rac1 mutant protein. Interestingly, consistent with the failure of these mutants to cooperate with Raf/CAAX in focus formation assays (Fig. 2A), we found that cells expressing the Rac61L/26D and Rac61L/43D mutants were unable to support growth in soft agar (Fig. 2B). Overall, we observed a direct relationship between these two transformation assays with all Rac1 mutants. Finally, cell lines expressing these mutants were also severely impaired in their ability to form foci of transformed cells in secondary focus formation assays (data not shown). Thus, we conclude that Rac1 interaction with PAK-1 is neither necessary nor sufficient to confer a transformed phenotype on NIH 3T3 cells.

PAK is dispensable for Rac1 activation of JNK. Several studies have implicated PAK in the activation of JNK by Rac. For example, kinase-inactive, dominant negative alleles of PAK blocked JNK activation by Rac, raising the possibility that PAK is the link between Rac and MEKK, and hence the JNK pathway (33). We have found, however, that both wild-type and kinase-inactive PAKs can block Rac signaling to the JNK pathway, suggesting instead that PAK overexpression is blocking the interaction of the bona fide effector for JNK activation (data not shown). Other studies have suggested that activated PAK alone is sufficient to enhance JNK activity (3, 38, 56). To address this question, we analyzed Rac1 effector domain mutants as well as Rac-Rho chimeras for their ability to activate JNK in transient-expression assays in COS-7 cells as well as in functional assays utilizing a Gal4-Jun fusion protein in tran-

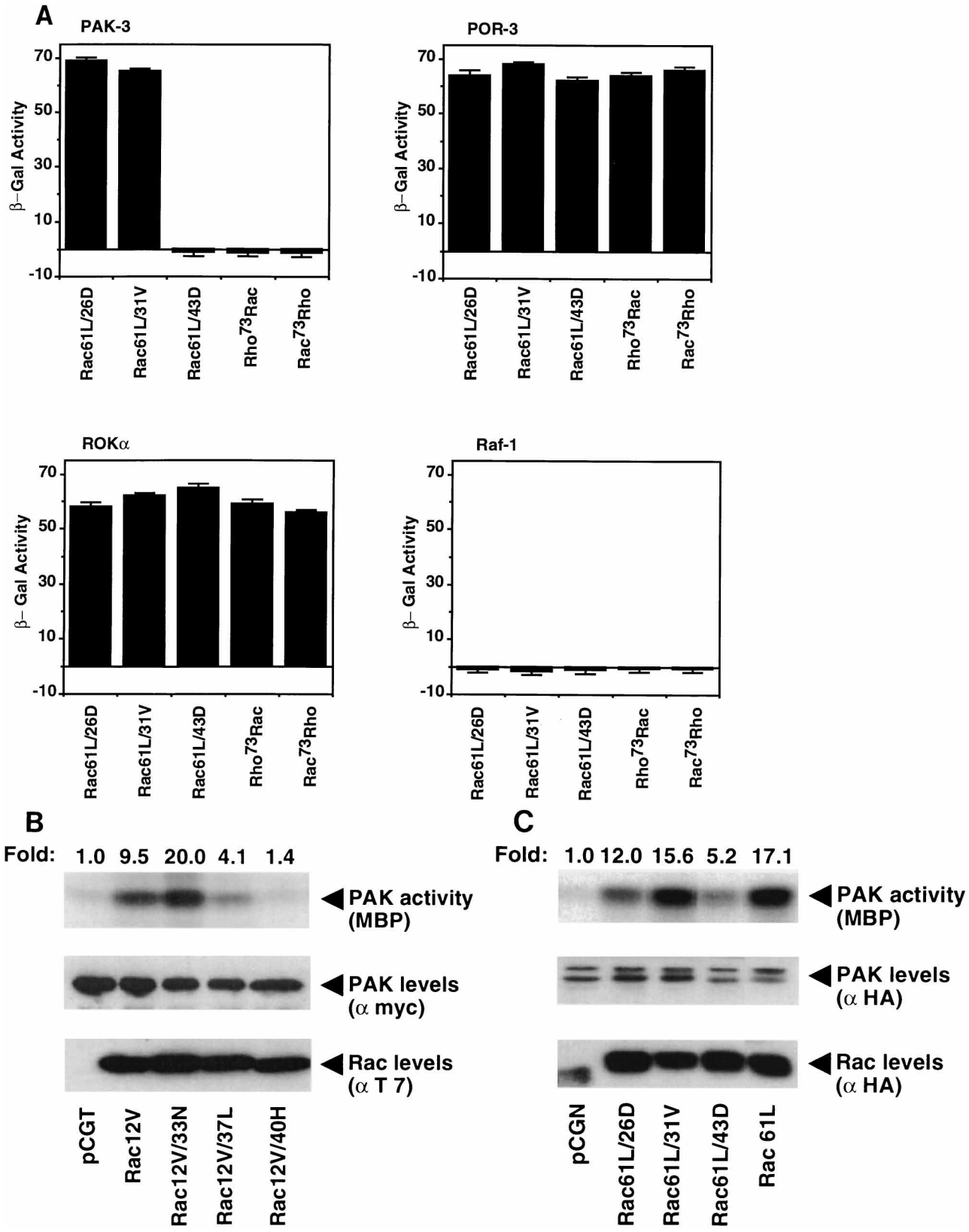


FIG. 1. Rac1 effector domain mutants are impaired in PAK binding and activation. (A) Two-hybrid analysis of Rac1 effector domain mutants and Rac-Rho chimeras. The indicated Rac effector domain mutant or chimeric Rac-Rho protein was expressed as a LexA fusion protein and subjected to yeast two-hybrid binding analysis with the indicated candidate effector (PAK-3, POR-3, ROK, or Raf expressed as a GAL4 DNA-binding domain fusion protein). β-Galactosidase (β-Gal) activity was quantitated, and data are expressed as the averages of three determinations (±SEM). (B) PAK activity is differentially stimulated by activated Rac1 effector domain mutants. COS-7 cells were transfected with an expression vector encoding Myc epitope-tagged wild-type PAK-1, along with the indicated effector domain mutants

siently transfected NIH 3T3 cells. As described above, Rac61L/43D is defective in its ability to bind to and activate PAK (Fig. 1A and C). This mutant displayed full activity, however, in activation of JNK as assessed in both the COS-7 and NIH 3T3 transient-transfection assays (Fig. 3A and C).

Utilizing reciprocal chimeras between Rac1 and RhoA, we found that neither of these proteins was capable of interacting with PAK in two-hybrid analyses, nor could they activate PAK catalytic activity (Fig. 1 and data not shown). This is consistent with published reports which indicate that multiple effector regions of Rac are required for interaction with PAK (12). Surprisingly, the Rho[73]Rac chimera stimulated JNK-dependent reporter gene activity as well as activated Rac1 (Fig. 3C), suggesting that RhoA sequences in the amino-terminal region can substitute effectively for Rac1 sequences in conferring this phenotype. Therefore, regions outside of the classical amino-terminal effector domain are involved in effector binding, leading to JNK and transcriptional activation. Furthermore, these observations provide further evidence that PAK binding is dispensable for Rac1 activation of JNK.

Rac1 activation of SRF is neither necessary nor sufficient for transformation. Rac1 is a strong activator of SRF, but the contribution of this activation to Rac1 function is not known (19). Rac1 mutants with amino acid substitutions at position 33 or 40 (Rac12V/33N, Rac12V/40H, and Rac61L/40C) were significantly impaired in SRF activation but still retained wild-type transforming potential (Fig. 4). In addition, Rac12V/37L was completely impaired in SRF activation yet still retained significant transforming capability (Table 2). In contrast, the Rac61L/26D mutant, which activated SRF significantly better than Rac61L in NIH 3T3 cells (Fig. 4), was impaired in transformation when assessed by focus formation and growth in soft agar (Fig. 2A and B, Table 2, and data not shown). Thus, Rac activation of SRF is neither necessary nor sufficient for mediating Rac transforming activity.

Since RhoA can activate SRF but cannot bind PAK, it had previously been assumed that PAK is not an effector for SRF activation, although it has been postulated that a PAK might link all Rho proteins to SRF (53). By using chimeras of Rac61L and RhoA(63L), it was found that both Rac[73]Rho and Rho[73]Rac activated SRF to the same degree as Rac61L and RhoA(63L) (Fig. 4). This indicated that Rac1 and RhoA sequences are interchangeable in conferring this phenotype in NIH 3T3 cells and also demonstrated that the Rac[73]Rho chimera, which is severely impaired in JNK activation (Fig. 3C), is biologically active in these cells. The differential activation of JNK and SRF by effector domain mutants as well as Rac-Rho chimeras further indicated that the JNK and SRF pathways are mediated by distinct effectors binding to Rac1.

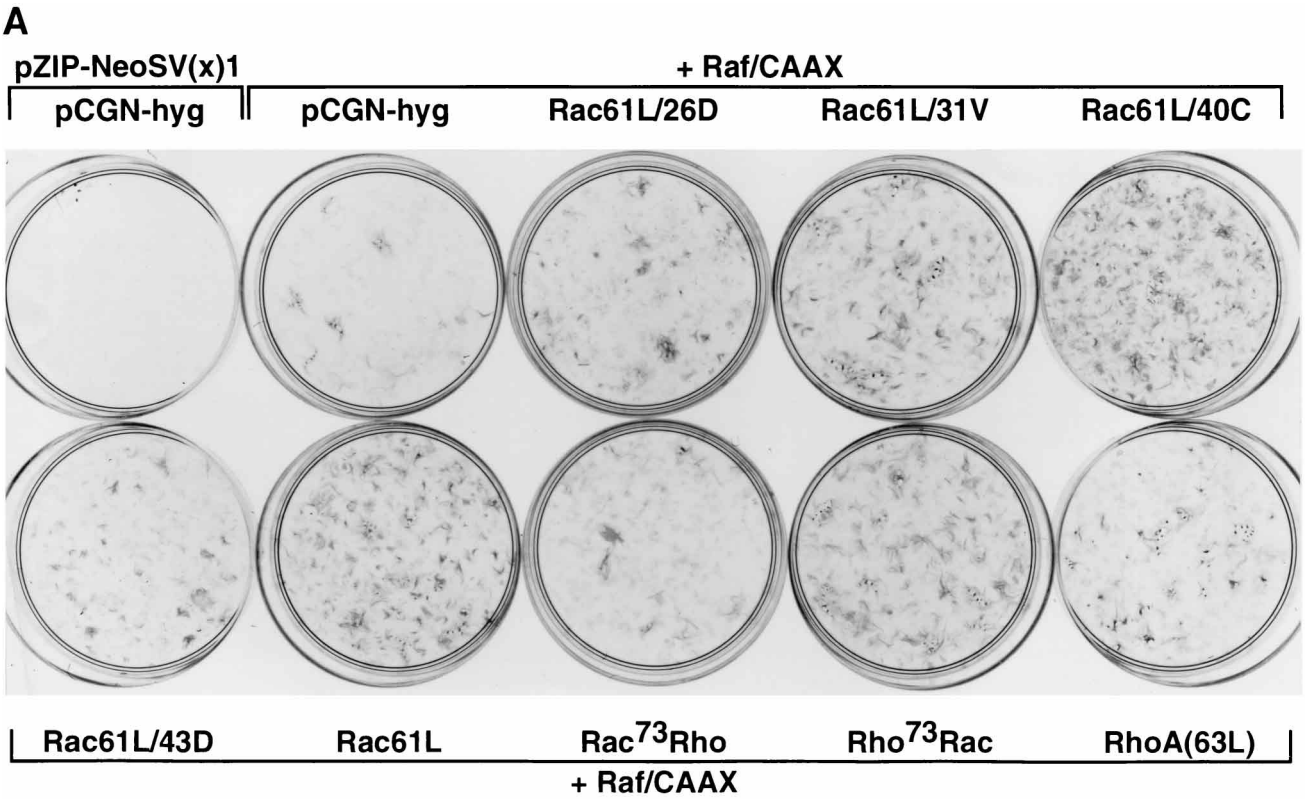
Rac1 interaction with PAK, induction of signaling cascades, and transformation are distinct from induction of lamellipodia. To assess the relationship between JNK and SRF activation and induction of lamellipodia, we measured the ability of effector domain mutants and chimeras to induce lamellipodia in PAE cells. Both Rac61L/40C and Rac12V/40H mutants were completely negative for PAK binding and activation and severely impaired in JNK and SRF activation, yet lamellipodia were induced with the same frequency as that seen with Rac61L (Fig. 5). In contrast, Rac61L/31V bound and activated PAK and activated JNK, yet it induced lamellipodia to only 20% the level of activated Rac1 (Fig. 5). Thus, Rac interaction with PAK and activation of JNK and SRF (as well as downstream targets [see below]) are activities distinct from those that promote Rac1 induction of lamellipodia.

Both the Rac61L/26D and Rac61L/43D mutants exhibited strong lamellipodium induction, yet both were impaired in their transforming capabilities in that they failed to promote proliferation of NIH 3T3 cells in anchorage-independent conditions (Fig. 2 and 5; Table 2). Therefore, Rac1-induced lamellipodium formation alone is not sufficient for full Rac1 transforming activity. Indeed, the Rac61L/31V mutant (partially inhibited in lamellipodium induction) and the Rac12V/37L mutant (completely incapable of inducing lamellipodia in these assays) were only mildly suppressed in their abilities to confer a transformed phenotype on NIH 3T3 cells, as measured in both focus formation and soft agar colony assays (Fig. 2A and data not shown). Thus, pathways leading to the induction of lamellipodia are unnecessary for Rac1 transformation of NIH 3T3 cells.

Stimulation of transcription from the cyclin D1 promoter correlates with Rac1 binding to PAK. Rac1 function has recently been shown to be necessary for progression through the G_1 phase of the cell cycle (36). Ras is also required for G_1 progression, in part by stimulating the expression of cyclin D1 (1). Since oncogenic Ras causes activation of Rac, we evaluated the possibility that Rac1 may stimulate cyclin D1 expression. For these analyses, we utilized a luciferase gene reporter plasmid where expression was controlled by the cyclin D1 promoter. Like Ras, activated Rac1 also stimulated cyclin D1 promoter expression (Fig. 6). Since both the 40C and 40H mutants, which retained strong transforming activity and induction of lamellipodia, were impaired in cyclin D1 activation, potent cyclin D1 activation is not required for these two Rac1 activities. Additionally, Rac12V/33N was impaired in SRF but not cyclin D1 stimulation, suggesting that Rac activates these two events via distinct pathways. With the exception of Rac61L/43D, we observed a direct correlation between JNK activation and cyclin D1 stimulation with the different Rac1 effector domain mutants.

In contrast, we observed a strong correlation between Rac1 binding to PAK and activation of the cyclin D1 promoter (Table 2). For example, we observed that Rac12V/33N exhibited significantly increased binding and activation of PAK and increased stimulation of cyclin D1 expression. Conversely, the 37L, 40C, 40H, and 43D mutants showed both impaired PAK interaction and impaired cyclin D1 stimulation. The only exception to this correlation was seen with the Rho[73]Rac chimeric protein, which failed to bind to or activate PAK yet strongly stimulated cyclin D1 expression. However, since RhoA(63L) also strongly stimulated cyclin D1 expression, PAK may serve as the effector that promotes Rac1, but not RhoA, activation of cyclin D1 expression. Consistent with this

derived by random mutagenesis and yeast two-hybrid screening. (Top panel) PAK was subsequently immunoprecipitated and used in an immune complex kinase assay with MBP as the substrate. Kinase reaction mixtures were subjected to SDS-PAGE, transferred to Immobilon filters, and exposed to film. (Middle panel) Phosphorylation of MBP was determined by PhosphorImager analysis and is expressed as fold activation relative to the level in PAK-plus-vector-transfected cells. The blot was then probed with antibody to the Myc epitope (α myc) to visualize the PAK levels in each immunoprecipitate. (Bottom panel) The level of Rac1 protein expressed in each extract was determined by Western blotting and probing with antibodies directed against the T7 epitope (α T7). (C) COS-7 cells were transfected with HA epitope-tagged wild-type PAK-1 and Rac1 effector domain mutants containing amino acid substitutions based on the Rho sequence. (Top panel) Phosphorylation of MBP by immunoprecipitated PAK. Activation of kinase activity relative to PAK-plus-empty-vector-transfected cells is indicated (Fold). (Middle panel) PAK levels in immunoprecipitates were visualized by probing the blot with an antibody directed at the PAK epitope tag (α HA). (Bottom panel) Rac levels in the extracts used for immunoprecipitation were visualized by Western blotting and probing with antibodies directed against the HA epitope (α HA). Similar results were obtained with the Myc-tagged PAK-1 expression vector with these mutants (data not shown).



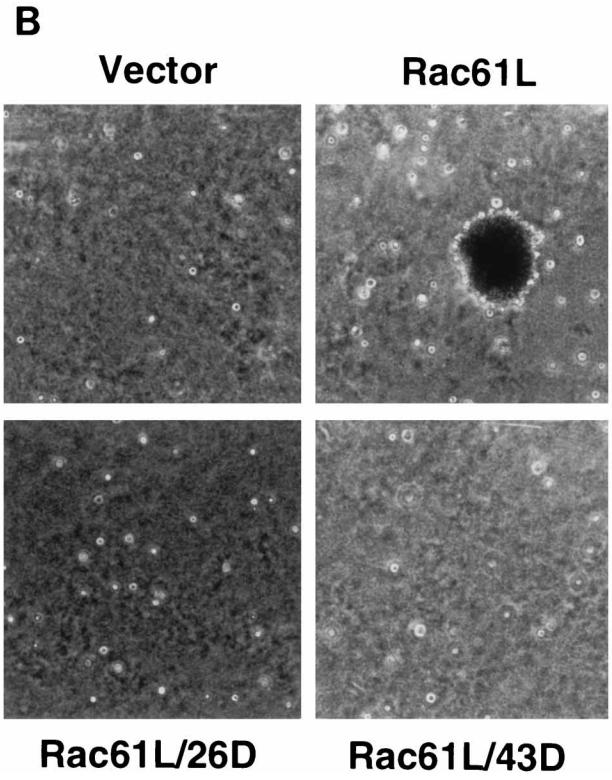
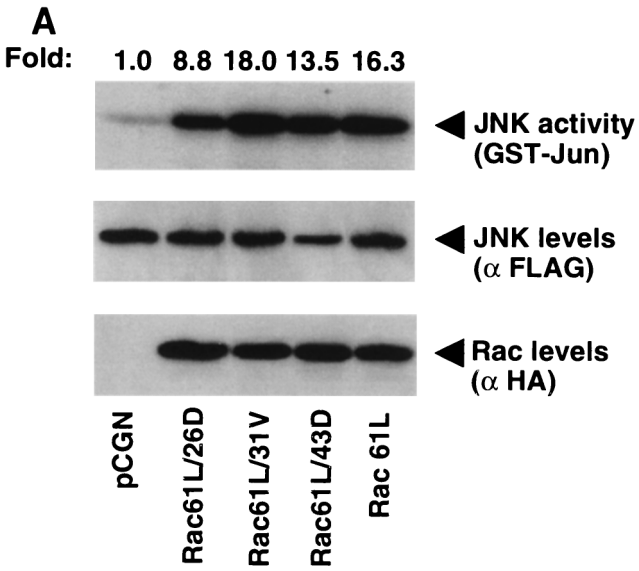


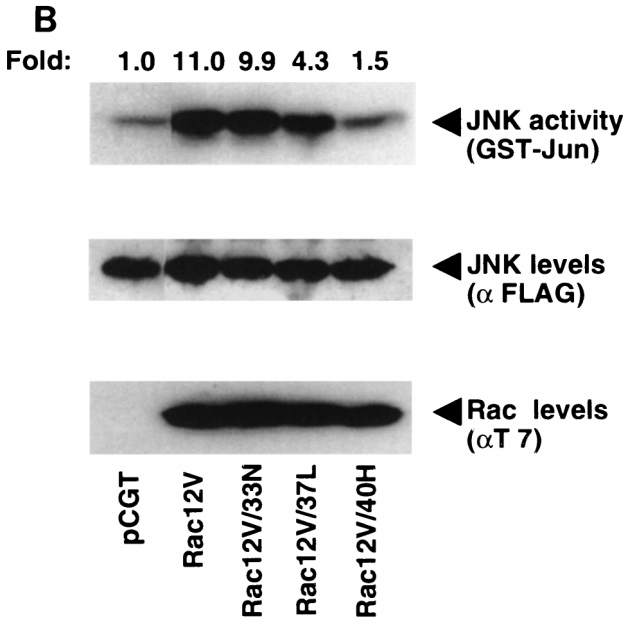
FIG. 2. Rac1 effector domain mutants and Rac-Rho chimeras show differential ability to transform NIH 3T3 cells. (A) Focus formation assay. NIH 3T3 cells were transfected with 10 ng of activated c-Raf1 (Raf/CAAX) along with the empty expression vector, which reproducibly yields a low level of focus formation in these cells (Raf/CAAX + pCGN-hyg). Where indicated, cells were cotransfected with 500 ng of the indicated expression vector and cultured for 14 days before being stained with 0.4% crystal violet. Results are representative of three assays performed in triplicate for each mutant. Similar results were obtained in cotransfection cooperation assays with another activated mutant of Raf (RafY340D; data not shown). (B) NIH 3T3 cells stably expressing the indicated Rac1 mutants or empty expression vector were assayed for their ability to proliferate under anchorage-independent conditions. Cells were seeded in growth medium containing 0.3% agar, and colonies were visualized after 18 days. Cells were photographed at ×40 magnification.

possibility, we have observed that distinct regions of the cyclin D1 promoter are required for Rac1 and RhoA stimulation (data not shown). Thus, PAK may serve as the key effector for Rac1 activation of cyclin D1.

DISCUSSION

Rac1 is now known to be a regulator of diverse cellular processes that include the control of actin organization (8), regulation of gene expression (53), and cell cycle progression through G_1 (36). Rac also regulates cell proliferation and may function downstream of Ras and be required for full Ras transformation (24, 41). The effectors by which Rac mediates these diverse activities, as well as the interrelationship between these events, remain poorly understood. In particular, while there is evidence that PAK is an effector for Rac function (3, 13, 56), the precise role of PAK in mediating the diverse actions of Rac has not been clearly established. We have utilized effector domain mutants of Rac1 to define the role of PAK in Rac1 function. Unexpectedly, we found that Rac interaction with PAK was dispensable for Rac1 functions except for cyclin D1 stimulation. Furthermore, we found that the signaling pathways by which Rac1 regulates gene expression are distinct from those that regulate actin organization. Finally, we determined that no single Rac1 activity correlated directly with Rac1 transforming potential. We conclude that Rac uti-





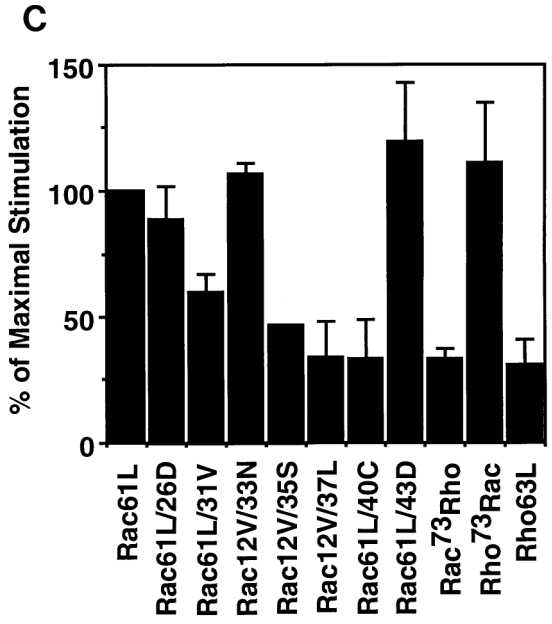


FIG. 3. Activation of JNK1 by Rac1 effector domain mutants. (A) COS-7 cells were transfected with a plasmid expressing Flag epitope-tagged JNK1 together with the indicated HA epitope-tagged Rac1 mutant or the cognate empty vector (pCGN-hyg). (Top panel) Cells were serum starved for 14 h, and JNK1 was immunoprecipitated from lysates for analysis in immune complex kinase assays with GST-Jun(1-79) as the substrate. (Middle panel) Activation of substrate phosphorylation by Rac is expressed relative to the level obtained in JNK1-plus-vector-transfected cells (Fold). Membranes were subsequently probed with anti-Flag antiserum to visualize JNK1 levels in the immunoprecipitates. (Bottom panel) Rac levels were determined by Western blotting as for Fig. 1. Data shown are representative of three independent experiments. (B) COS-7 cells were transfected as for panel A, but with the indicated mutants expressed as T7-tagged proteins. JNK1 activity (top panel) and JNK1 levels (middle panel) were determined as for panel A; Rac levels were determined by probing Western blots of extracts with T7 epitope antiserum (bottom panel). Data shown are representative of three independent experiments. (C) JNK-dependent transcriptional activation by Rac1 effector domain mutants and Rac-Rho chimeras. NIH 3T3 cells were transiently transfected with Gal-Jun(1-223), composed of the yeast Gal4 DNA-binding domain fused to the amino-terminal activation domain of the c-Jun protein (0.25 μg), and the reporter construct 5XGal-luciferase (2.5 μg). This reporter system provides a functional readout for total cellular JNK activity. Cells were cotransfected with the empty expression vector or the indicated Rac mutant (0.5 μg), cultured for 30 h, and then serum starved (0.5% calf serum) for 14 h before extract preparation. Luciferase activity was determined and is expressed as the percentage of activation relative to the level of activation (100%) seen with Rac61L and Rac12V. Average activation by activated (nonmutated) Rac1 was 18.6-fold in these assays. Data shown are representative of three independent experiments for each mutant performed in duplicate.

lizes at least four distinct downstream effector pathways to mediate its diverse actions (Fig. 7).

Effector domain mutants of Ras have been very useful reagents for deciphering the complex nature of Ras signaling involved in Ras transformation. In particular, the demonstration that Ras effector domain mutants, which no longer bound to nor activated the Raf-1 serine/threonine kinase, nevertheless retained the ability to cause tumorigenic transformation of NIH 3T3 cells clearly indicated that Raf-independent effector pathways were also important for Ras regulation of cell growth (24, 55). In the present study, we sought to generate equivalent effector domain mutants of Rac1 which no longer bound to or activated PAK. Although genetic and biochemical evidence suggested that PAK was an important effector for Rac activation of JNK and regulation of actin cytoskeletal organization, a verification of these roles has not been accomplished. Thus, our identification of Rac1 mutants, such as the Rac61L/43D mutant that exhibited greatly impaired PAK binding and activation, provided important reagents to address this question. Rather unexpectedly, the loss of PAK interaction did not impair Rac61L/43D induction of lamellipodia or activation of JNK or SRF. Similar observations were also seen with the Rho^{73}Rac chimeric protein. Furthermore, a second PAK-binding deficient mutant, Rac61L/40C, still retained potent transforming activity. Therefore, PAK is also dispensable for Rac1 transforming activity. Thus, Rac1 mediates these actions through PAK-independent signaling pathways. The role of the Rac interaction with PAK in Rac1 function is therefore unclear at present. However, we did observe a direct correlation between the abilities of Rac1 to activate PAK and to stimulate cyclin D1 expression. Whereas a mutant of Rac1 with significantly heightened PAK binding and activation displayed increased activation, mutants with impaired PAK activation were also impaired in cyclin D1 activation.

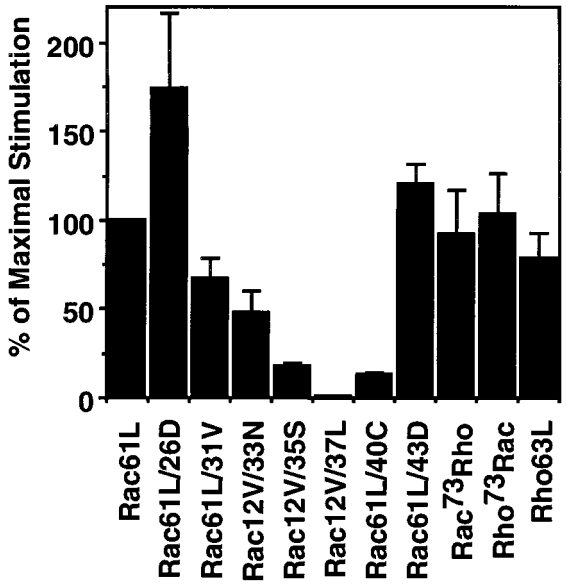


FIG. 4. Activation of SRF by Rac1 effector domain mutants and Rac-Rho chimeras in NIH 3T3 cells. Cells were cotransfected as for Fig. 3C with the reporter construct $(SREm)_2$-luciferase to measure Rac1 activation of SRF, either with the empty expression vector or with one encoding the indicated Rac mutant. Average activation by nonmutated Rac1 [Rac12V or Rac61L], relative to empty-vector-plus-reporter-transfected controls, was 67-fold, and data are expressed as described in the legend to Fig. 3C. Data shown are representative of three independent experiments performed in duplicate for each mutant.

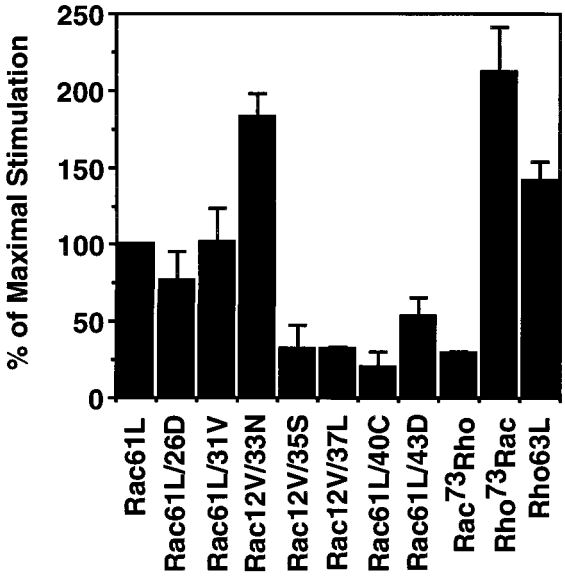


FIG. 6. Activation of the cyclin D1 promoter by Rac1 mutants and Rac-Rho chimeras. NIH 3T3 cells were cotransfected with the human cyclin D1-luciferase reporter construct and the indicated Rac mutant. Results are expressed as for Fig. 3C and 4. Average activation by Rac1 was 7.2-fold relative to reporter-plus-empty-vector-transfected controls. Results shown represent the average of at least three independent experiments performed in duplicate (±SEM).

Our observation that Rac interaction with PAK was dispensable for activation of JNK contrasts with previous observations, where PAK was suggested to be required for this Rac activity (3, 56). Furthermore, a PAK dominant negative was shown to block Rac activation of JNK (33). However, as suggested by the authors of that study, since the PAK dominant negative constituted the GTP-dependent Rac-binding domain of PAK, its activity may be nonspecific in that it is likely to block Rac interaction with PAK as well as with other candidate effectors. Thus, this does not provide definitive evidence for the involvement of PAK in Rac activation of JNK. Finally, the limited ability of constitutively activated PAKs to activate JNKs suggests that Rac may cause JNK activation primarily through

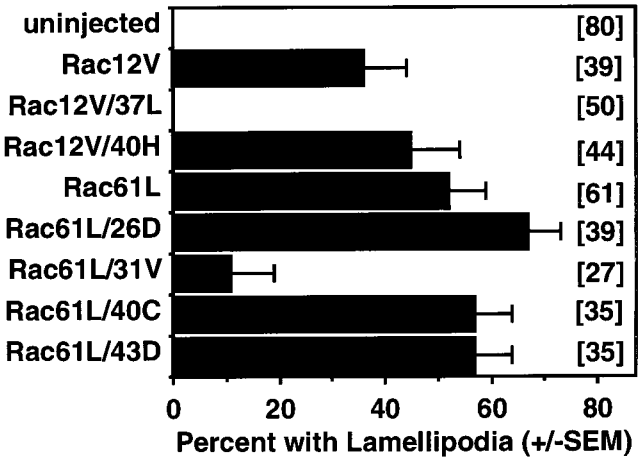


FIG. 5. Lamellipodium induction in PAE cells by Rac1 mutants and chimeras. PAE cells were microinjected with Rac1 expression constructs as indicated, along with a GFP expression vector to identify injected cells. Cells were serum starved after injection, and lamellipodium formation was assessed at 13 to 15 h following rhodamine-phalloidin staining. The numbers of cells injected are indicated in brackets. Expression of mutant proteins was confirmed by immunohistochemical staining of cells (data not shown). Data represent mean counts of lamellipodia in three independent analyses (±SEM).

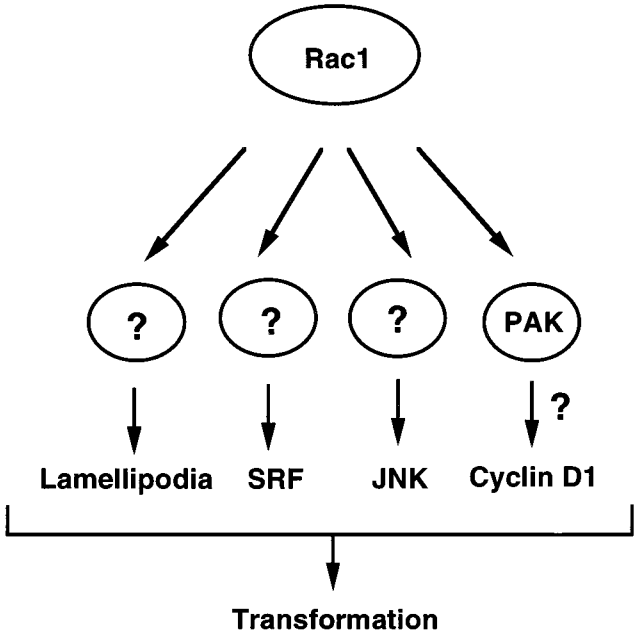


FIG. 7. Model of Rac1 effector interactions and downstream pathways. Our data suggest that Rac1 mediates a PAK-dependent pathway that causes stimulation of transcription from the cyclin D1 promoter and three distinct, PAK-independent effector pathways leading to induction of lamellipodia, activation of JNK and Jun, and activation of SRF. The effectors for the PAK-independent pathways are not known.

PAK-independent pathways. Since Rac61L/43D retained binding to other candidate Rac effectors (e.g., POR-3 and ROK), Rac may mediate JNK activation via these or other Rac binding proteins.

Previous studies showed that Ras function was necessary for cell cycle progression through G_1 (36, 47). While the precise signaling pathways that mediate this function are not known, the ability of Ras to stimulate transcription of cyclin D1 suggests one mechanism for this action. Cyclin D1 mRNA levels are elevated in Ras-transformed cells (6), and Ras stimulates transcription from the cyclin D1 promoter (1, 27). Recently, Rho family protein function was also found to be necessary for G_1 progression (36). Thus, it is possible that Ras regulates G_1 progression via a pathway that involves Rac. Consistent with this, in the present study we observed that constitutively activated Rac1 also stimulated transcription from the cyclin D1 promoter. However, a recent report showed that the Raf-MEK-MAPK pathway positively controlled cyclin D1 expression, whereas p38 antagonized this expression (27). One possible explanation for this apparent difference in their observations is that their study used a fragment of the cyclin D1 promoter that lacked the responsive element by which Rac activation of JNK and p38 may cause stimulation of cyclin D1 expression. Instead, our promoter fragment contains an AP-1 site (at −954 of the human sequence) where JNK activation of Jun may cause stimulation of cyclin D1 expression (1). In support of this, when this AP-1 site was mutated, Rac activation of the reporter was impaired by greater than 50% (data not shown). As we have described recently, a distinct region of the cyclin D1 promoter is sensitive to stimulation by MAPKs (1). In NIH 3T3 cells, activated Rac proteins were significantly better activators of the cyclin D1-luciferase reporters than activated Ras. Therefore, Ras activation of MAPKs and JNKs constitutes distinct pathways for regulation of cyclin D1 expression and cell cycle progression.

Although it is clear that Rac1, as well as RhoA and CDC42Hs, is a regulator of both actin reorganization and gene expression, whether these two events are controlled by independent or interrelated signaling events has not been determined. Since Rac-mediated induction of lamellipodia and membrane ruffling occurs on a time scale of minutes, it has been suggested that they occur independently of Rac-mediated events in the nucleus (53). Our observation that the introduction of 40C or 40H mutations into Rac1 resulted in mutant proteins that retained strong lamellipodium induction yet were greatly impaired in their ability to activate JNK and SRF activity or cyclin D1 transcription demonstrates that the pathways of Rac1-regulated gene expression characterized to date are not required for induction of lamellipodia. Furthermore, since the Rac61L/31V mutant showed impaired lamellipodium induction, without a concomitant loss of JNK activation or cyclin D1 stimulation, Rac regulation of gene expression is not a consequence of actin reorganization. Thus, we suggest that Rac stimulates distinct, independent signaling pathways that regulate gene expression and actin organization.

To date, essentially nothing is known concerning the mechanism by which Rho family proteins activate SRF. Since a substitution at amino acid 33 of Rac1 significantly impaired SRF activity yet resulted in enhanced PAK activation, SRF activation is not likely to be mediated through PAK. Additionally, since Rac^{73}Rho activated SRF but not cyclin D1, the pathways by which Rac mediates these two functions are distinct.

The mechanism by which Rac causes alterations in cell proliferation and promotes tumorigenic transformation has not been determined. Unexpectedly, our analyses of a spectrum of Rac effector domain mutants failed to demonstrate that any one particular Rac-mediated event was essential for Rac transforming activity. One interpretation of these data is that Rac transformation is mediated by some as-yet-unidentified Rac function. Alternatively, it is possible that multiple Rac functions contribute to Rac transformation but that an impairment in any one may not cause a significant loss of transforming potential. Support for this second possibility is provided by studies evaluating the requirement for the Raf-MEK-MAPK signaling pathway in Ras transformation. The ability of dominant negative mutants of Raf, MEK, and MAPKs to block Ras transformation supports the important contribution of this kinase cascade to Ras transformation (30). Nevertheless, the fact that effector domain mutants of Ras that are impaired in the ability to bind to and activate Raf can still cause potent transformation argues that multiple Ras pathways contribute to Ras transformation (25, 55). Thus, Rac1 may also cause transformation by multiple mechanisms.

The ability of mutations in Rac1 amino-terminal sequences, which are strongly homologous (~70%) to the well-defined Ras effector domain (Ras residues 26 to 48), to impair Rac signaling and function has clearly established this region as an important domain for Rac effector interaction (12). In agreement with previous studies, we also observed that at least one amino acid substitution in this region impaired each Rac activity that we analyzed, although each mutation caused differential impairment of a particular function. Recent studies with chimeric proteins between Rac1 and RhoA also identified a second, carboxy-terminal region of Rac1 (residues 143 to 175) important for Rac effector function. Diekmann and colleagues showed that both amino- and carboxy-terminal regions of Rac1 were required for PAK and p67phox binding, as well as for Rac-induced lamellipodium formation (13). In agreement with their observations, we also observed that the Rac^{73}Rho chimera did not bind PAK or induce lamellipodia but instead induced stress fiber formation (Table 2). Furthermore, we observed a loss of JNK activation, suggesting that the effector required for JNK activation also requires interaction with carboxy-terminal Rac sequences. Interestingly, in their studies, they found that the Rho^{73}Rac chimeric protein lacked biological activity in microinjection analyses. Thus, they suggested that the amino-terminal Rac1 effector sequences cannot be replaced functionally by the Rho effector domain sequences. However, in the present study, we found that Rho^{73}Rac showed the ability to activate JNK and Jun and to induce lamellipodia. Rho^{73}Rac also activated cyclin D1 and was potently transforming in NIH 3T3 cells. Thus, our observations suggest that RhoA amino-terminal sequences can substitute for Rac1 amino-terminal effector sequences to promote Rac-specific functions.

In summary, we have demonstrated that Rac effector domain mutants, as well as chimeric proteins between Rac and Rho, provide important reagents to decipher the complex signaling pathways and activities regulated by Rac. Following the paradigm established for Ras, it has been anticipated that Rho family proteins also utilize multiple effectors to mediate their complex array of functions. Our studies suggest that Rac1 mediates at least four distinct effector pathways, one of which may involve PAK activation of cyclin D1. Further analyses of additional Rac effector mutants, coupled with the identification of additional Rac effector proteins and their functions, will be required to fully unravel the complex nature and interplay of signaling pathways that regulate the actions of Rac and other Rho family proteins.

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