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Rheb Interacts with Raf-1 Kinase and May Function To Integrate Growth Factor- and Protein Kinase A-Dependent Signals

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Rheb is a recently described member of the Ras family that was originally identified as an immediate-early gene in brain but is also widely expressed in other tissues. Here we demonstrate that Rheb interacts with and appears to regulate Raf-1 kinase, an essential component of the H-Ras signaling pathway. In direct contrast to H-Ras, however, the interaction of Rheb with Raf-1 is potentiated by growth factors in combination with agents that increase cyclic AMP (cAMP) levels. Protein kinase A-dependent phosphorylation of serine 43 within the regulatory domain of Raf-1 reciprocally potentiates its interaction with Rheb and decreases its interaction with H-Ras. A single amino acid in the G2 effector domain is critical for the differential properties of Rheb. Since Rheb is an immediate-early gene, our studies suggest that Rheb functions in concert with H-Ras to dynamically integrate cAMP and growth factor signaling.

Several lines of evidence indicate that establishment of long-term neuronal plasticity is dependent on mRNA and protein synthesis that is rapidly induced during a brief time window following stimulation (23). To further understand the molecular processes that contribute to neuronal plasticity, we have used differential cloning techniques to identify molecules rapidly induced in neurons by excitatory transmitter stimulation. The identified genes include those encoding immediate-early gene transcription factors (1, 11, 45, 54), growth factors (2, 8, 30, 50, 63), secreted enzymes such as tissue plasminogen synthase that can modify the extracellular matrix (52), and enzymes involved in intracellular signaling, such as prostaglandin synthase (72). One of the novel cDNAs identified in our screen encodes a member of the Ras family of small GTP-binding proteins, termed Rheb (Ras homolog enriched in brain) (73). Rheb mRNA is rapidly induced in hippocampal granule neurons by *N*-methyl-D-aspartate-dependent synaptic activity and is highly expressed in the developing and adult cortex. Rheb mRNA is also rapidly induced in cultured pheochromocytoma (PC12) cells by epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). While Rheb is enriched in the central nervous system, it is also widely expressed in peripheral tissues, including muscle, lung, intestine, and cultured fibroblasts.

The deduced amino acid sequence of Rheb permitted several predictions regarding its function. Rheb is most similar to human Rap 2, yeast RAS1, and human H-Ras. It is identical to H-Ras in six of nine amino acids in the G2 effector domain, which is critical for downstream signaling activity (73). Rheb also encodes a CAAX box motif that predicts posttranslational farnesylation and membrane targeting similar to H-Ras (26, 35). Consistent with these homologies, we report in the present study that Rheb shares many biochemical and signaling properties with H-Ras.

H-Ras plays an essential role in the transduction of growth factor signals. Growth factors activate receptor tyrosine kinases, leading to the sequential binding of Grb2 and the guanine nucleotide exchange factor mSOS, which in turn converts H-Ras to its active, GTP-bound state. Active H-Ras binds the amino terminus of Raf-1 serine/threonine kinase and, by virtue of this physical interaction, recruits Raf-1 to the plasma membrane (39, 59), where Raf-1 is activated by tyrosine and/or serine phosphorylation (14). Additional membrane-associated kinases and cofactors have been implicated in the activation of Raf-1 (70). Recent studies indicate that activation of Raf-1 involves a multiprotein complex of membrane-associated proteins, possibly including 14-3-3 proteins (19, 29, 59). H-Ras also binds the closely related Raf family member B-Raf (43), which is enriched in neural tissues. Upon membrane recruitment and activation, Raf-1 and B-Raf phosphorylate and activate MEK1 (28, 32) and trigger a kinase cascade, leading to phosphorylation and activation of mitogen-activated protein (MAP) kinase and downstream transcription factors. Mutations in the G1 domain which increase the level of H-Ras in the GTP state also increase Raf-1-dependent signaling and are oncogenic in fibroblasts (74), while mutations in the G2 effector domain which inhibit the H-Ras–Raf-1 interaction reduce H-Ras oncogenicity (10, 57, 74). In contrast to the proliferative effects mediated by oncogenic H-Ras in fibroblasts, expression of constitutively active H-Ras results in neuronal differentiation and neurite outgrowth in PC12 cells (6, 49). This effect is again linked to the ability of H-Ras to bind Raf-1 (57). These proliferative and differentiative responses have become important bioassays of Ras-dependent signaling.

The interaction between H-Ras and Raf-1 represents a regulated nodal point for cross talk between growth factor- and cyclic AMP (cAMP)-dependent signaling cascades. Treatment of fibroblasts with growth factors has been shown to activate Raf-1 and MAP kinase signaling (12, 71), while pretreatment of cells with agents that increase the intracellular cAMP level blocks this growth factor-mediated kinase activation (12, 37, 56, 71). Phosphorylation of Raf-1 by protein kinase A (PKA) has been shown to inhibit its binding to H-Ras (10, 71) and thereby effectively interfere with Raf-1 activation (25) and subsequent downstream kinase activation (67).

In the present study, we report that Rheb interacts with Raf-1 kinase. Like H-Ras, the affinity of the Rheb–Raf-1 in-

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teraction is regulated by growth factors and second messengers. In marked contrast to H-Ras, however, binding of Raf-1 to Rheb is potentiated by agents that raise intracellular cAMP levels. The novel Raf-1 binding properties of Rheb appear relevant to cellular function, since Rheb acts in synergy with Raf-1 in transforming the growth properties of NIH 3T3 fibroblasts and Rheb potentiates neurite outgrowth of PC12 cells selectively under conditions of combined growth factor and cAMP stimulation. These studies suggest that Rheb mediates a novel, H-Ras-divergent signaling pathway.

MATERIALS AND METHODS

Yeast two-hybrid system. Wild-type Rheb was amplified by using PCR primers to create a *Bam*HI restriction site 5′ of the start Met in frame with the GAL4 transcription activation domain of pGADGH and a *Eco*RI site 3′ of the truncated Rheb carboxy terminus, which lacked the CAAX box to ensure nuclear localization in the yeast. The final construct was verified by sequencing the entire reading frame. Wild-type Raf-1 kinase, RafΔN1 (lacking residues 26 to 302), RafΔN2 (lacking residues 1 to 375), and RafΔC1 (lacking the carboxy terminus from residues 380 to 648) were previously subcloned in frame with the GAL4 DNA binding domain in pGBT9 (66). The Hf7c strain of *Saccharomyces cerevisiae* (*ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3 URA3*::*GAL4*) was transformed with pGADGH-Rheb by the lithium acetate method (3). Transformants were grown on solid media lacking Leu, and isolated colonies were selected for further transformation with pGBT9-Raf, pGBT9-RafΔN1, pGBT9-RafΔN2, or pGBT9-RafΔC2. Double transformants were grown on media lacking Leu and Trp. pGADGH- and pGBT9-interacting inserts grow on media lacking His. Isolated colonies were streaked onto media lacking Leu, Trp, and His to identify transformants expressing interacting proteins. Yeast interactions were further confirmed by β-galactosidase assays. β-Galactosidase filter assays were performed as described previously (9).

Cell culture. COS and HEK 293 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin–streptomycin (GIBCO-BRL). NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (GIBCO-BRL) and 1% penicillin–streptomycin. PC12 cells (passages 5 to 13, originally obtained from Lloyd Greene) were maintained in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% heat-inactivated horse serum (Hyclone), 5% fetal bovine serum, and 1% penicillin–streptomycin. All cells were maintained in an atmosphere of 5% CO_2 at 37°C.

Focus formation assays. A subline of NIH 3T3 fibroblasts, which had been previously selected for low spontaneous transformation under maintenance medium conditions, was split 1:6 from an 80% confluent plate into 10-cm dishes 16 h prior to transfection. Full-length wild-type Rheb under cytomegalovirus promoter control (pMT2-Rheb), wild-type Raf-1 under Rous sarcoma virus promoter control (pBSK-RSPA-cRaf), and H-Ras under H-Ras promoter control (3625N) expression constructs were transfected with 5 μg/plate, and the G418 resistance vector pMT-neo was transfected with 2 μg/plate. Cells were transfected by the calcium phosphate method (3) for 8 h, rinsed, trypsinized, and split 1:6 into 10-cm dishes. They were allowed to recover in maintenance medium overnight and then were rinsed and refed with maintenance medium containing 500 μg of G418 per ml. They were fed every 3 days with G418 medium, and foci were scored 18 days posttransfection.

Focus formation was quantitated based on the following criteria: (i) identification of colonies visible to the unaided eye which (ii) also exhibited a dense, multilayered phenotype consistent with the reported appearance of colonies isolated from oncogenic transformation assays under G418 selection. The expression of the Rheb- or Ras-specific transgene was confirmed in randomly selected foci. Random foci were aspirated, triturated, plated into 10-cm dishes, and passed twice under G418 selection. Cells were harvested into 600 μl of 100 mM sodium acetate (pH 5.2)–10 mM EDTA–0.5% sodium dodecyl sulfate (SDS) (diethylpyrocarbonate-treated water). This material was extracted four times with water-buffered phenol. RNA was precipitated and resuspended in 100 μl of diethylpyrocarbonate-treated water. RNA concentrations were determined from optical density readings at 280 nm and were normalized to 1 μg/μl. Residual DNA in these samples, if present, was eliminated with RNase-free DNase. Reverse transcription (RT)-PCR was performed to demonstrate the presence of Rheb mRNA in transfected cells forming foci. RNA (2 μg) from untransfected or transfected NIH 3T3 cells was reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (GIBCO-BRL), and the subsequent cDNA products were directly amplified with primers to either Rheb or the Rheb transgene. RT-PCRs were carried out with RNA samples separately representing at least two foci from each condition (control, G418, H-Ras, H-Ras–Raf-1, Rheb, Rheb–Raf-1, and Raf-1 foci). Rheb-specific primers bound to (i) the sense strand of the 5′ untranslated region directly adjacent to the amino terminus of the Rheb open reading frame; (ii) the antisense strand of the 3′ untranslated region adjacent to the carboxy terminus of the Rheb open reading frame; or (iii) exon 2 of the dihydrofolate reductase gene which forms a fusion to the 3′ untranslated region of Rheb in the pMT2 expression construct. Then 80% of the total RT-PCR volume was electrophoresed and visualized. Controls for the RT reactions included (i) no RNA, no oligo(dT); (ii) RNA, no oligo(dT); and (iii) no RNA, oligo(dT). Controls for the PCRs included (i) no cDNA, no primers; (ii) cDNA, no primers; (iii) no cDNA, primers; and (iv) pMT2-Rheb expression construct with primers.

In vitro binding assays: NIH 3T3 fibroblasts and PC12 cells. (i) Bead preparation and GMP-PNP loading. Activated *N*-hydroxysuccinimide beads (70 mg) were washed once with distilled water at 4°C and twice with chilled 20 mM morpholinepropanesulfonic acid (MOPS; pH 7.4)–1 mM $MgCl_2$–200 mM sucrose–0.1 mM EDTA buffer (p21 buffer). The beads were resuspended in 400 μl of p21 buffer and allowed to settle at 4°C. Then 400 μl of 1-mg/ml recombinant wild-type Rheb, N41D Rheb, or H-Ras was added and allowed to couple to the beads overnight at 4°C. The beads were pelleted at 1,500 × *g* for 5 min at 4°C, washed three times with p21 buffer, and resuspended in 300 μl of p21 buffer. Coupling efficiency was determined by analyzing the amount of [α-^{32}P]GTP bound per aliquot of protein-coupled bead (22). To ensure comparable levels of bead-linked Rheb and H-Ras and to confirm nucleotide loading, (i) identical amounts of bacterial fusion proteins were mixed with active silica beads, (ii) the supernatant of the protein-loading step was assayed for H-Ras–Rheb to ensure quantitative linkage to the beads, (iii) [^{32}P]GTP binding assays were performed on all newly prepared batches of bead-linked H-Ras/Rheb to confirm the presence of comparable levels of GTP binding, and (iv) in all experiments, the binding of cell extracts to GDP- and 5′-guanylylimidodiphosphate (GMP-PNP)-loaded beads was prepared. A robust difference in the amount of Raf binding to GDP- and GMP-PNP-loaded beads was interpreted as indicative of effective nucleotide loading. Beads were loaded with either GDP or GMP-PNP, a nonhydrolyzable GTP analog, immediately prior to incubation with cytosolic extracts from cells or brain samples (15). Bovine serum albumin-linked beads were used as a negative control to confirm that Raf-1 binding was dependent on the presence of Rheb or H-Ras. Wild-type Rheb and N41D Rheb were expressed as recombinant fusion proteins in *Escherichia coli* BL21 DE3 (Invitrogen). Hexahistidine-tagged wild-type and N41D Rheb were prepared with purified nickel agarose affinity resins as described previously (73). Glutathione-*S*-transferase (GST)-Ras protein was purified as previously described (68). The N41D Rheb fusion construct was created by generating an A→G point mutant at base 244 of full-length Rheb by PCR mutagenesis. Mutations introduced by PCR were verified by sequencing, and N41D Rheb was subcloned into the pTrcHis expression construct (Invitrogen).

(ii) NIH 3T3 fibroblast growth factor stimulation. Soluble NIH 3T3 extracts were prepared 5 min after the addition of saline or EGF (200-ng/ml final concentration). After 5 min, the cells were pelleted for 3 min at 100 × *g* and 4°C. The supernatants were removed, and the cells were resuspended in 1 ml of chilled p21 buffer–10 mM sodium vanadate–1.5 mM *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate)–25 mM NaF–0.2 mM sodium pyrophosphate–1 mM dithiothreitol (DTT)–25 μg each of aprotinin and leupeptin per ml–40 μg of phenylmethylsulfonyl fluoride per ml–1.7 μg of pepstatin A per ml. The cells were sonicated on ice for two 30-s bursts with a Branson Sonifier 450 at setting 2, 50% duty cycle. Samples were pelleted for 15 min at 300,000 × *g* and 4°C. Supernatant samples were removed, split in half, and incubated with 40 μl of protein-coupled *N*-hydroxysuccinimide bead suspension for 1 h at 4°C.

The beads were separated from cell or tissue extracts by centrifugation at 5,000 × *g* for 5 min at 4°C and layered on top of 1 ml of chilled 5× p21 buffer. After 5 min, samples were centrifuged for 20 s at 5,000 × *g* and the sucrose buffer was removed. The beads were washed twice with ice-cold p21 buffer–1 mM DTT–0.01% Nonidet P-40 and twice with ice-cold p21 buffer alone. They were resuspended in 30 μl of 3× SDS loading buffer with pyronin Y dye, boiled for 3 min, and pelleted. Supernatant samples were loaded onto a denaturing SDS–12.5% polyacrylamide gel and run at 50 V for 16 h.

(iii) PC12 cell depolarization and growth factor stimulation. PC12 cells were split (10^5 cells/15-cm^2 flask) in a total volume of 2 ml of medium per flask 24 h prior to treatment. In experiments examining the effects of depolarization and calcium channel antagonists on the Rheb–Raf-1 interaction, either 20 μl of 500 μM nifedipine (Sigma) in 100% ethanol (5 μM final nifedipine concentration) or 20 μl of ethanol alone was added to each flask. After a 15-min incubation at 37°C under 5% CO_2, 600 μl of a 170 mM KCl–1.8 mM $CaCl_2$–0.8 mM $MgCl_2$ depolarizing solution (10) was added to all the flasks (50 mM KCl, final concentration). After a 3-min incubation, the cells were pelleted for 3 min at 1,500 × *g* and 4°C.

In experiments examining the effects of increased intracellular cAMP concentrations on the Rheb/Raf-1 interaction, 100 μl of a stock solution of forskolin (1 mM in dimethyl sulfoxide [Research Biochemicals Inc.]) or vehicle alone was added to each flask (50 μM final forskolin concentration). After a 15-min incubation, 20 μl of a 20-ng/μl EGF (GIBCO-BRL) solution in sterile phosphate-buffered saline (200 ng of EGF or nerve growth factor [NGF] per ml, final concentration) was added to all flasks for 5 min prior to cell harvesting. In vitro binding assays were carried out as described above.

(iv) Immunoblot analysis. Protein samples were transferred to supported nitrocellulose and examined for Raf-1 binding by immunoblotting a 1:1,000 dilution of α-Raf1 rabbit polyclonal antisera (Raf 3-8; R. Schatzman, Syntex). The blots were then washed and visualized as described above. Certain blots

were stripped with 5% SDS–50 mM β-mercaptoethanol–150 mM Tris (pH 8.0) for 15 min at 50°C, washed extensively with Tris-buffered saline–0.1% Tween 20, and reprobed with α-MEK1 (Kinetek) or α-MAP kinase (UBI) polyclonal antibodies. Western blots were scanned and relative band intensities were determined with the Molecular Dynamics ImageQuant program and Adobe Photoshop version 3.0.

In vitro phosphorylation of Raf-1. (i) Kinase-defective Raf-1. Kinase-defective Raf-1 (K375M Raf-1) was expressed in Sf11 insect cells by a recombinant baculovirus. Sf11 cells were sonicated to extract K375M Raf-1, and cell lysates were pelleted to remove insoluble material. The K375M Raf-1 preparation (5 μg) was phosphorylated at 30°C for 30 min in a total volume of 100 μl of PKA phosphorylation buffer as described previously (71) with or without PKA (25 mU of the catalytic subunit [Boehringer Mannheim]). Reactions were quenched with 20 mM (final concentration) EDTA. To control for the presence of PKA, 25 mU of enzyme was added to the tube containing non-PKA-phosphorylated K375M Raf-1 following EDTA addition. Aliquots of these reaction mixtures were incubated with immobilized Rheb or H-Ras preloaded with GDP or GMP-PNP for in vitro binding analysis as described above. Bound K375M Raf-1 was detected by immunoblotting. Western blots were scanned and relative band intensities were determined with the Molecular Dynamics ImageQuant program and Adobe Photoshop version 3.0.

(ii) Amino-terminal fragments of Raf-1. GST-tagged Raf-1(1–149) and [S43D]Raf-1(1–149) were expressed in BL21 bacteria and purified as described above. A 2-μg portion of either Raf-1(1–149) or [S43D]Raf-1(1–149) was phosphorylated in PKA buffer as described above and labeled in the presence of [γ-^{32}P]ATP by PKA. Control reactions either omitted the catalytic subunit of PKA or contained 100 ng of PKI peptide (TTYADFIASGRTRRNAIHD; no. P0300, Sigma). After reactions had been quenched, Rheb- or H-Ras-linked succinimide beads in p21 buffer (see above) plus an additional 200 μl of p21 buffer were added directly to the reaction mixtures and incubated with tumbling at 4°C for 1 h. In vitro bound protein complexes were washed as described above and split into two aliquots. Samples either were electrophoresed by denaturing SDS polyacrylamide gel electrophoresis (PAGE) and processed for autoradiography and phosphorimaging or were processed for Western blot analysis as described above. Phosphorimaging analysis was done with a PhosphorImager and the ImageQuant analysis program (Molecular Dynamics).

In vitro kinase assays. (i) MEK1 phosphorylation assay. Recombinant polyhistidine-tagged MEK-1 was purified from induced bacterial extracts and dialyzed into 50 mM MOPS–200 mM NaCl–1 mM EDTA. Soluble cell extracts were prepared from PC12 cells as described above. Extracts from EGF-only cells were incubated with Ras · GMP-PNP-linked beads while extracts from forskolin- and EGF-stimulated cells were incubated with wild-type Rheb · GMP-PNP-linked beads for 1 h at 4°C with tumbling, and protein complexes were washed as described above in the section on in vitro binding. Associated proteins, including Raf-1, were eluted in 100 μl of 1 M glycine (pH 4.5). The elution buffer was immediately neutralized with 400 μl of 500 mM MOPS (pH 7.5), and Raf-1 was immunoprecipitated from this mixture with polyclonal α-Raf-1 (C terminus) (no. sc-520; Santa Cruz Biotechnology) and immobilized protein A beads (Pierce) at 4°C for 1 h. Immunoprecipitates were washed three times with p21 buffer supplemented with antiprotease and antiphosphatase agents, as described above, and once with kinase assay buffer (50 mM MOPS, 7 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT, 15 μM ATP). Kinase assay buffer (40 μl) containing 20 μCi of [γ-^{32}P]ATP was added, and immunoprecipitates were incubated at 25°C for 20 min in the presence or absence of autokinase-inactive MEK-1. Autokinase-inactive MEK-1 was prepared with 5′-*p*-fluorosulfonylbenzoyladenosine hydrochloride (18). Kinase assays were terminated with 3× SDS loading buffer, and proteins were resolved by SDS-PAGE (12.5% polyacrylamide). Autoradiography was used to identify ^{32}P-labeled phosphoproteins.

(ii) Coupled MAP kinase phosphorylation assay. Recombinant polyhistidine-tagged MEK-1 was purified from induced bacterial extracts and dialyzed into 50 mM MOPS–200 mM NaCl–1 mM EDTA. Soluble cell extracts were prepared from control and stimulated PC12 cells as described above in the section on in vitro binding assays. Extracts were incubated with Rheb · GMP-PNP- and Ras · GMP-PNP-linked beads for 1 h at 4°C with tumbling, and protein complexes were washed as described above in the section on in vitro binding assays. Associated proteins, including Raf-1, were eluted in 100 μl of 1 M glycine (pH 4.5). The elution buffer was immediately neutralized with 400 μl of 500 mM MOPS (pH 7.5), and Raf-1 was immunoprecipitated from this mixture with polyclonal α-Raf-1 (C terminus) (no. sc-520; Santa Cruz Biotechnology) and immobilized protein A beads (Pierce) at 4°C for 1 h. Immunoprecipitates were washed three times with p21 buffer supplemented with antiprotease and antiphosphatase agents, as described above, and once with kinase assay buffer (50 mM MOPS, 7 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT, 15 μM ATP). Immunoprecipitates were then split into four aliquots of equal volume. A 40-μl volume of kinase assay buffer containing 20 μCi of [γ-^{32}P]ATP was added, and immunoprecipitates were incubated at 30°C for 30 min in the presence of 100 ng of MEK1 and 2 μg of inactive MAP kinase (obtained from M. Weber and from Upstate Biotechnology) and also in control reactions, which lacked inactive MAP kinase. Kinase assays were terminated by addition of 3× SDS loading buffer, and proteins resolved by SDS-PAGE (12.5% polyacrylamide). Autoradiography was used to identify ^{32}P-labeled phosphoproteins. Phosphorimaging analysis was done with the Molecular Dynamics PhosphorImager and ImageQuant analysis program. All experimental reactions were normalized to their respective control reactions. Relative phosphorylation was determined normalized to the H-Ras-bound Raf-1 isolated from control PC12 cells and averaged with data from three independent experimental series.

PC12 neurite outgrowth assays. PC12 cells were transfected with expression constructs for LacZ and Rheb or for H-Ras. The cells were incubated in serum-free RPMI 1640 medium for 30 min prior to transfection and were incubated for 6 h in RPMI 1640 medium containing DNA-Lipofectamine (GIBCO-BRL) solution at 37°C under 5% CO_2. Then 12-well plates of cells were transfected with 2 μg of LacZ reporter construct alone or in addition to 5 μg of pMT2-Rheb or 5 μg of 3625N (H-Ras) per well. Lipofectamine was neutralized with PC12 maintenance medium containing 20% heat-inactivated horse serum, 10% fetal bovine serum, and 2× penicillin-streptomycin. The cells were allowed to recover overnight and then were rinsed with PBS and refed. At 24 h after transfection, the cells were fed with PC12 maintenance medium alone or containing 15 ng of NGF per ml, 10 μM forskolin, 1 mM dibutyryl cAMP, NGF plus forskolin, or NGF plus dibutyryl cAMP and assayed for neurite extension 48 h later. The cells were fixed with 2% paraformaldehyde–0.4% glutaraldehyde and stained with a 0.5-mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; GIBCO-BRL)–1.5-mg/ml potassium ferricyanide–2.1-mg/ml potassium ferrocyanide solution overnight in a darkened, humidified environment. LacZ-positive cells bearing neurites at of least one cell body length were counted by an experimenter blind to the experimental design, and the percentage of LacZ-positive cells bearing neurites was calculated. The average transfection efficiency was 5 to 8% of each wild-type PC12 cell population, as indicated by the percentage of cells expressing the LacZ marker.

RESULTS

Rheb binds Raf-1 kinase. We examined the possibility that Rheb directly interacts with Raf-1. Rheb possesses regions, including the G2 effector domain, that are highly homologous to H-Ras (73). This region is critical for interactions between H-Ras and Raf-1 kinase, as well as other Ras targets. We began our analysis by using the yeast two-hybrid system (Fig. 1A). Yeast expressing GAL4 fusion constructs of Rheb (Rheb-GAL4 transcription activation domain) and Raf-1 (Raf-1–GAL4 DNA binding domain) grew on medium lacking histidine, indicating a physical interaction between Rheb and Raf-1. Controls confirmed that growth was dependent on both the Rheb and Raf-1 inserts in their respective expression vectors (data not shown). To estimate the relative affinity of the Rheb–Raf-1 and H-Ras–Raf-1 interactions, we assayed the production of the β-galactosidase reporter transgene (9). Yeast colonies expressing either the Rheb–Raf-1 or H-Ras–Raf-1 combination of constructs turned blue within 1 h, suggesting similar affinities of interaction.

The amino terminus of Raf-1 has been shown to contain a "Ras-binding domain," which forms stable interactions with the G2 effector domain of H-Ras or other Ras-like proteins (47). To initially map the domains of Raf-1 required for its interaction with Rheb, we examined carboxy- and amino-terminal deletion mutants of Raf-1 in the two-hybrid system. A Raf-1 mutant (Raf1ΔC1) lacking the carboxy-terminal half of the molecule supported growth on media lacking histidine when expressed in combination with Rheb, similar to wild-type Raf-1. In contrast, Rheb did not interact with the two amino-terminal deletion mutants Raf1ΔN1 and Raf1ΔN2, indicating that it, like H-Ras (5, 66, 68, 69, 74), requires the amino terminus of Raf-1 for direct interaction.

All Ras-like proteins require GTP for full activation and interaction with downstream targets. To examine the GTP dependence of the interaction between Rheb and Raf-1, full-length Rheb was expressed in bacteria as an N-terminal hexahistidine fusion and linked to *N*-hydroxysuccinimide beads. We have previously shown that Rheb binds GTP and possesses GTP hydrolase activity (73). Rheb-linked succinimide beads were loaded with either GDP or the nonhydrolyzable GTP analog GMP-PNP and incubated with extracts from control or EGF-stimulated NIH 3T3 fibroblasts (Fig. 1B). Proteins bound by the Rheb-linked beads were analyzed by SDS-PAGE and

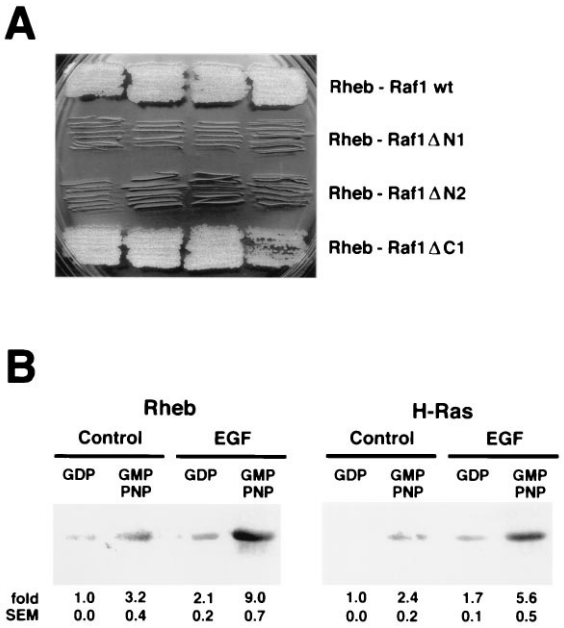

FIG. 1. Rheb interacts with Raf-1. (A) Rheb interacts with Raf-1 in the yeast two-hybrid system, as shown by growth on selective medium. Rheb interacts with the wild type (wt) and carboxy-terminal deletion mutant (ΔC1, lacking the carboxy terminus from residues 380 to 648), but not with Raf-1 variants lacking either a portion (ΔN1, lacking residues 26 to 302) or the complete (ΔN2, lacking residues 1 to 375) amino terminus. Four independent yeast colonies expressing both Rheb and the Raf-1 constructs were selected from medium plates lacking Leu and Trp and streaked onto medium lacking Leu, Trp, and His. (B) Rheb binds Raf-1 from control and EGF-stimulated NIH 3T3 fibroblasts in a GTP-dependent manner. Rheb- and Ras-linked beads were loaded with either GDP or GMP-PNP, a nonhydrolyzable GTP analog, and incubated with soluble cell lysates. Bound proteins were analyzed by SDS-PAGE and immunoblotted to detect Raf-1. Rheb, like H-Ras, demonstrates preferential binding for Raf-1 in its GTP-dependent conformation. The intensity of the Raf-1 band (apparent molecular mass, 74 kDa) was estimated by densitometry, and the numbers below the lanes indicate relative intensities. EGF stimulation of NIH 3T3 fibroblasts for 20 min prior to preparation of cell extracts increased the amount of Raf-1 that binds either Rheb · GMP-PNP and H-Ras · GMP-PNP by two- to threefold, relative to unstimulated cells.

Western blotting with anti-Raf-1 antibody. In parallel assays, we also examined Raf-1 interactions with bead-linked H-Ras. Identical amounts of Rheb and H-Ras were loaded on beads, as confirmed by binding of radiolabelled GTP, to permit a comparison of their relative binding properties to Raf-1. Both Rheb- and H-Ras-linked beads bound Raf-1 from NIH 3T3 fibroblasts in a GTP-dependent manner with similar efficacy. The GMP-PNP-loaded Rheb and H-Ras beads bound at least threefold more Raf-1 than did the GDP-loaded proteins. Stimulation of NIH 3T3 fibroblasts with EGF 5 min before the preparation of cell extracts resulted in increased binding of Raf-1 to both bead-linked Rheb and H-Ras (Fig. 1B). Stimulus-induced changes in Raf-1 binding to Rheb and H-Ras are examined in detail with PC12 cells in studies described below. Previous studies with Rat1hER cell extracts and in vitro binding assays, have demonstrated that the H-Ras–Raf-1 interaction is GTP dependent but did not find increased binding following growth factor stimulation (71). Differences in the cell types and/or stimulation conditions may underlie this difference.

Rheb and Raf-1 synergistically transform NIH 3T3 fibroblasts. To assess the possible physiological relevance of the Rheb–Raf-1 interaction, we examined the effect of Rheb transgene expression on the growth properties of NIH 3T3 fibroblasts. Cultured fibroblasts expressing oncogenic H-Ras lose contact inhibition and form dense foci (51, 74). Wild-type H-Ras is relatively ineffective in promoting focus formation but acts synergistically with Raf-1 (13), consistent with the notion that H-Ras and Raf-1 act in concert to generate transformation. To determine whether Rheb might also act in concert with Raf-1 in this assay, we cotransfected NIH 3T3 cells with wild-type Rheb and Raf-1 under antibiotic selection for 2 weeks and scored the number of foci. Consistent with previous studies, cotransfection of wild-type H-Ras and Raf-1 resulted in a substantial increase in focus number compared to expression of either alone (Fig. 2A). Similar results were obtained with Rheb. Focus formation in fibroblasts cotransfected with Rheb and Raf-1 was four- to fivefold greater than in fibroblasts transfected with either Rheb or Raf-1 alone, indicating a synergistic interaction. Rheb transformed fibroblasts with an efficacy comparable to H-Ras, suggesting similar activation of Raf-1-dependent signaling. Expression of the Rheb transgene was confirmed in identified foci by RT-PCR (Fig. 2B). Interestingly, the expression of endogenous Rheb appeared to be increased in foci from cells transfected with either H-Ras or Rheb singly or in combination with Raf-1.

Rheb–Raf-1 and H-Ras–Raf-1 interactions are regulated and differentially modified by forskolin and growth factor stimulation of PC12 cells. PC12 cells are derived from the neural crest tumor pheochromocytoma and respond to treatment with NGF by differentiating into a neuronal phenotype. In PC12 cells, H-Ras-dependent signaling plays a role in the transduction of multiple stimuli including growth factors, second messengers, and membrane depolarization with associated calcium influx (27, 37, 42, 48, 53, 62, 65). This multiplicity of signals transduced by H-Ras in PC12 cells suggests that PC12 cells possess mechanisms either parallel or downstream of H-Ras that can mediate differential responses to various stimuli. Rheb is endogenously expressed in PC12 cells and is induced by growth factor stimulation (73), suggesting that mechanisms which distinguish between Rheb- and H-Ras-dependent signaling may exist. Accordingly, we used PC12 cells and in vitro binding assays to examine and compare effects of various stimuli on Rheb–Raf-1 and H-Ras–Raf-1 interactions.

Raf-1 from extracts of PC12 cells maintained in control medium binds Rheb and H-Ras in a GTP-dependent manner (Fig. 3A). The amounts of Raf-1 that bound either Rheb or H-Ras were comparable, again suggesting similar affinities of interaction. The GTP binding capacity of bead-linked Rheb and H-Ras was routinely assayed to confirm similar loadings (Fig. 3D). Previous studies indicated that depolarization of PC12 cells can activate MEK and MAP kinases via the influx of extracellular calcium through voltage- and dihydropyridine-sensitive calcium channels (53). To determine if calcium influx modulates Raf-1 binding, we depolarized PC12 cells with potassium chloride for 10 min prior to harvesting cells. To confirm that the amount of Raf-1 used for in vitro binding was comparable between different conditions, crude cell extracts were assayed by Western blot analysis (Fig. 3E). Depolarization of PC12 cells produced increases in Raf-1 binding to both Rheb and H-Ras (Fig. 3A). The depolarization-induced increase in Raf-1 binding was blocked by prior treatment of cells with the calcium channel antagonist nifedipine, suggesting a role for calcium influx in this response. Moreover, nifedipine reduced Raf-1 binding to both Rheb and H-Ras under control conditions, suggesting that spontaneous activation of the calcium channel regulates basal Raf-1 binding activity.

We next examined the effects of growth factor treatment on the binding of Raf-1 by Rheb. Treatment of PC12 cells with EGF has little effect on the Rheb–Raf-1 interaction, but in

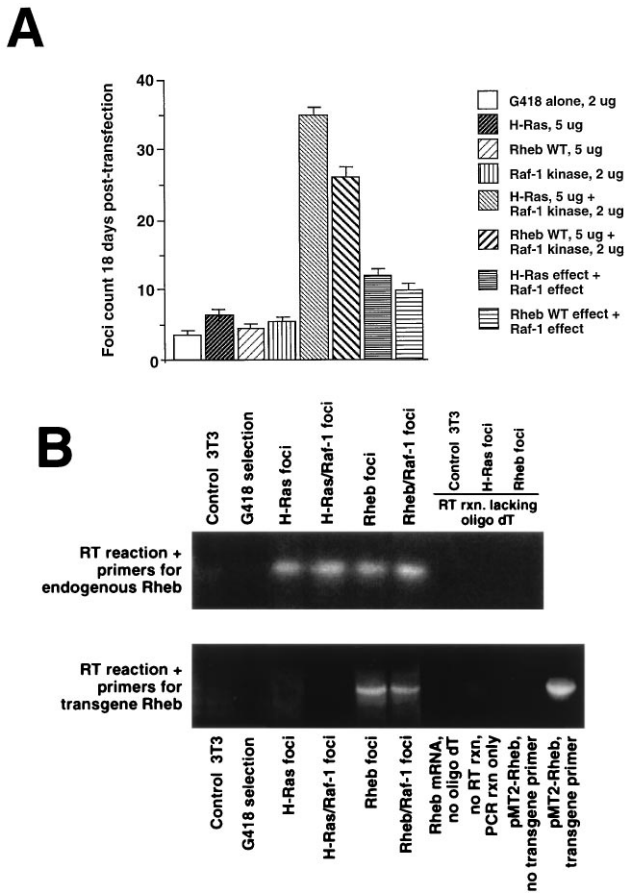

FIG. 2. Rheb and Raf-1 kinase synergistically act to transform NIH 3T3 fibroblasts. (A) NIH 3T3 fibroblasts were transfected with expression constructs for Rheb or H-Ras, either alone or in combination with Raf-1, and maintained under G418 selection for 2 weeks, at which time the number of foci was determined. Fibroblasts transfected with Rheb, H-Ras, or Raf-1 alone formed foci comparable in number to those formed by cells transfected with the G418 resistance expression vector alone. Cotransfection of Raf-1 in combination with Rheb produced four- to fivefold more foci than either alone. Similar levels of focus formation were observed in the H-Ras–Raf-1 series of parallel experiments. Each focus formation assay was performed twice in duplicate. Focus counts are expressed as mean ± SEM. (B) Foci were randomly selected from fibroblasts transfected with Rheb either alone or in combination with Raf-1 and were assayed to confirm the expression of the Rheb transgene. Expression of endogenous and transgene Rheb mRNA was determined by RT-PCR. Rheb-transfected foci demonstrate the presence of a Rheb-specific transgene mRNA (lower panel). Six of seven foci selected from the Rheb alone set and seven of seven foci from the Rheb–Raf-1 set demonstrated Rheb transgene expression. Control and G418 resistance vector-transfected NIH 3T3 foci demonstrated a very low basal expression of endogenous Rheb mRNA, while foci originating from cells transfected with H-Ras, H-Ras–Raf-1, Rheb, or Rheb–Raf-1 demonstrated increased endogenous Rheb (top panel).

combination with pretreatment with forskolin, which activates adenylate cyclase and increases intracellular cAMP concentrations, EGF produces a marked potentiation of the Rheb–Raf-1 interaction (Fig. 3B). Forskolin treatment alone had little effect on Rheb–Raf-1 binding, indicating a synergistic activation of Raf-1 binding with Rheb by combined forskolin and EGF treatment. In direct contrast to the Rheb–Raf-1 interaction, the H-Ras–Raf-1 interaction is potentiated by EGF, and this EGF effect is inhibited by forskolin pretreatment. The inhibitory effect of forskolin on EGF-potentiated H-Ras–Raf-1 binding is consistent with previous studies which have demonstrated that growth factor induction of Raf-1 binding by H-Ras and subsequent MAP kinase activation is inhibited by agents which raise intracellular cAMP levels (12, 56, 71).

We next examined the effect of NGF stimulation of PC12 cells on Raf-1 binding to Rheb and H-Ras. NGF treatment alone increased GTP-dependent binding of Raf-1 to both Rheb and H-Ras (Fig. 3C). Forskolin pretreatment potentiated NGF-induced increases in Rheb–Raf-1 binding but blocked H-Ras–Raf-1 binding. These observations generally parallel the results of experiments performed with EGF stimulation, in that forskolin potentiates Rheb–Raf-1 binding and blocks H-Ras–Raf-1 binding. It is notable, however, that NGF stimulation alone increases Rheb–Raf-1 binding, while EGF stimulation alone has little effect (Fig. 3B), indicating that the Rheb–Raf-1 interaction may be differentially regulated by these growth factors. Together, these observations indicate that Rheb–Raf-1 and H-Ras–Raf-1 interactions are reciprocally regulated by cAMP-dependent mechanisms.

Raf-1, as isolated from cell extracts, is part of a complex of proteins which includes other members of the MAP kinase cascade, notably MEK1 (32, 38, 44, 66) and B-Raf. Like Raf-1, B-Raf is activated by growth factors via H-Ras and can be inhibited by increased cAMP levels (37, 64). Western blot analysis of the in vitro binding pellets determined that MEK1 (Fig. 3A and B) and B-Raf (Fig. 3B) are also constituents of the complex of proteins associated with Rheb- and H-Ras-linked beads in a GTP-dependent manner. B-Raf has been reported to bind directly to H-Ras (31, 43), while MEK1 is presumed to be a secondary constituent of the binding complex by virtue of its interaction with Raf-1 and B-Raf. The levels of MEK1 and B-Raf paralleled those of Raf-1. Notably, the presence of MEK1 and B-Raf in the H-Ras-associated complex is potentiated by EGF stimulation and blocked by forskolin, while their presence in the Rheb-associated complex is not increased by EGF or forskolin alone but is potentiated by combined EGF and forskolin stimulation.

The most direct interpretation of the prior experiments is that the amount of Raf-1 which binds to H-Ras or Rheb varies as a function of the pretreatment of the cells. An alternative explanation might be that Rheb and Ras bind different isoforms of Raf in stimulus-specific ways and are detected with different sensitivities by the Raf-1 C-terminal antibody. In favor of the first hypothesis, the apparent size of Raf-1 did not appear to change as a function of the various cell treatments. In crude cell extracts, a single major band of 74 kDa, as well as less intense bands at ~85 and ~65 kDa, is detected with the C-terminal antibody. The 74-kDa band corresponds to the reported size of Raf-1 and is also detected with an antibody generated against the N terminus of Raf-1 (data not shown). Western blots performed after in vitro binding to H-Ras–Rheb show a single band of 74 kDa (Fig. 3A to C). Identical results were obtained with the N-terminal Raf-1 antibody.

Previous studies have reported that NGF stimulation does not induce H-Ras–Raf-1 binding in PC12 cells (31). While the NGF-induced increase in H-Ras–Raf-1 binding was less robust than the EGF-induced binding, it seems likely that factors other than technical considerations may underlie the difference in experimental results. NGF stimulation of PC12 cells has been reported to result in increases in intracellular cAMP levels, and certain NGF-dependent responses can be blocked by inhibitors of PKA (34). If NGF produces a coordinate activation of cAMP, it might mimic the action of combined growth factor and forskolin stimulation in our assays and block the H-Ras–Raf-1 interaction. Modest differences in the signaling properties of different PC12 cell lines may therefore underlie the different observations. Consistent with the notion that NGF may produce a coordinate activation of growth fac-

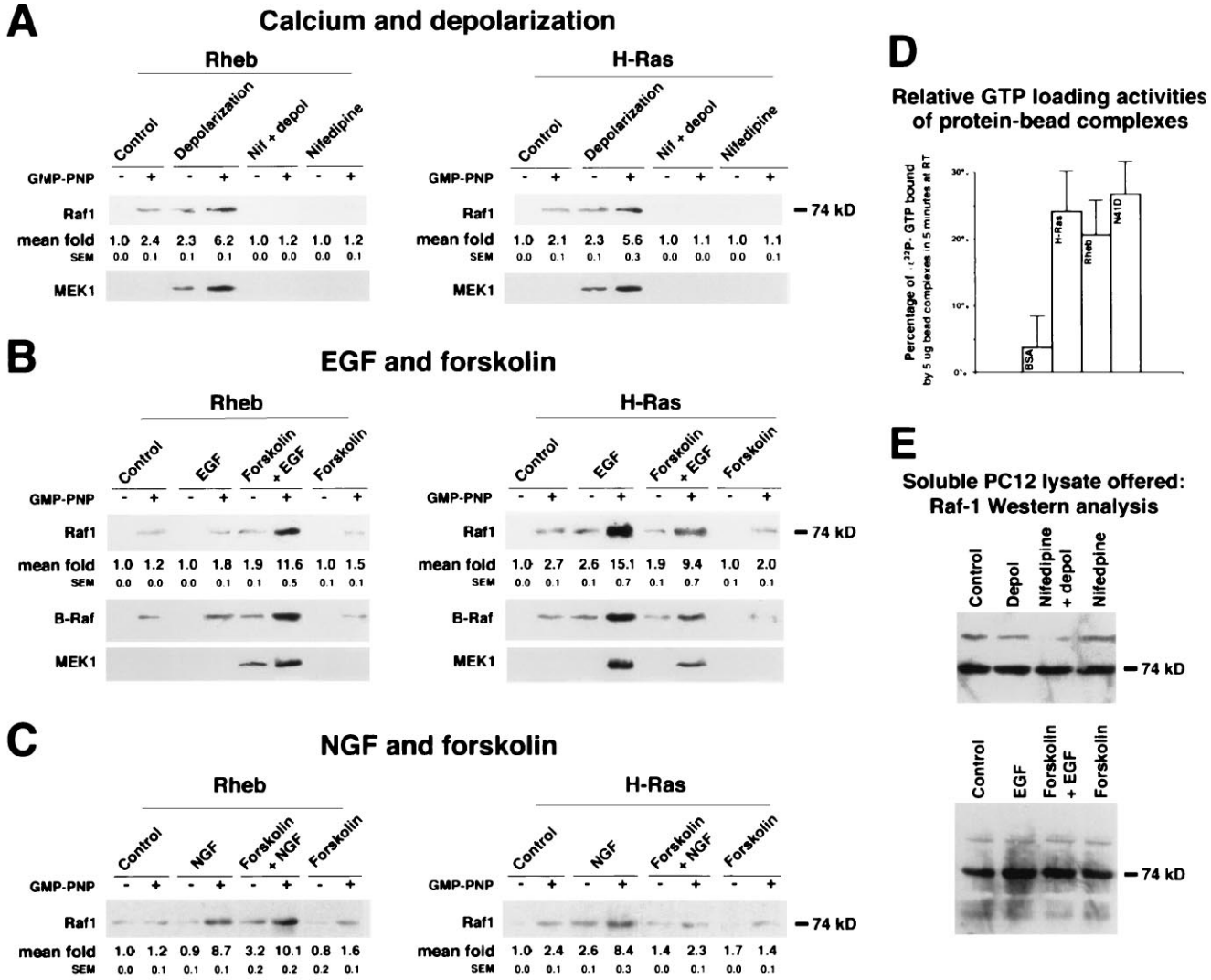

FIG. 3. Binding of Raf-1 from PC12 cells to recombinant Rheb and H-Ras is differentially regulated by growth factor and forskolin stimulation but not by depolarization. (A) Depolarization of PC12 cells increases the binding of Raf-1 to Rheb-linked beads in a GTP-dependent manner. PC12 cells were depolarized by the addition of potassium chloride (50 mM final concentration) for 10 min prior to preparation of cell extracts. Equal amounts of soluble extracts were incubated with Rheb- or Ras-linked beads loaded with either GDP (referred to as GMP-PNP − in the figure) or GMP-PNP (GMP-PNP +), and bound proteins were analyzed by immunoblotting. Immunoblot analysis of the offered soluble PC12 lysates confirms that equivalent amounts of Raf-1 were presented in each binding reaction. Raf-1 binding to both Rheb and H-Ras is GTP dependent and is increased following depolarization. The intensity of the Raf-1 band (apparent molecular mass, 74 kDa) was estimated by densitometry, and numbers below the lanes represent relative intensities. We observed only one species of Raf-1 in our immunoblot analysis, in contrast with the three Raf-1 immunoreactive bands observed in PC12 whole-cell extract. Raf-1 binding was GTP dependent. Comparison of Raf-1 in the GMP-PNP-bound lanes for Rheb and H-Ras indicates a two- to threefold increase in Raf-1 binding following depolarization. Pretreatment of PC12 cells with nifedipine (5 μM), a voltage-sensitive calcium channel blocker, for 15 min prior to potassium chloride depolarization inhibits the increased binding of Raf-1 to both Rheb and H-Ras. Reprobing the same blot with MEK1 selective antiserum demonstrates that MEK1 (apparent molecular mass, 45 kDa) is also present in the protein complexes bound by Rheb- or H-Ras-linked beads. Similar results were observed in three additional experiments. (B) Raf-1 differentially binds Rheb and H-Ras following stimulation with EGF and forskolin. PC12 cells were stimulated with EGF (200 ng/ml) for 20 min prior to harvesting, and soluble extracts were used for in vitro binding to Rheb- and H-Ras-linked beads, as described for panel A. Raf-1 binding to H-Ras · GMP-PNP increased fivefold following addition of EGF, while Raf-1 binding to Rheb · GMP-PNP was essentially unchanged. Pretreatment of PC12 cells with forskolin (50 μM) for 15 min prior to addition of EGF inhibited the EGF-induced increase of Raf-1 binding to H-Ras but increased Raf-1 binding to Rheb · GMP-PNP by six- to ninefold compared to either EGF-stimulated or unstimulated control cells. Forskolin treatment alone did not change Raf-1 binding to either Rheb · GMP-PNP or H-Ras · GMP-PNP. B-Raf (apparent molecular mass, 68 kDa) and MEK1 (apparent molecular mass, 45 kDa) are also present in the in vitro binding pellets, and their levels parallel those of Raf-1. Similar results were observed in an additional three experiments. (C) Raf-1 differentially binds Rheb and H-Ras following stimulation with NGF and forskolin. PC12 cells were stimulated with NGF (200 ng/ml) for 5 min, and cell extracts were assayed for Raf-1 binding to Rheb and H-Ras, as described for panel B. Raf-1 binding to Rheb · GMP-PNP and H-Ras · GMP-PNP increased eight- and threefold, respectively, following addition of NGF. Pretreatment of PC12 cells with forskolin for 15 min prior to the addition of NGF reduced the NGF-induced increase of Raf-1 binding to H-Ras to levels present in control cells but further increased Raf-1 binding to Rheb · GMP-PNP compared to NGF alone. Forskolin treatment alone did not change Raf-1 binding to either Rheb · GMP-PNP or H-Ras · GMP-PNP. Similar results were observed in two additional experiments. (D) GTP-binding capacity of bead-linked Rheb and H-Ras was assayed to ensure similar loadings. The relative amount of [α-^{32}P]GTP bound by 5 μg of bead complexes (based on volume) with H-Ras, Rheb, N41D Rheb (used in Fig. 6), or BSA was determined for each newly prepared batch. Beads with linked Ras proteins typically exhibited fivefold higher binding than beads with BSA. Average binding data for five different preparations of bead-linked proteins are illustrated. (E) Raf-1 Western analysis of PC12 cell lysates. Raf-1 used for in vitro binding assays was monitored to ensure equal amounts in extracts from the different conditions. The major band is a 74-kDa band and corresponds to the reported size of Raf-1. Minor bands of ~85 and ~65 kDa were not detected following in vitro binding and are presumed to be nonspecific.

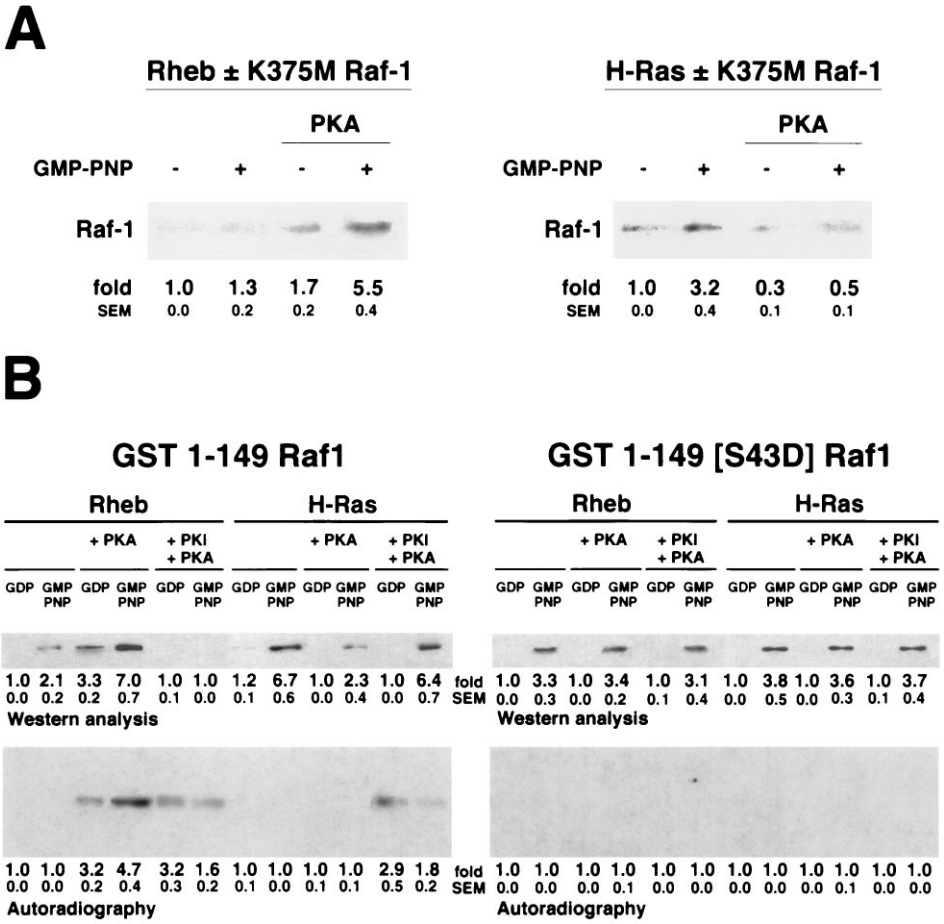

FIG. 4. PKA phosphorylation of Raf-1 increases binding to Rheb and decreases binding to H-Ras. (A) PKA in vitro phosphorylation of Raf-1 increases its binding to Rheb. Recombinant kinase-deficient Raf (K375M Raf-1) was expressed in Sf11 insect cells and was phosphorylated in vitro by PKA. Control or phosphorylated K375M Raf-1 was incubated with Rheb- or H-Ras-linked beads preloaded with GMP-PNP and evaluated for binding by Raf-1 immunoblotting. The intensity of the Raf-1 band was estimated by densitometry, and numbers below the lanes represent relative intensities. Raf-1 binding was GTP dependent. PKA treatment increased Raf-1 binding to Rheb · GMP-PNP by fourfold and decreased binding to H-Ras · GMP-PNP by approximately sixfold. Similar results were obtained in an additional two experiments. (B) PKA in vitro phosphorylation of the amino-terminal region of Raf-1 increases its binding to Rheb. Purified GST-tagged truncated Raf-1(1–149) and [S43D]Raf-1(1–149) was combined with PKA and [γ-^{32}P]ATP in in vitro kinase reactions and subsequently assayed for binding to Rheb and H-Ras by Raf-1 immunoblotting and autoradiography. Binding of ^{32}P-labeled Raf-1(1–149) was GTP dependent, and PKA phosphorylation increased Raf-1 binding to Rheb · GMP-PNP by nearly fivefold and decreased binding to H-Ras · GMP-PNP by twofold. Note that the Raf which binds Rheb is phosphorylated. The [S43D]Raf-1(1–149) form contains a disrupted consensus site for PKA phosphorylation and was not phosphorylated. Binding of [S43D]Raf-1(1–149) to Rheb was not potentiated by PKA, and PKA did not inhibit binding to H-Ras. The intensity of autoradiographic Raf-1 bands was determined with a PhosphorImager and the immunoblot bands were estimated by densitometry. The numbers below the lanes represent intensities subtracted from unstimulated GDP lanes. Similar results were obtained in an additional three experiments.

tor receptor-dependent and cAMP-dependent signaling, we note that EGF alone does not induce Rheb–Raf-1 binding while NGF alone does. The initial growth factor-dependent responses to EGF and NGF share many mechanisms, and their differential effects on Rheb–Raf-1 binding are consistent with the effect of NGF to partially activate cAMP-dependent signaling.

Rheb–Raf-1 binding and H-Ras–Raf-1 binding are differentially modified by PKA phosphorylation of Raf-1. Modifications of the Raf-1 complex are likely to be responsible for the growth factor-dependent changes observed in Rheb–Raf-1 binding (Fig. 3), since the bead-linked Rheb and H-Ras used in the in vitro-binding assays are in vitro recombinant proteins not subjected to the in vivo conditions from which Raf-1 is extracted. Raf-1 function is regulated by phosphorylation (17, 36, 46), and phosphorylation of Raf-1 by PKA blocks its interaction with H-Ras (10, 12, 71). In view of the differential effect of forskolin on Raf-1 binding to Rheb or H-Ras, we examined the hypothesis that PKA phosphorylation of Raf-1 might modify its interaction with Rheb. Recombinant full-length K375M Raf-1 kinase, which is kinase deficient, was expressed in Sf11 insect cells and harvested for presentation to bead-linked Rheb. In vitro phosphorylation of Raf-1 by PKA resulted in a threefold increase in the binding of Raf-1 by Rheb (Fig. 4A). In contrast, in vitro phosphorylation of K375M Raf-1 by PKA resulted in decreased binding to H-Ras, consistent with earlier reports. As expected, both Rheb and H-Ras bind their respective preferred forms of Raf-1 in a GTP-dependent manner.

To further examine the specific interaction between phosphorylated and nonphosphorylated forms of Raf-1 and Rheb or H-Ras, we used GST fusion proteins of truncated forms of Raf-1 (residues 1 to 149) representing the Ras-binding domain (5) (Fig. 1). A mutant Raf-1(1–149), which contains a substitution of an aspartate for serine (S43D), in the only known PKA phosphorylation site in Raf-1 (46), was also used in these in vitro binding experiments. In contrast to experiments involv-

ing insect cell-derived Raf-1, these truncated forms were expressed in *E. coli* and purified. Similar to our observations made with the full-length kinase-deficient Raf-1, Rheb preferentially binds PKA-phosphorylated Raf-1(1–149) (Fig. 4B). The effect of PKA on the Rheb–Raf-1(1–149) interaction was typically more robust than that seen with insect cell-derived Raf-1 and was blocked by PKI, a specific peptide inhibitor of PKA. In contrast, the H-Ras–Raf-1(1–149) interaction was blocked by prior PKA phosphorylation of Raf-1(1–149) and restored when PKI was present in the phosphorylation buffer. In identical experiments with [S43D]Raf-1(1–149), PKA was ineffective in either increasing Rheb–Raf-1 binding or decreasing H-Ras–Raf-1 binding, indicating an essential role for serine 43. As further confirmation that the PKA effect was due to phosphorylation, kinase reaction mixtures included [^{32}P]ATP and binding-reaction mixtures were analyzed by autoradiography. Following PKA treatment, the Raf-1 that bound to Rheb was phosphorylated. Together, these data indicate that PKA phosphorylation of Raf-1 differentially modifies its interaction with Rheb and with H-Ras and suggest that rapid changes in the phosphorylation state of Raf-1 may be important in targeting Raf-1 to selectively bind either H-Ras or Rheb.

Raf-1 bound to Rheb is catalytically active. PKA phosphorylation of Raf-1 has been reported to inhibit the catalytic activity of Raf-1 kinase (25). This suggested that the Raf-1 bound to Rheb from forskolin- and EGF-treated extracts might be catalytically inactive. To examine this possibility, we prepared extracts from PC12 cells that were stimulated with either EGF alone or in combination with forskolin and first performed in vitro binding with bead-linked H-Ras or Rheb. Bound proteins were eluted from the beads, and Raf-1 was immunoprecipitated with a Raf-1-specific polyclonal antibody and incubated with autokinase-inactive MEK1. MEK-1 is reported to be phosphorylated by active Raf-1 in the MAP kinase pathway and can serve as a substrate for Raf-1 in in vitro kinase activity assays (17, 38). In this assay, MEK1 was phosphorylated equally well (Fig. 5A) by Raf-1 prepared from forskolin- and EGF-treated PC12 cells that initially bound to Rheb as it was by Raf-1 prepared from cells stimulated by EGF and that initially bound to H-Ras.

To address the possibility that kinases other than Raf-1 phosphorylate MEK1 in the above assay, we performed coupled phosphorylation assays which use inactive MAP kinase as the end substrate (32). The greater specificity of this assay is because MEK1 can be phosphorylated at multiple sites by kinases other than Raf-1 but only Raf-1 is known to activate MEK1 kinase activity. MAP kinase is used as a substrate for activated MEK1. PC12 cell extracts from control and growth factor- and/or forskolin-stimulated conditions were again presented to bead-linked Rheb or H-Ras. Bound proteins were then eluted from the in vitro-binding pellets, and Raf-1 was immunoprecipitated with a Raf-1-specific polyclonal antibody and incubated with MEK1 and inactive MAP kinase. Control reactions confirmed that the extent of MAP kinase phosphorylation in the assay was linearly associated with the amount of added Raf-1 and was not due to phosphorylation of coprecipitated endogenous MAP kinase (data not shown). Raf-1 immunoprecipitated from Rheb in in vitro binding reactions was at least as active as Raf-1 immunoprecipitated from H-Ras in in vitro binding reactions. Raf-1 activity was highest from immunoprecipitates isolated from Rheb-binding reaction mixtures with PC12 cell lysates following combined forskolin and EGF stimulation. For both Rheb and H-Ras, Raf-1 activity was decreased by pretreatment with forskolin alone. This is consistent with reports that forskolin can inhibit Raf-1 kinase activity (10, 12, 71), although it is important to note that previous studies have assayed Raf-1 activity in the total-cell extract while our assays examined the activity of Raf-1 that selectively bound Rheb or H-Ras. We conclude that the population of Raf-1 kinase bound to Rheb under conditions of combined EGF and PKA stimulation is catalytically active.

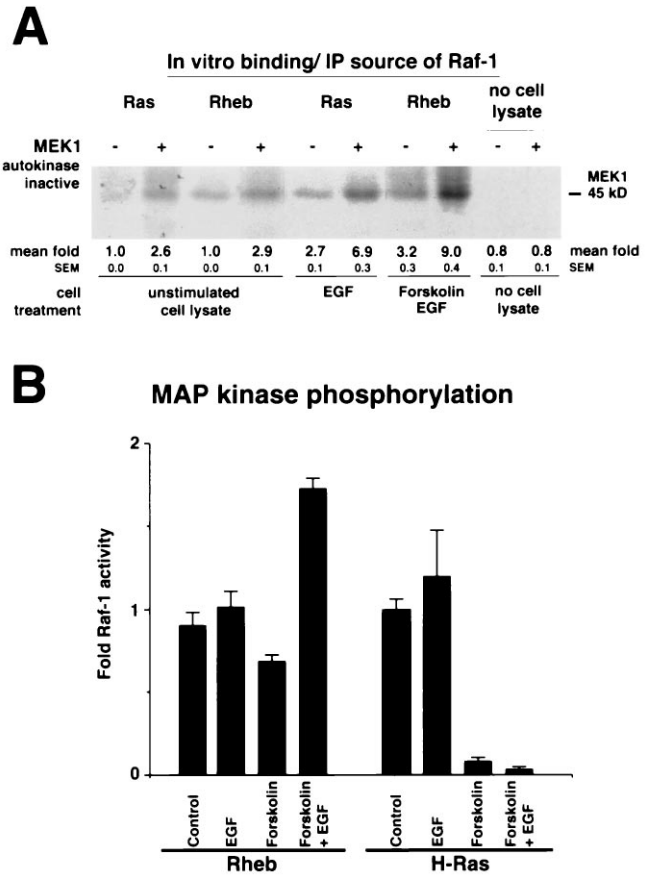

FIG. 5. Raf-1 that binds Rheb is catalytically active. (A) Raf-1 bound by Rheb from forskolin- and EGF-stimulated PC12 lysates is catalytically active and phosphorylates MEK1. PC12 cells were stimulated with EGF and forskolin, and cell extracts were incubated with bead-linked Rheb · GMP-PNP, as in Fig. 3. In parallel experiments, extracts were prepared following EGF stimulation alone and mixed with H-Ras · GMP-PNP. Stimulation conditions were selected that yielded maximal binding of Raf-1 to Rheb and H-Ras. Raf-1 was then immunoprecipitated from bound complexes and incubated in kinase reaction mixtures containing [γ-^{32}P]ATP and 100 ng of autokinase-inactivated recombinant MEK1 (MEK1 +). Control reaction mixtures omitted either MEK1 (MEK1 −), cell lysates, or both. Kinase reactions were analyzed by SDS-PAGE and autoradiography of ^{32}P-labeled proteins. Raf-1 from all three conditions demonstrated similar catalytic activity by phosphorylating MEK1. Reaction mixtures which lacked MEK1 demonstrated detectable but very low levels of MEK1 phosphorylation, attributable to endogenous MEK1 present in the Raf-1 immunoprecipitation pellets. Reaction mixtures lacking cell lysate demonstrated no detectable MEK1 phosphorylation, confirming that the inactivated recombinant MEK1 lacked autokinase activity. (B) Raf-1 bound by Rheb from cAMP- and EGF-stimulated PC12 lysates is catalytically active in a coupled MEK1/MAP kinase assay. PC12 cells were stimulated with saline (control), forskolin, EGF, and forskolin plus EGF, and soluble cell lysates were incubated with bead-linked Rheb · GMP-PNP or H-Ras · GMP-PNP. Raf-1 was then immunoprecipitated from bound complexes and incubated in kinase reaction mixtures containing [γ-^{32}P]ATP, 100 ng of recombinant MEK1, and 2 μg of kinase-inactive recombinant GST-tagged MAP kinase (62 kDa) as the end substrate. Kinase reactions were analyzed by SDS-PAGE and autoradiography of ^{32}P-labeled GST-MAP kinase. Similar levels of Raf-1 activity were detected in binding assays to Rheb and to H-Ras with control PC12 extracts. Combined forskolin and EGF stimulation of PC12 cells increased Raf-1 activity associated with Rheb but reduced Raf activity associated with H-Ras, while forskolin stimulation alone reduced Raf activity associated with both Rheb and H-Ras. MAP kinase bands were quantitated with a Molecular Dynamics PhosphorImager and the ImageQuant program. Relative phosphorylation is compared to control H-Ras reactions and was averaged over three experimental series.

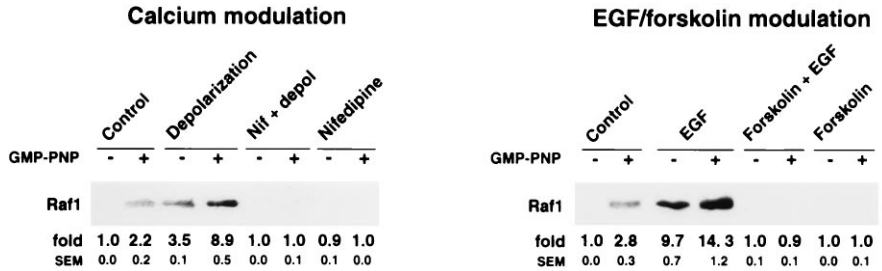

FIG. 6. Asparagine 41 of Rheb is critical for cAMP potentiation of Raf-1 binding. (A) Asparagine 41 of Rheb was mutated to an aspartate (N41D Rheb), and its binding properties for Raf-1 were examined. Soluble extracts were prepared from PC12 cells as described in Fig. 3 following potassium chloride depolarization or EGF and forskolin stimulation. Potassium chloride depolarization resulted in a fourfold increase in binding of Raf-1 to N41D Rheb · GMP-PNP that was blocked by pretreatment with nifedipine. This stimulation is similar to depolarization-induced increases in Raf-1 binding to both Rheb and H-Ras as illustrated in Fig. 3. EGF treatment increased the binding of Raf-1 to N41D Rheb · GMP-PNP by approximately fourfold, and pretreatment with forskolin blocked EGF-induced increases in binding. Thus, the binding properties of N41D Rheb in response to EGF and forskolin are similar to those of H-Ras and distinct from those of Rheb. EGF stimulation resulted in a substantial increase in binding to the GDP form of N41D Rheb that was not seen with H-Ras in parallel experiments. Similar results were observed in an additional two experiments. These data indicate that asparagine 41 of Rheb, which is the single nonconserved amino acid in the G2 effector domain, is critical for the unique Raf-1 binding properties of Rheb.

The point mutant, N41D Rheb, identifies an amino acid critical for the unique Raf-1 binding properties of Rheb. We focused on the G2 effector domain to identify amino acid residues of Rheb that would distinguish it from H-Ras in its binding properties to Raf-1. As noted above, six of nine amino acids in the G2 effector domain are identical between Rheb and H-Ras, while two of the nonidentical amino acids represent conservative changes. The single nonconservative amino acid at residue 41 of Rheb (equivalent to residue 38 of H-Ras) encodes an asparagine while H-Ras encodes an aspartate. Mutation of the aspartate at position 38 of H-Ras to an asparagine (D38N) abolishes the transforming activity of oncogenic H-Ras and inhibits its interaction with Raf-1 (10, 57, 74). Moreover, a recent crystal structure analysis of the interaction between the G2 effector domain of Rap (which is identical to the G2 domain of H-Ras) and Raf-1 indicates that the charged side chain of aspartate 38 of H-Ras has an important stabilizing effect on this interaction. These observations suggested that the asparagine at position 41 of Rheb may be important in defining the unique binding properties of Rheb. Accordingly, we generated the N41D point mutant of Rheb to test its in vitro binding to Raf-1 from PC12 cell lysates.

N41D Rheb binds Raf-1 in a GTP-dependent manner, and this binding is increased by depolarization of PC12 cells (Fig. 6), similar to both wild-type Rheb and H-Ras. Depolarization-induced increases in Raf-1 binding to N41D Rheb were blocked by pretreatment with nifedipine, indicating a role for calcium influx through voltage-sensitive calcium channels. In contrast to wild-type Rheb, binding of Raf-1 to N41D Rheb is markedly potentiated by EGF and is blocked by forskolin pretreatment (Fig. 5A). Thus, N41D Rheb mimics the binding properties of H-Ras in this assay. EGF stimulation resulted in a substantial increase in binding to the GDP form of N41D Rheb that was not seen with H-Ras in parallel experiments. It is not certain whether this represents a technical difficulty in preparing the GDP form of N41D Rheb or is a true biological property of the mutant protein. Nevertheless, Raf-1 binding to both the GDP and the GTP forms of N41D Rheb is strongly inhibited by forskolin pretreatment, which differentiates it from wild-type Rheb. We conclude that asparagine 41 plays an important role in the increase in Rheb–Raf-1 binding produced by combined EGF and forskolin stimulation. In view of the striking potentiation of the N41D Rheb–Raf-1 interaction by EGF stimulation, the asparagine 41 residue may also play a role in limiting the interaction between wild-type Rheb and Raf-1 under these conditions. Interestingly, calcium-induced increases in the binding of Raf-1 to Rheb are not sensitive to this mutation.

Rheb potentiates neurite outgrowth from PC12 cells. To examine the cellular correlates of our biochemical observations, we monitored the effects of Rheb transgene expression in PC12 cells. Oncogenic H-Ras mutants induce neuritic outgrowth and neuronal differentiation in PC12 cells and mimic the effects of NGF (6, 49). Neuritic outgrowth has been linked to the ability of H-Ras mutants to bind Raf-1 and consequently transfer Raf-1 to the membrane, where its kinase and signaling properties are activated. For example, oncogenic valine 12 H-Ras persists in the active GTP-bound state in which it binds Raf-1 and strongly induces neurite outgrowth in PC12 cells (21, 57). Introduction of a second mutation (D38N) in the G2 domain of valine 12 H-Ras, which inhibits its binding to Raf-1, produces a form of Ras that does not induce neuritic outgrowth (57). Consistent with the notion that mechanisms that increase the activity of Raf-1 result in neurite outgrowth, expression of a constitutively active form of Raf-1 also induces neurite outgrowth (70a).

We assayed the ability of Rheb to induce neurite outgrowth in PC12 cells under growth factor conditions that were identified in biochemical assays to regulate the Rheb–Raf-1 interaction. Neurite outgrowth was assayed 60 h after transient transfection and 48 h after the addition of NGF or second-messenger agents to the media. Transfected cells were identified by using a cotransfected β-galactosidase expression construct and LacZ staining. Neurites were assayed at this relatively early time point to reduce the possible contribution of endogenously expressed Rheb. Endogenous Rheb can be induced in PC12 cells by NGF treatment, but its mRNA time course is relatively delayed compared to the mRNA response induced by either EGF or bFGF. Rheb mRNA is only modestly induced 48 h after addition of NGF but is strongly induced within 1 h of addition of EGF or bFGF (73). By assaying cells 48 h after addition of NGF or second-messenger agents, we anticipated that cells transfected with the Rheb construct would express differentially high levels of Rheb compared to control cells and would permit an assessment of the effects of Rheb expression on PC12 cell phenotype.

Transfection of PC12 cells with Rheb or H-Ras resulted in modest increases in the number of cells with processes when maintained in standard growth media (Fig. 7). Under these control conditions, neurites are evident on 7.6% ± 2.0% (H-Ras; mean ± standard error of the mean [SEM]) and 12.8% ± 1.5% (Rheb) of PC12 cells coexpressing the LacZ marker. Less

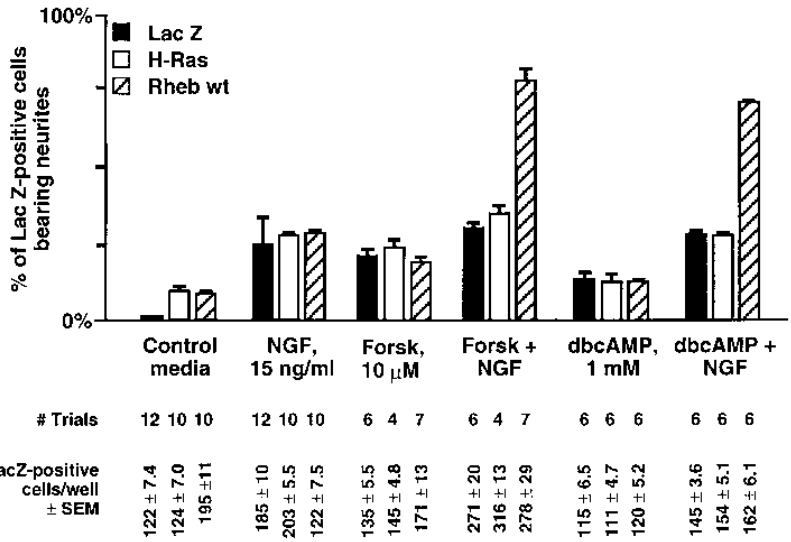

FIG. 7. Rheb induces neurite outgrowth that is selectively potentiated by NGF and cAMP. PC12 cells were transiently transfected with Rheb or H-Ras expression constructs in combination with a β-galactosidase marker. At 12 h after transfection, the medium was supplemented with 10 μM forskolin (Forsk), 1 mM dibutyryl cAMP (dbcAMP), and/or 15 ng of NGF per ml. The percentage of LacZ-positive cells presenting neurites after 48 h of treatment was compared. Both Rheb and H-Ras produced a modest increase in the number of cells with neurites when grown in control medium (control, <2%; Rheb, 9.0%; H-Ras, 10.0%). Rheb, H-Ras, and control transfected cells grown in medium supplemented with forskolin, dibutyryl cAMP, or NGF alone exhibited similar increases in neurite outgrowth to that of cells grown in control medium. By contrast, Rheb-transfected PC12 cells treated with combined NGF and forskolin (79.3%) or with combined NGF and dibutyryl cAMP (72.0%) produced at least twofold more cells with neurites than did control cells (30.5 and 28.0%, respectively) or PC12 cells transfected with H-Ras (34.9 and 28.0%, respectively). The mean neurite counts, mean number of observed LacZ-positive PC12 cells per well, and number of trials per transfection and treatment are reported below each bar graph.

than 2% of the cells transfected with the LacZ marker alone possessed neurites. Treatment of cells with either forskolin (10 μM) or dibutyryl cAMP (1 mM) alone produced a moderate increase in the number of cells with neurites. In contrast, combined forskolin-NGF (15 ng/ml) treatment induced neurite outgrowth in 79.3% ± 4.2% of cells transfected with Rheb, 34.9% ± 2.6% of cells transfected with H-Ras, and 30.5% ± 1.9% of cells transfected with the control vector. Dibutyryl cAMP (1 mM) was equally effective as forskolin in this assay, supporting the notion that these agents act to increase intracellular cAMP-dependent signaling. Additional statistical analysis of the neurite assays is presented in Fig. 7.

These observations are consistent with our biochemical data and suggest that Rheb functions in PC12 cells to activate Raf-1. In biochemical assays, activators of PKA act in conjunction with growth factors to increase Rheb–Raf-1 binding, while in cell culture assays, PKA activators in conjunction with NGF selectively potentiate neurite outgrowth from PC12 cells transfected with Rheb. Nevertheless, it is possible that Rheb acts indirectly to mimic the phenotype of activated Raf, and additional experiments will be required to determine whether Rheb directly activates Raf-1 in vivo.

DISCUSSION

Our observations indicate that Rheb interacts with Raf-1 in a novel way that is regulated by growth factor and second-messenger activation. Rheb shares many properties with its closest homolog, H-Ras, including a C-terminal CAAX box and a conserved G2 effector domain (73). Consistent with this sequence homology, Rheb binds and hydrolyzes GTP and interacts with Raf-1 in a GTP-dependent manner. Like H-Ras, Rheb is synergistic with Raf-1 in transforming the growth properties of NIH 3T3 fibroblasts. The Rheb–Raf-1 interaction, like the H-Ras–Raf-1 interaction, requires the amino-terminal one-third of Raf-1 and is sensitive to mutation in the G2 domain of Rheb. In addition, the in vitro binding of recombinant Rheb to cellular Raf-1 is potentiated by prior growth factor stimulation. The latter observation, in conjunction with evidence that in vitro phosphorylation of Raf-1 by PKA modifies both Rheb–Raf-1 and H-Ras–Raf-1 binding, suggests that these interactions can be modified by signal-dependent phosphorylation of Raf-1 (see below). Since Rheb is regulated as an immediate-early gene and is induced by growth factor stimulation, these observations are consistent with the notion that Rheb functions as part of a positive-feedback response to enhance Raf-1-dependent growth factor signaling.

Despite its many shared properties with H-Ras, Rheb possesses several unique features that suggest that it functions to modify, rather than simply amplify, Raf-1-dependent signaling. Analysis of the in vitro binding of cellular Raf-1 to recombinant Rheb or H-Ras indicates that the Rheb–Raf-1 and H-Ras–Raf-1 interactions are reciprocally regulated by concomitant growth factor and cAMP stimulation. Consistent with in vitro biochemical observations, Rheb potentiates neurite outgrowth from PC12 cells under conditions of combined NGF and forskolin or dibutyryl cAMP stimulation. Because of the close association between Raf-1 activation and neurite outgrowth in this paradigm, the ability of Rheb to potentiate these effects suggests that it is able to increase the activation of Raf-1 under specific conditions which distinguish its signaling function from that of H-Ras. Together, these observations suggest a functional distinction in which H-Ras transduces growth factor stimuli in the absence of concomitant cAMP stimulation, while Rheb transduces growth factor stimuli in the presence of cAMP stimulation (Fig. 8).

The current model of the H-Ras pathway suggests that H-Ras functions to translocate inactive Raf-1 to the cell membrane, where Raf-1 interacts with additional proteins which stimulate its kinase and signaling activity (39, 59). Our studies

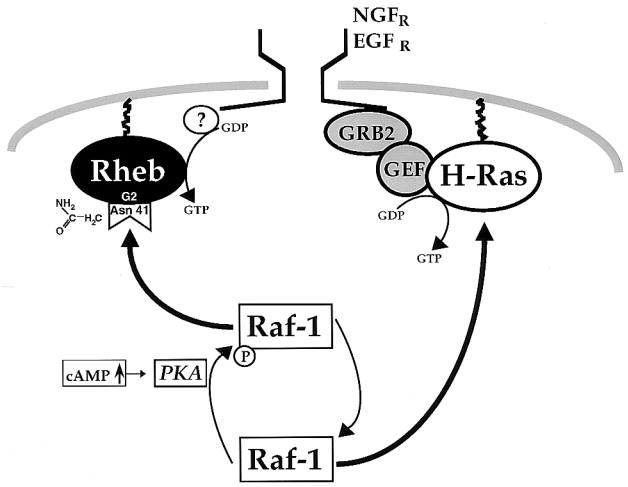

FIG. 8. Proposed model of Rheb interaction with Raf-1 kinase. Rheb signals downstream of growth factor receptors in a pathway that is divergent from H-Ras and is differentially activated by concomitant growth factor stimulation and increased intracellular cAMP. Growth factor-induced Rheb signaling is potentiated in this model by phosphorylation of Raf-1 and the consequent increase in Raf-1 binding to Rheb. In contrast, growth factor-induced H-Ras signaling via Raf-1 is blocked by PKA activation due, in part, to phosphorylation of Raf-1 and consequent inhibition of Raf-1 binding to H-Ras. An important implication of this model is that Raf-1 can be selectively targeted to interact with either Rheb or H-Ras by the cAMP second-messenger pathway. The model indicates that asparagine in position 41 of Rheb is critical for the unique Raf-1 binding properties of Rheb. Upstream mechanisms regulating the activation of Rheb remain to be determined.

suggest a novel regulatory mechanism for Ras-dependent signaling that confers differential targeting of Raf-1 to either Rheb or H-Ras. Changes in Raf-1 binding to H-Ras and Rheb are inferred to involve posttranslational modifications of Raf-1, as opposed to changes in the Ras proteins, since the Rheb and H-Ras used in the in vitro binding assay are recombinant proteins that are not subjected to the in vivo stimulation conditions. Consistent with a phosphorylation mechanism, in vitro phosphorylation by PKA of Sf11 cell-derived Raf-1 or purified, bacterially expressed GSTRaf(1–149) increases their interaction with Rheb and decreases their interaction with H-Ras, mimicking the effects of the in vivo stimulation on Raf-1 binding. Accordingly, rapid changes in the phosphorylation state of Raf-1 may contribute to the selective activation of the H-Ras and Rheb signaling pathways.

It should be noted that these PKA-dependent changes in Raf binding do not represent the complete picture of mechanisms controlling the Rheb-Raf interaction, since the in vitro binding data generated from PC12 cell extracts indicate a requirement for combined growth factor and PKA activation. Additional mechanisms that may contribute to signal-dependent changes in Raf-1-binding activity are suggested by recent studies indicating that 14-3-3 proteins bind Raf-1 and assist in its activation in cell-free systems (40) and in yeast (19, 29). Analysis of proteins that bind to Rheb and H-Ras in our in vitro assays demonstrates the presence of the Raf-1-associated proteins MEK-1 and 14-3-3 (unpublished observation). Signal-dependent changes in Raf-1 binding may therefore involve additional interacting proteins that might allosterically regulate the binding properties of Raf-1. It will be important to understand how such allosteric effects or, alternatively, direct phosphorylation of Raf-1 can differentially affect its binding to Rheb and to H-Ras. As a final caveat, we note that the potentiating effect of activators of PKA in combination with growth factors may be cell type specific, since we have been unable to detect this effect with NIH 3T3 fibroblasts.

The demonstration that Rheb interacts with B-Raf and that the interaction is regulated in parallel with the Rheb–Raf-1 interaction may also be important for understanding the signaling function of Rheb. Recent reports indicate that B-Raf, and not Raf-1, is the kinase primarily responsible for the phosphorylation and activation of MEK1 in brain and PC12 cell extracts (31, 43). Our data are consistent with the notion that Rheb, like H-Ras, may utilize both Raf-1 and B-Raf. As with Raf-1, Rheb would play a distinct role in activating B-Raf signaling in response to convergent growth factor and cAMP stimulation.

Rheb is widely and abundantly expressed in rodent tissues, and we anticipate that Rheb is a general partner to H-Ras in many cell types. Recently, a human Rheb amino acid sequence (GenBank accession number Z29677) cloned from keratinocytes has been reported and is identical to the rat sequence with the single exception of I170M, demonstrating evolutionary conservation of Rheb. Although the specific contribution of Rheb to natural cellular responses remains to be determined, there are numerous reports of "Ras-independent" growth factor signaling (24, 33, 41), which might be mediated by Rheb. In PC12 cells, stable expression of dominant negative H-Ras (N17 Ras) blocks NGF-induced neurite outgrowth (61) but does not block outgrowth induced by combined NGF and PKA activators (62). Because mechanisms regulating the GTP state of Rheb remain to be determined, particularly whether N17 Ras might also inhibit Rheb activation, it is not yet possible to determine the signaling properties of Rheb in the absence of functional H-Ras.

Regulation of Rheb expression in the intact animal has been examined, to date, only in the brain. Rheb is rapidly induced in neurons of the hippocampus following *N*-methyl-D-aspartate-dependent synaptic stimuli that induce long-term potentiation, a form of synaptic plasticity associated with specific structural changes of the dendrite and synapse (4). It is interesting that the late phase of long-term potentiation is dependent on persistent activation of cAMP-dependent processes, presumably including PKA (20). Neural plasticity in *Aplysia* (7, 55, 60) and *Drosophila melanogaster* (16, 58) similarly appears to involve persistent activation of PKA. The unique activation of Rheb in the presence of increased cAMP levels and its demonstrated effect on neurite outgrowth in PC12 cells suggest that Rheb may participate in events linking neuronal activity with neuritic remodeling or cytodifferentiation, particularly under conditions of persistent activation of PKA.

In summary, we propose that Rheb functions in concert with H-Ras in the transduction of extracellular stimuli. Specifically, Rheb contributes a unique, positive integration of coincident growth factor and cAMP signals. We hypothesize that the ratio of Rheb to H-Ras in a cell, and hence the ability of the cell to potentiate or inhibit growth factor signals that are coincident with PKA activation, may be dynamically regulated, since Rheb expression is rapidly induced by growth factor and transmitter stimulation. Rheb should provide new insights into cell signaling and mechanisms that underlie long-term adaptive cellular responses.

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