

The Ras-related GTPase Rac1 Binds Tubulin*

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The Ras-related Rho family are involved in controlling actin-based changes in cell morphology. Microinjection of Rac1, RhoA, and Cdc42Hs into Swiss 3T3 cells induces pinocytosis and membrane ruffling, stress fiber formation, and filopodia formation, respectively. To identify target proteins involved in these signaling pathways cell extracts immobilized on nitrocellulose have been probed with [γ -³²P]GTP-labeled Rac1, RhoA, and Cdc42Hs. We have identified two 55-kDa brain proteins which bind Rac1 but not RhoA or Cdc42Hs. These 55-kDa proteins were abundant, had pI values of around 5.5, and could be purified by Q-Sepharose chromatography. The characteristics on two-dimensional gel analysis suggested the proteins comprised α - and β -tubulin. Indeed, β -tubulin specific antibodies detected one of the purified 55-kDa proteins. Rac1 bound pure tubulin (purified by cycles of polymerization and depolymerization) only in the GTP-bound state. The GTPase negative Rac1 point mutants, G12V and Q61L, did not significantly affect the ability of Rac1 to interact with tubulin while the “effector-site” mutant D38A prevented interaction. These results suggest that the Rac1-tubulin interaction may play a role in Rac1 function.

The Ras superfamily of small molecular weight GTP-binding proteins (p21s) are molecular switches that control a variety of cellular processes, including growth and proliferation, protein trafficking, and changes in morphology (Hall, 1990). p21 proteins possess intrinsic GTPase and GDP/GTP exchange activity which is affected by regulatory proteins such as *n*-chimaerin (GTPase activating protein, GAPs¹; Diekmann *et al.* (1991)) and DBL (GDP/GTP exchange factor; Hart *et al.* (1991)) and allows them to cycle between “on” (GTP-bound) and “off” (GDP-bound) states. Members of the Rho family, Rac1, RhoA, and Cdc42Hs are implicated in inducing morphological changes associated with actin polymerization (Hall, 1992). More specifically, Rac1 induces cortical actin polymerization seen as the process of membrane ruffling and lamellipodia formation (Ridley *et al.*, 1992), RhoA induces stress fiber formation possibly by catalyzing the formation of focal adhesions (Paterson *et al.*, 1990; Ridley and Hall, 1992), and Cdc42Hs induces the formation of peripheral actin microspikes and filopodia (Kozma *et al.*, 1995). Rac1 also activates pinocytosis (Ridley *et al.*, 1992) and superoxide formation by the neutrophil NADPH oxidase (Abo *et*

al., 1991). A common theme that runs through all these events is the requirement for Rho proteins to translocate between the cytosol and the membrane/cytoskeleton followed by the formation of protein complexes with specific functions.

Target proteins that act downstream in these p21 signaling pathways have been isolated. In mammalian cells, Raf (Moodie *et al.*, 1993; Van Aeist *et al.*, 1993; Votjek *et al.*, 1993; Zhang *et al.*, 1993; Warne *et al.*, 1993) and phosphatidylinositol 3'-kinase (Rodriguez-Viciano *et al.*, 1994) are two such proteins that act directly downstream of Ras in the mitogenic pathway. Cdc42Hs and Rac1-binding proteins, p120^{ack} (Manser *et al.*, 1993), p65^{pak} (Manser *et al.*, 1994), and p67^{phox} (Diekmann *et al.* 1994; Prigmore *et al.*, 1995), have been identified in brain and in neutrophils by probing cell extracts and recombinant proteins with [γ -³²P]GTP-labeled p21 proteins. p120^{ack} and p65^{pak} are protein kinases that may play a role in Rac1 and Cdc42Hs induced morphology changes while p67^{phox} is a component of the neutrophil NADPH oxidase.

In the present study, we were interested in isolating binding proteins specific for individual members of the Rho family. Brain cell extracts were separated on SDS-PAGE, transferred to nitrocellulose, and probed with [γ -³²P]GTP-labeled p21 proteins, Rac1, RhoA, and Cdc42Hs. Two 55-kDa proteins were identified as Rac1-specific binding proteins and purified by column chromatography. The biochemical properties of these proteins suggested that they may be α - and β -tubulin and this was confirmed by probing purified tubulin with Rac1 and using tubulin-specific antibodies. Rac1 interacted with purified tubulin only in the GTP-bound state and the Rac1 “effector site” mutation D38A prevented interaction. These results suggest that the Rac1-tubulin interaction may have physiological significance.

MATERIALS AND METHODS

Expression of p21s and Rac1 Mutants—The p21s, Rac1, Cdc42Hs, and RhoA, were purified as glutathione *S*-transferase fusion proteins from *Escherichia coli* on glutathione-Sepharose (Pharmacia), cleaved with thrombin, and stored at -70°C in aliquots with 15% glycerol (Ahmed *et al.*, 1993, 1994). Rac1, Cdc42Hs, and RhoA were greater than 95% pure as judged by Coomassie Blue staining on one-dimensional SDS-PAGE. Rac1 point mutants (Q61L, G12V, and D38A) were generated using the Clontech Transformer mutagenesis kit with Rac1 cloned in pBluescript (Stratagene) as a template. Once generated, mutant Rac1 cDNAs were subcloned into p265 (a pGEX-2T derivative with *Bam*HI, *Kpn*I, *Sma*I, *Hinc*II, *Spe*I, *Nsi*I, *Hind*III, and *Eco*RI sites in the polylinker) for expression in *E. coli*. Mutant cDNAs were sequenced to confirm encoded amino acid change. The following oligonucleotide primers were used for mutagenesis: Q61L, 5'-CATAATCTTCTAGTC-CAGCTGTAT-3'; G12V, 5'-TTTACCTACAGCTACGTCTCCAC-3'; and D38A, 5'-CAGAATAATTGGCAAAGACAGT-3'. The GTPase activity of mutants G12V and Q61L compared to wild-type was reduced as measured by the hydrolysis of Rac1-[γ -³²P]GTP using a nitrocellulose filtration assay (see GAP assay below for method). The % hydrolysis of Rac1-GTP and mutants over 10 min at 15°C , was 30% (G12), 5% (G12V), and no hydrolysis (Q61L). Cdc42Hs and RhoA were poor substrates for thrombin. Cleavage was significantly improved by subcloning Cdc42Hs and RhoA cDNAs into p265polyG (a p265 derivative with

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¹ The abbreviations used are: GAP, GTPase activating protein; MES, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PAK, serine/threonine protein kinases.

a polyglycine spacer immediately downstream of the thrombin cleavage site; Guan and Dixon (1991)). p265polyG was made by cloning an oligonucleotide cassette into the *Bam*HI/*Sma*I sites of p265. The oligonucleotides used were: 5'-GGGTAGCATGCGGATCCACCACCACCGGAAATTCCTCGGA-3' and 5'-GATCTCCGGGAATTTCCTGGTGTGGTGGATCCGCATGTACCC-3'. This cassette removes the original *Bam*HI site in the polylinker, creates a new *Bam*HI site for subcloning 3' of the polyglycine spacer, and also converts the *Kpn*I site in the polylinker to a new *Sph*I site for selection purposes.

Polymerase Chain Reaction Cloning of the GAP Domain of p190—The GAP domain of p190 was expressed using polymerase chain reaction of rat brain cDNA with the following oligonucleotides: 5'-GGGGATCTCTATTTTGGGGTGCTTTTAAACAACAGTGGTG-3' and 5'-GGGGAATTCAAGAAGACAACATGATTTCCTGCTTCCT-3'. The polymerase chain reaction product was digested with *Bam*HI and *Eco*RI, cloned into pBluescript for sequencing to confirm encoded amino acid sequence, and then subcloned into p265polyG for expression in *E. coli*. Amino acids expressed from 1246 to 1514 of p190 (Settleman *et al.*, 1992). p190 GAP^{1246–1514} domain was purified on glutathione-Sepharose as described previously (Ahmed *et al.*, 1994) and stimulated the GTPase activity of Rac1, RhoA, and Cdc42Hs.

Tissue Sample Preparation—Rat tissues were homogenized in 4 volumes of homogenization buffer (10 mM Tris, pH 7.4, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin). The homogenates were centrifuged at 100,000 *g* for 1 h, glycerol added (final concentration of 5%) to the supernatants, and aliquots stored at -70°C .

Detection of p21 Binding Proteins—Protein samples were mixed with equal volumes of sample buffer (1% SDS, 125 mM Tris, pH 6.8, 30% glycerol, 0.01% bromophenol blue, and 2% β -mercaptoethanol) and separated on 9% SDS-PAGE gels containing 8% glycerol, 1 mM dithiothreitol, and 0.5 mM MgCl_2 . Proteins were transferred onto nitrocellulose using a Bio-Rad semi-dry blotter in modified Bjerrum and Schafer-Nielsen buffer (48 mM Tris, 39 mM glycine, 10% methanol, 0.025% SDS, 0.5 mM MgCl_2). Blots were incubated in renaturation buffer (phosphate-buffered saline containing 3% bovine serum albumin, 0.1% Triton X-100, 0.5 mM MgCl_2 , 50 μM ZnCl_2 , and 5 mM dithiothreitol) for at least 2 h at 4°C before use. Blots were probed [γ - ^{32}P]GTP-labeled p21 proteins as described previously (Manser *et al.*, 1992), washed, and exposed to X-Omat film (Kodak) overnight at -70°C . Two-dimensional SDS-PAGE was carried out as described previously (Prigmore *et al.*, 1995).

GAP Assay—The assay was performed essentially as described by Ahmed *et al.* (1995). Briefly, 2 μg of Rac1 was loaded with 12 μCi of [γ - ^{32}P]GTP (30 $\mu\text{Ci}/\text{mmol}$; DuPont NEN) in 25 μl of exchange buffer for 10 min at 30°C . Exchange was stopped by the addition of MgCl_2 to 5 mM final concentration. 3 μl of loaded Rac1 mixture was diluted into 30 μl of incubation buffer in the presence or absence of the above additions and 5- μl aliquots removed at 0, 5, 10, and 15 min. The amount of Rac1-[γ - ^{32}P]GTP remaining was estimated by rapid filtration on nitrocellulose followed by 2×5 -ml washes with ice-cold buffer. Filters were air dried and then counts/min estimated by liquid scintillation counting in Beckman Ready Safe scintillant.

Purification of Rac1 Binding Protein—Rat brain cytosol, 1 ml (approximately 5 mg/ml), was loaded onto a Q-Sepharose column (Pharmacia). Proteins were eluted with 50 mM Tris, pH 7.5, with a step NaCl gradient from 0.1 to 0.5 M in 0.1 M increments. Fractions were dialyzed against 50 mM Tris, pH 7.5, overnight (2×2 liters) and proteins concentrated 5-fold with a Microcon unit (Amicon). Aliquots (30 μl) were electrophoresed on 9% SDS-PAGE gels and analyzed by silver staining or Rac1-[γ - ^{32}P]GTP binding.

Tubulin Purification—Tubulin was purified by the method of Shelanski *et al.* (1973). Rat brains were homogenized in an equal volume of assembly buffer (0.1 M MES, pH 6.5, 1 mM EGTA, 0.5 mM MgCl_2 , 1 mM GTP, 2 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ each of aprotinin, leupeptin, and pepstatin A). The homogenate was centrifuged at $100,000 \times g$ for 1 h at 4°C and the supernatant was mixed with an equal volume of 8 M glycerol in assembly buffer. Tubulin was polymerized at 37°C for 30 min, pelleted at $100,000 \times g$ at 25°C for 1 h, and resuspended in assembly buffer with the aid of a ground glass homogenizer. Microtubules were depolymerized on ice for 30 min and insoluble material pelleted at 4°C . The supernatant was mixed with an equal volume of 8 M glycerol in assembly buffer and tubulin repolymerized at 37°C for 30 min. Microtubules were re-pelleted, resuspended in PEM buffer (100 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM MgCl_2) and de-polymerized by incubation on ice for 30 min. Tubulin was further purified on a P11-cellulose phosphate column equilibrated with PEM buffer. The tubulin, which eluted in the void volume, was aliquoted and stored at -20°C . Microtubule-associated proteins were

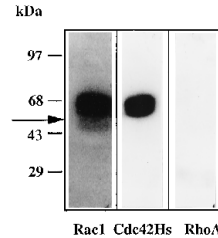


FIG. 1. Detection of p21-[γ - ^{32}P]GTP-binding proteins from rat brain cytosol. The filters were probed with: lane 1, Rac1; lane 2, Cdc42Hs; lane 3, RhoA. Arrow corresponds to 55-kDa Rac1-specific binding proteins. Aliquots containing 100 μg of protein from rat brain extracts were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with p21 proteins labeled with [γ - ^{32}P]GTP as described under "Materials and Methods."

eluted with 0.8 M NaCl in PEM buffer, dialyzed against PEM buffer, and stored at -70°C .

Proteolysis of Tubulin—Chymotrypsin (Sigma) was stored at 1 mg/ml in 10 mM sodium phosphate, pH 7.0, at -20°C . For limited proteolysis 50 μg of tubulin was incubated in PMG buffer (10 mM sodium phosphate, pH 7.0, 0.1 mM GTP, and 0.5 mM MgCl_2) for 20 min at 25°C with 0.5 μg of chymotrypsin (Serrano *et al.*, 1986). Phenylmethylsulfonyl fluoride was then added to the samples to a final concentration of 2 mM with an equal volume of sample buffer.

Tubulin Polymerization—Polymerization was followed by examining OD_{350} as a function of time at 37°C in a dual beam spectrophotometer (Pye-Unicam Sp8-100). Tubulin (36 mg; 0.5 mg/ml) was incubated at 4°C in 46 ml of PEM buffer with or without Rac1 (5 μg ; 1 mg/ml) and then preincubated at 37°C . To initiate polymerization, Taxol was added to 10 mM final concentration and optical density followed for 20 min.

Cell Culture and Immunostaining—Swiss 3T3 cells were grown and immunostained as described previously (Kozma *et al.*, 1995). Anti-Rac1 and anti-Cdc42Hs antibodies were purchased from Santa Cruz Biotechnology Inc.

RESULTS

Rac1 Binds to two 55-kDa Proteins in Brain Cell Extracts—We have previously detected Cdc42Hs/Rac1-binding proteins of around 62–68 kDa in brain (Manser *et al.*, 1992) and neutrophil cell extracts (Prigmore *et al.*, 1995) using a "Western-type" analysis by probing nitrocellulose immobilized proteins with p21-[γ - ^{32}P]GTP. These 62–68-kDa proteins comprise a family of serine/threonine protein kinases (PAKs) that are activated by Cdc42Hs and Rac1 and therefore potential targets for these p21s (Manser *et al.*, 1994). The 62–68-kDa PAKs bind Cdc42Hs and Rac1 with high affinity and give strong signals with PAK amounts as low as 100 ng (Prigmore *et al.*, 1995). With Rac1-[γ - ^{32}P]GTP as probe a 55-kDa signal is detected but sometimes masked by the signal from the PAK family of proteins (see arrow in Fig. 1). This 55-kDa signal can be resolved into two distinct bands and is not seen when cell extracts from testes, spleen, muscle, lung, liver, kidney, or heart are probed with Rac1 (data not shown). Cdc42Hs-[γ - ^{32}P]GTP and RhoA-[γ - ^{32}P]GTP probes do not detect these 55-kDa proteins (Fig. 1). These results suggest that the 55-kDa proteins may be degradation products of the PAKs or a brain-enriched Rac1-specific binding protein.

Purification of the 55-kDa Proteins—Rat brain cytosol was fractionated on Q-Sepharose as a first step in the purification of the 55-kDa proteins. As previously reported the p65^{pak} eluted at 0.2–0.3 M NaCl (Manser *et al.*, 1994). The 55-kDa proteins eluted at 0.5 M NaCl with >95% purity (Fig. 2, lane 7). No other Rac1-binding proteins were detected in this fractionation. The 55-kDa proteins were abundant (at least 3% of total protein), but the amount of Rac1 binding was significantly less than that obtained with an equivalent amount of the recombinant p65^{pak}. Thus it is unlikely that the 55-kDa proteins are breakdown products of the PAKs.

The 55-kDa Proteins Are α - and β -Tubulin—To establish the

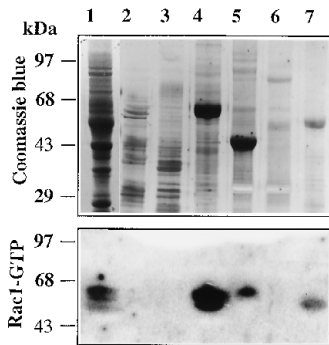


FIG. 2. **Purification of the 55-kDa Rac1-binding proteins.** Lane 1, rat brain cytosol. Lane 2, flow-through. Lanes 3–7, 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl elutions, respectively. Proteins from rat brain cytosol were loaded onto a Q-Sepharose column and elution carried out with a step gradient of NaCl. Fractions were separated by SDS-PAGE and analyzed by silver staining and Rac1-[γ - 32 P]GTP binding as described under "Materials and Methods."

identity of the 55-kDa proteins, a two-dimensional SDS-PAGE analysis was carried out with rat brain cytosol and the purified 55-kDa proteins (Fig. 3). The major Rac1 binding activity seen on two-dimensional SDS-PAGE, as with one-dimensional SDS-PAGE, were the PAKs which streak across a range of pI values. With lower exposure two main PAK spots are seen with basic pI values (Prigmore *et al.*, 1995). The 55-kDa proteins migrated as two overlapping spots with pI values of around 5.5 and both spots appeared to bind Rac1 (the spots seen on Coomassie Blue staining of the 55-kDa protein co-migrated with the spots seen in the Rac1-binding analysis). The high salt elution on Q-Sepharose, abundance, and pI values of the 55-kDa proteins (as seen on two-dimensional SDS-PAGE) suggested that they may be α - and β -tubulin. To address this possibility the 55-kDa proteins were probed with β -tubulin specific antibodies. Fig. 3 (last two panels) shows that the lower of the two spots did indeed react with β -tubulin antibodies. Further evidence that the 55-kDa proteins were tubulin was obtained by examining Rac1 binding to tubulin purified by cycles of polymerization and depolymerization (Shelanski *et al.*, 1973). Tubulin present in rat brain cytosol was pelleted after glycerol induced polymerization, depolymerized by incubation on ice, repolymerized, and pelleted. Fig. 4 shows the one-dimensional SDS-PAGE Coomassie Blue and Rac1-binding analysis of this experiment. During the purification of tubulin the PAK-binding proteins are eliminated. The remaining Rac1 binding activity and tubulin co-purify. We also probed samples from the Q-Sepharose purification (Fig. 2) with actin and β -tubulin specific antibodies. Again Rac1 binding activity and tubulin co-purified while actin (a 45-kDa band) was eluted at 0.3 M NaCl (data not shown) and did not bind Rac1.

Rac1 Binds Tubulin Isolated from Liver and Testes—The data presented above show that Rac1 binds to tubulin present in rat brain and that 55-kDa signals are not seen from a range of other tissues. This observation suggested either that there was too little tubulin in these tissue cell extracts for detection (at least 2 μ g of protein is required for detection, see Fig. 5) or that the brain tubulin was unique in its ability to bind Rac1. To address these possibilities tubulin was purified from liver and testes using Q-Sepharose chromatography and probed with Rac1. Tubulin prepared from both liver and testes possessed Rac1 binding activity (Fig. 5) which is therefore not specific to brain. Tubulin purified from spleen also binds Rac1 (not shown). Rac1 binding to tubulin is dependent on the amount of tubulin immobilized on nitrocellulose (2–20 mg; Fig. 5). The strength of Rac1 binding to tubulin was quantified by scintillation counting and the (cpm/mg protein) ratio found to be

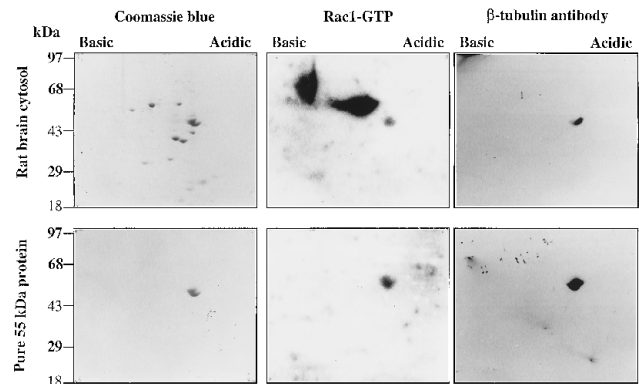


FIG. 3. **Two-dimensional SDS-PAGE analysis of the 55-kDa Rac1-binding proteins.** 25 μ g of rat brain cytosol protein or 5 μ g of the purified 55-kDa proteins were resolved by two-dimensional SDS-PAGE as described under "Materials and Methods." Gels were analyzed by Coomassie Blue staining (left panels), Rac1-[γ - 32 P]GTP binding (middle panels), and immunoblotting with β -tubulin specific antibodies (right panels) as indicated on the figure. Isoelectric focusing (left to right) and molecular weight separation (top to bottom).

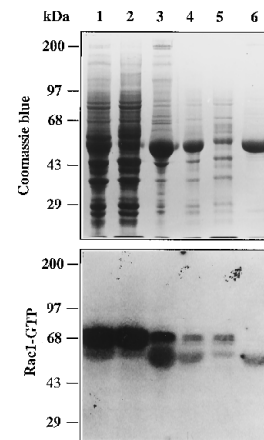


FIG. 4. **Rac1 binds to tubulin purified by the Shelanski method.** Lane 1, rat brain cytosol. Lanes 2 and 3, supernatant and pellet from first polymerization. Lanes 4 and 5, pellet and supernatant from second polymerization. Lane 6, pellet from third polymerization. Coomassie Blue staining (top) and Rac1-[γ - 32 P]GTP probed (bottom). Tubulin was purified by 3 cycles of polymerization and depolymerization (Shelanski *et al.*, 1973), see "Materials and Methods" for details and samples analyzed.

similar to ratios obtained with Rac1 binding to the neutrophil NADPH oxidase component p67^{phox} (Prigmore *et al.*, 1995).

Specificity of Rac1 Binding to Tubulin—Initially, the nucleotide preference of Rac1 (Rac1-GTP versus Rac1-GDP) for binding to tubulin was examined (Fig. 6A, lanes 2, 4, 6, and 8) with rat brain cytosol, which contains the major Rac1-binding PAK proteins, as control (Fig. 6A, lanes 1, 3, 5, and 7). The PAK signals had to be overexposed to allow clear visualization of the tubulin signals. Rac1 was loaded with GTP labeled either at the γ or α positions and used as a probe in binding assays. Rac1-[γ - 32 P]GTP (Fig. 6A, lanes 1 and 2) and Rac1-[α - 32 P]GTP (Fig. 6A, lanes 5 and 6) probes were used immediately or after 60 min incubation of these probes at room temperature (Fig. 6A, lanes 3, 4 and 7, 8, respectively). The high intrinsic GTPase activity of Rac1 removes the γ -phosphate group leaving Rac1 bound to GDP after 60 min. In the case of Rac1-[α - 32 P]GTP this generates a labeled Rac1 probe (Rac1-[α - 32 P]GDP). Fig. 6A shows that the Rac1 interaction with brain PAKs (as previously reported; Manser *et al.* (1994)) and purified tubulin is dependent on Rac1 being in the GTP bound state. As with tubulin present in brain extracts (Fig. 1), purified tubulin did not

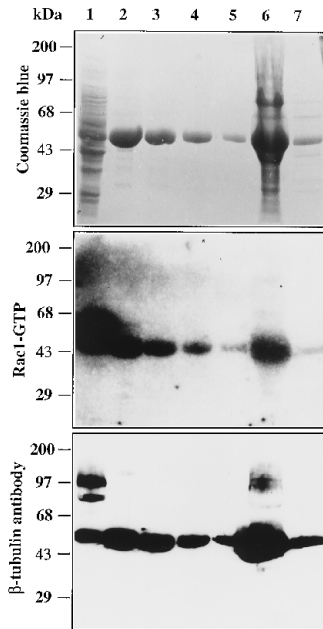


FIG. 5. **Rac1 binds tubulin isolated from liver and testes.** Lane 1, rat brain cytosol. Lanes 2–5, 20, 10, 5, and 2 μ g of the 55-kDa purified proteins from rat brain cytosol. Lane 6, liver proteins eluted in 0.5 M NaCl fraction. Lane 7, testes proteins eluted in 0.5 M NaCl fractions. Soluble extracts from rat tissues were loaded onto Q-Sepharose columns and eluted with a step gradient of NaCl. The 0.5 M NaCl fractions were separated by SDS-PAGE and analyzed by Coomassie Blue staining (top panel), Rac1- $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ binding (middle panel), and β -tubulin antibody (bottom panel).

interact with Cdc42Hs or RhoA (data not shown).

The Ras point mutations, G12V, Q61L, and D38A, have been used to determine specificity of interaction with potential targets. The G12V and Q61L mutants are GTPase negative and therefore trapped in the on state while D38A does not interact with Ras effectors such as Raf (Warne *et al.*, 1993). Similarly, Rac1-G12V and Rac1-Q61L are GTPase negative (see “Materials and Methods”) and Rac1-D38A does not induce membrane ruffling² (Xu *et al.*, 1994) or activate neutrophil oxidase (Xu *et al.*, 1994; Diekmann *et al.*, 1994). These Rac1 proteins were loaded with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and used to probe both rat brain cytosol and purified tubulin (Fig. 6B). The signal for tubulin was not significantly affected by the GTPase negative mutations but was eliminated by the D38A mutation. Interestingly, the signal generated in rat brain cytosol from the PAK proteins was not eliminated but rather enhanced by the D38A mutation.

To determine the region binding Rac1 we subjected tubulin to limited proteolysis with chymotrypsin. This protease cleaves β -tubulin specifically (leaving α -tubulin intact) generating two polypeptides of 30 and 16 kDa (N-terminal and C-terminal, respectively). Rac1 was found to bind to the intact α -tubulin and the N-terminal polypeptide of β -tubulin but not its C-terminal (Fig. 6C).

Rac1 Localization in Swiss 3T3 Cells—To examine whether Rac1 interacted with tubulin polymers/microtubules Swiss 3T3 cells were double stained *in situ* with either anti-Rac1 or anti-Cdc42Hs antibodies and anti- β -tubulin antibodies. There was a colocalization of Rac1 with microtubules (Fig. 7). Anti-Cdc42Hs antibodies strongly stained the cell periphery and filopodia but did not stain microtubules (data not shown). These results support the data with the Rac1 overlay assay and suggest that Rac1 interacts with tubulin *in vivo*.

Effect of Tubulin on Rac1 GTPase Activity and Rac1-p190

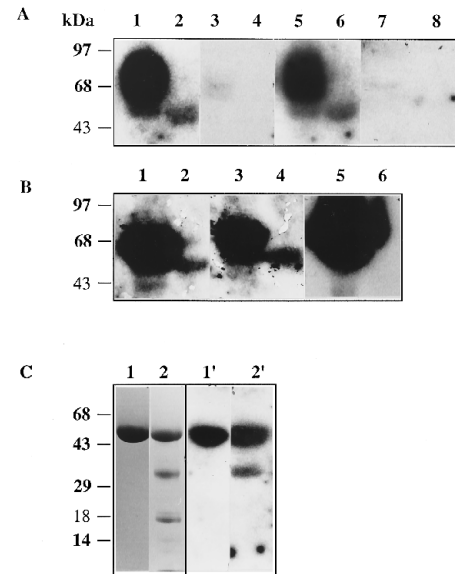


FIG. 6. **Specificity of Rac1 binding to rat brain cytosol and purified tubulin.** A, 100 μ g of rat brain cytosol protein (lanes 1, 3, 5, and 7) and 20 μ g of purified rat brain tubulin (lanes 2, 4, 6, and 8) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with Rac1-GTP or GDP. Probes used were as follow: lanes 1 and 2, Rac1- $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. Lanes 3 and 4, Rac1-GDP (produced by allowing $\gamma\text{-}^{32}\text{P}$ group to be hydrolyzed). Lanes 5 and 6, Rac1- $[\alpha\text{-}^{32}\text{P}]\text{GTP}$. Lanes 7 and 8, Rac1- $[\alpha\text{-}^{32}\text{P}]\text{GDP}$. B, 100 μ g of rat brain cytosol protein (lanes 1, 3, and 5) and 20 μ g of purified rat brain tubulin (lanes 2, 4, and 6) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with Rac1 point mutants. Lanes 1 and 2, Rac1-G12V. Lanes 3 and 4, Rac1-Q61L. Lanes 5 and 6, Rac1-D38A. C, 50 μ g of tubulin (lanes 1 and 1') and tubulin digested with chymotrypsin at 25 $^{\circ}\text{C}$ for 20 min (lanes 2 and 2'). The polypeptides were separated on 12% SDS-PAGE and analyzed by Coomassie Blue staining (lanes 1 and 2) and Rac1- $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ binding (lanes 1' and 2').

Interaction—p65^{pak} proteins prevent GAPs such as Bcr from interacting with Rac1 suggesting that these two proteins cannot bind to Rac1 at the same time. Furthermore, they inhibit Rac1-GTPase activity. To investigate whether tubulin could interfere with GAPs interacting with Rac1 or its GTPase activity we assayed the GTPase activity of Rac1 in the presence of tubulin and/or the Ras-GAP-binding protein p190 GAP domain (Fig. 8). The p190 GAP is active on at least three members of the Rho family: RhoA, Cdc42Hs, and Rac1. The intrinsic Rac1 GTPase activity is high under the conditions of the assay with a half-life for Rac1-GTP of 10 min (Fig. 8). Tubulin did not affect the intrinsic Rac1-GTPase activity or the ability of p190 GAP domain to stimulate its GTPase activity.

Function of the Rac1-Tubulin Interaction—The biochemical characteristics of the Rac1-tubulin interaction resemble those of the Ras-Raf (Warne *et al.*, 1993) and Ras-phosphatidylinositol kinase (Rodriguez-Viciana *et al.*, 1994) interactions, suggesting that tubulin is a target for Rac1. What then is the physiological function of the Rac1-tubulin interaction? One possibility is that Rac1 affects the ability of tubulin to polymerize. To examine this an *in vitro* polymerization assay was used. Addition of taxol to tubulin induces an increase OD₃₅₀ caused by formation of microtubules. Fig. 9 shows that Rac1-GTP did not induce tubulin polymerization by itself or affect Taxol-induced tubulin polymerization.

Rac1 is an activator of membrane ruffling and pinocytosis in Swiss 3T3 cells (Ridley *et al.*, 1992). Translocation of Rac1 between the membrane/cytoskeleton and cytosol is likely to be required for Rac1 to activate these events. It is therefore possible that the function of the Rac1-tubulin interaction may be to allow Rac1 to use the microtubule network for the process of translocation. To examine this possibility we treated Swiss 3T3

² R. Kozma, A. Best, and S. Ahmed, unpublished data.

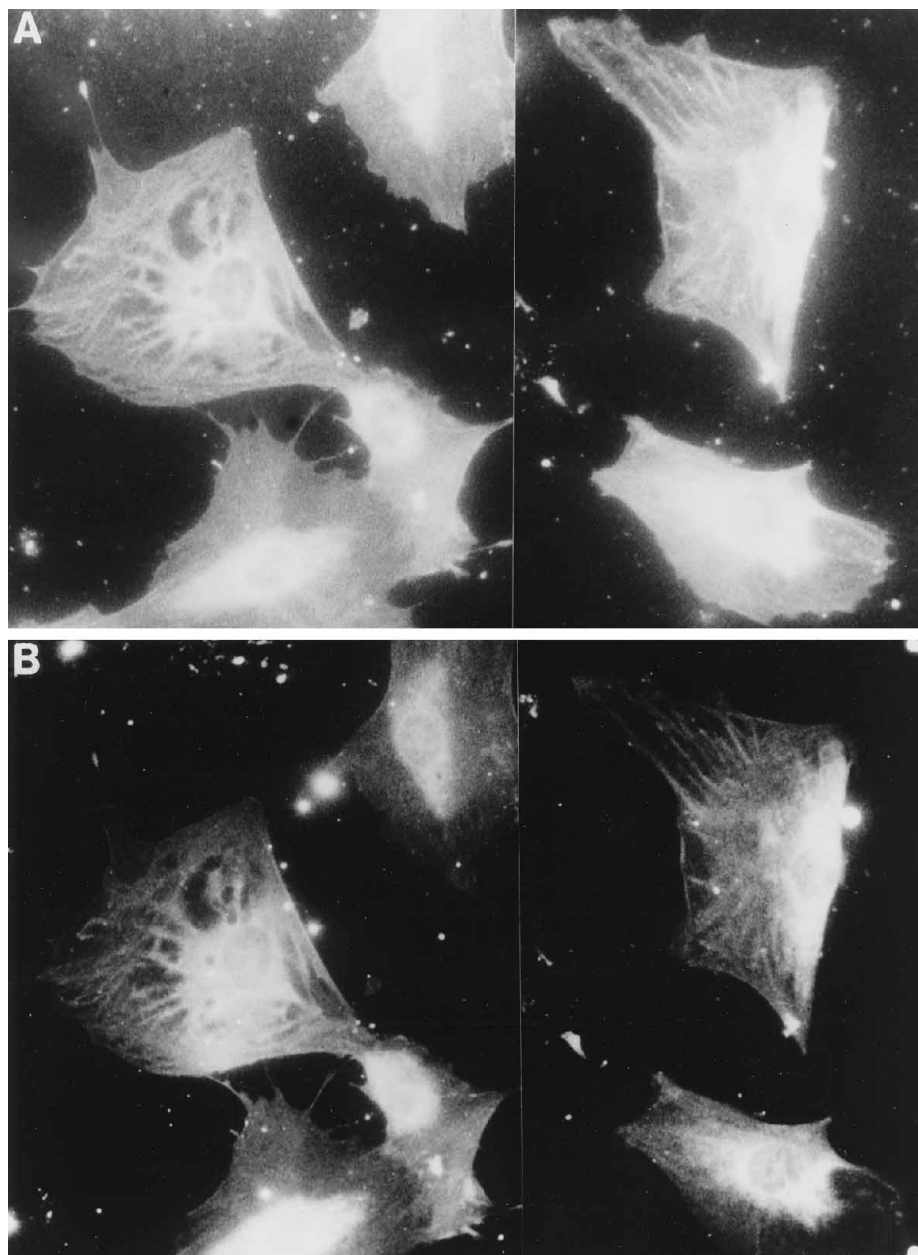


FIG. 7. **Localization of Rac1 in Swiss 3T3 cells.** Swiss 3T3 cells were stained with either anti- β -tubulin antibodies (A) or anti-Rac1 antibodies (B) as described under "Materials and Methods."

cells with colchicine to disrupt the microtubule network and then monitored pinocytosis by lucifer yellow uptake and membrane ruffling by phalloidin staining of actin. In serum starved control cells (without colchicine treatment) fetal calf serum stimulated pinocytosis seen by diffuse cytosolic lucifer yellow staining. Interestingly, lucifer yellow uptake in cells treated with colchicine was very different. Almost all cells contained intense particulate staining throughout the cytoplasm (data not shown). Furthermore, colchicine-treated cells did not undergo membrane ruffling on stimulation with fetal calf serum, platelet-derived growth factor, or phorbol myristyl acetate.³

DISCUSSION

Growth factors activate Rac1, RhoA, and Cdc42Hs to induce specific changes in the actin-based cell morphology of mammalian cells. Platelet-derived growth factor induces Rac1-mediated membrane ruffling and lamellipodia formation (Ridley *et al.*, 1992), lysophosphatidic acid induces RhoA-mediated stress fiber formation (Ridley and Hall, 1992) and bradykinin induces

Cdc42Hs-mediated peripheral actin microspike formation and filopodia formation (Kozma *et al.*, 1995). The mechanism(s) by which these factor-induced changes in morphology involving Rho family proteins occurs is unclear. As one step in elucidation of the p21 mechanism of action, proteins have been isolated that interact directly with Rac1 and Cdc42Hs and therefore may be components of these morphological pathways. p120^{ack} and p65^{pak} are protein kinases that bind to Cdc42Hs and Rac1/Cdc42Hs, respectively (Manser *et al.*, 1993, 1994). Rac1 and Cdc42Hs also bind to p67^{phox} (Diekmann *et al.*, 1994; Prigmore *et al.*, 1995).

In the present study we have identified two 55-kDa proteins from brain that bind Rac1 but not RhoA or Cdc42Hs. These are the first Rac1-specific binding proteins to be isolated. The 55-kDa proteins were shown to be tubulin by using β -tubulin-specific antibodies to probe purified protein on two-dimensional SDS-PAGE. Rac1 bound α - and β -tubulin, tubulin purified from brain, liver, testes, and spleen, and tubulin purified by cycles of polymerization and depolymerization. The characteristics of this binding were as follows: (i) Rac1 bound tubulin

³ A. Best, S. Ahmed, and R. Kozma, unpublished data.

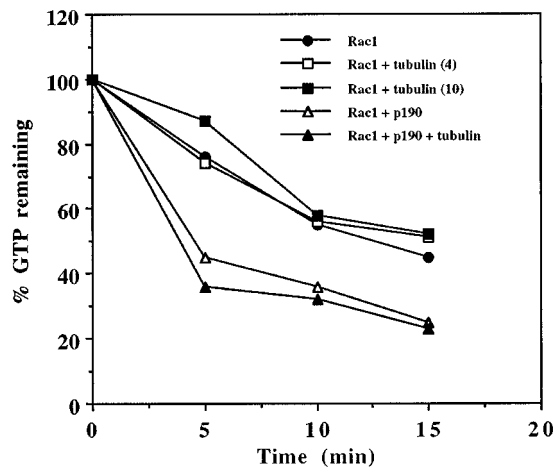


FIG. 8. Effect of tubulin on the Rac1-p190 interaction. Rac1-GTPase activity, with no additions (●), with 4 μ g of tubulin (□), and with 10 μ g of tubulin (■). Rac1-GTPase activity with 25 ng of p190 GAP domain in the absence (△) or presence of 4 μ g of tubulin (▲). Results are expressed as a % of bound GTP (cpm) present at zero time. Rac1 was loaded [γ - 32 P]GTP as described under "Materials and Methods" and GTP hydrolysis followed by filtration on nitrocellulose. Similar results were obtained in one other experiment.

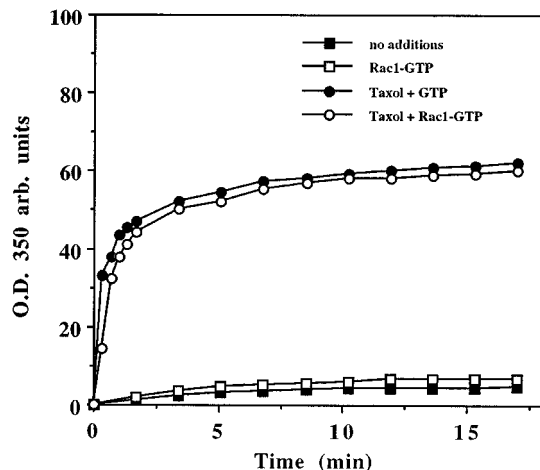


FIG. 9. Effect of Rac1 on tubulin polymerization. Tubulin (approximately 36 μ g), with no additions (■), with 5 μ g of Rac1 (●), with 10 μ M taxol with GTP (○) and 10 μ M taxol with 5 μ g of Rac1-GTP (□). Tubulin polymerization was induced with taxol and the optical density followed as described under "Materials and Methods."

only in its GTP-bound form, (ii) the strength of Rac1 binding to tubulin (cpm/mg protein) was similar to that seen with Rac1 binding to the NADPH oxidase component p67^{phox}, (iii) the GTPase negative mutations G12V or Q61L did not significantly affect binding while the effector site mutation D38A eliminated binding, and (iv) Rac1 bound to the N-terminal (30 kDa) of β -tubulin. Furthermore, anti-Rac1 antibodies stained microtubules indicating that Rac1 can interact with both tubulin monomers and polymers. Taken together, these results show that the interaction between Rac1 and tubulin is specific and that it may have physiological relevance.

Rac1-D38A does not induce membrane ruffling (Xu *et al.*, 1994) or activate the neutrophil oxidase (Diekmann *et al.*, 1994; Xu *et al.*, 1994) and Cdc42Hs-D38A does not induce peripheral actin microspike formation, however, both D38A mutants bound the PAK proteins from cell extracts and recombinant p65^{pak}.⁴ This result makes it unlikely that the PAK proteins are the sole downstream targets of Rac1 and Cdc42Hs respon-

sible for the changes in actin-based cell morphology.

Tubulin does not contain any regions of obvious sequence identity to the known Rac1 binding proteins: p65^{pak}, p67^{phox}, the Bcr family of GAPs, or the Rho-GDP dissociation inhibitor, suggesting the existence of a unique binding domain. Unlike the other Rac1 interacting proteins tubulin did not affect the GTPase activity of Rac1 or compete with GAP proteins (p190 was used here) for interaction with Rac1. This implies that p190 and tubulin can bind to Rac1 simultaneously and this possibility is supported by the fact that the D38A mutation, while eliminating tubulin binding, does not affect the ability of Rac to act as a substrate for p190 GAP domain (Xu *et al.*, 1994). However, we cannot rule out the possibility that the binding constants for interaction with Rac1 of tubulin and p190 GAP domain are such that competition would be difficult to observe. In preliminary experiments, the K_d of Rac1 binding tubulin was found to be in the micromolar range making the latter possibility unlikely. Further work will be necessary to resolve this issue.

What are the physiological implications of Rac1 binding to tubulin? Rac1 bound to the N-terminal 30 kDa of tubulin while proteins such as tau and the microtubule-associated proteins, which stimulate tubulin polymerization, bind to the C-terminal. It is therefore unlikely that the role of the Rac1-tubulin interaction is to affect tubulin polymerization (microtubule formation). Indeed, Rac1 did not influence taxol induced polymerization. The growth factor mediated induction of membrane ruffling and pinocytosis in Swiss 3T3 cells is rapid and probably requires Rac1 to translocate between cytosol and plasma membrane (Ridley *et al.*, 1992). Additionally, stimulation of neutrophils leads to a rapid translocation of Rac (Benna *et al.*, 1994) from the cytosol to the membrane and cytoskeleton where components (p67^{phox}, p47^{phox}, p40^{phox}, and cytochrome b) of the NADPH oxidase form a complex which is capable of generating superoxide. Thus, cellular translocation is likely to be a key event in Rac1 function. It is therefore possible that tubulin binding allows Rac1 to use the microtubule network for translocation. This possibility was tested by examining the effect of colchicine (and nocadazole) on pinocytosis and membrane ruffling. Disruption of the microtubule network with these drugs affected factor induced pinocytosis and inhibited membrane ruffling.³ Two recent studies also support a role for microtubules in Rac1 function. Gloushankova *et al.* (1994) have observed that lamellipodia formation induced by transfection of N-ras, possibly via activation of Rac1 (Ridley *et al.*, 1992), in IAR epitheliocytes is inhibited by the microtubule network disrupting agents taxol and colcemid. Tanaka *et al.* (1995) have found that disruption of the microtubule network of neural growth cones with vinblastine leads to a loss of lamellipodia and ruffled edges while leaving filopodia intact. On washing out vinblastine from these neurons lamellipodia and ruffled edges re-emerge. Further work examining cellular movement of Rac1 upon factor stimulation in the presence/absence of colchicine is needed to establish whether microtubules are required for Rac1 function.

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