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Increased Expression of the Ras Suppressor Rsu-1 Enhances Erk-2 Activation and Inhibits Jun Kinase Activation

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Studies were undertaken to determine the effect of the Ras suppressor Rsu-1 on Ras signal transduction pathways in two different cell backgrounds. An expression vector containing the mouse *rsu-1* cDNA under the control of a mouse mammary tumor virus promoter was introduced into NIH 3T3 cells and the pheochromocytoma cell line PC12. Cell lines developed in the NIH 3T3 background expressed p33^{rsu-1} at approximately twice the normal endogenous level. However, PC12 cell clones which expressed p33^{rsu-1} at an increased level in a regulatable fashion in response to dexamethasone were isolated. Analysis of proteins involved in regulation of Ras and responsive to Ras signal transduction revealed similar changes in the two cell backgrounds in the presence of elevated p33^{rsu-1}. There was an increase in the level of SOS, the guanine nucleotide exchange factor, and an increase in the percentage of GTP-bound Ras. In addition, there was an increase in the amount of p120 Ras-specific GTPase-activating protein (GAP) and GAP-associated p190. However, a decrease in Ras GTPase-activating activity was detected in lysates of the Rsu-1 transfectants, and immunoprecipitated p120 GAP from the Rsu-1 transfectants showed less Ras GTPase-activating activity than GAP from control cells. Activation of Erk-2 kinase by growth factor and tetradecanyol phorbol acetate was greater in the Rsu-1 transfectants than in control cells. However, c-Jun amino-terminal kinase activity (Jun kinase) was not activatable by epidermal growth factor in Rsu-1 PC12 cell transfectants, in contrast to the PC12 vector control cell line. Transient expression of p33^{rsu-1} in Cos1 cells following cotransfection with either hemagglutinin-tagged Jun kinase or hemagglutinin-tagged Erk-2 revealed that Rsu-1 expression inhibited constitutive Jun kinase activity while enhancing Erk-2 activity. Detection of in vitro binding of Rsu-1 to Raf-1 suggested that in Rsu-1 transfectants, increased activation of the Raf-1 pathway occurred at the expense of activation of signal transduction leading to Jun kinase. These results indicate that inhibition of Jun kinase activation was sufficient to inhibit Ras transformation even in the presence of activated Erk-2.

One approach to deciphering the necessary cellular changes that occur during the transformation process has been to identify genes which inhibit the process and determine their contribution to maintenance of the nontransformed state. This approach has been successfully applied in the case of transformation by the *ras* oncogene, in which case a number of genes with this property have been studied (6, 21–23, 30, 32). One approach for identification of Ras suppressor genes has been use of an expression cloning assay for the isolation of genes capable of suppressing the Ras-transformed phenotype. Using this approach, the *rsu-1* cDNA was isolated by expression cloning on the basis of its ability to suppress transformation by Ki-Ras (11). Subsequent experiments demonstrated that the expression of the *rsu-1* gene under the control of a heterologous promoter could suppress transformation by the v-*ras* but not the v-*raf*, v-*mos*, or v-*src* oncogene. *rsu-1* is a highly conserved single-copy gene (11, 43) which encodes a protein of 33 kDa. p33^{rsu-1} contains a series of 23-amino-acid leucine-based amphipathic repeats homologous to the repeats found in yeast adenylyl cyclase in the region through which Ras activates adenylyl cyclase in *Saccharomyces cerevisiae* (5, 13). Similar repeats are also found in the *Drosophila* adhesion proteins Toll and Chaoptin (24) as well as in the von Willebrand factor receptor proteins. Proteins with extensive homology to the carboxy-terminal region of Rsu-1 have not been detected in databases; this region of the protein contains putative sites for phosphorylation by serine/threonine kinases.

Recent studies examining the function of the small GTP-binding proteins Rho, Rac, and Cdc42Hs have indicated that Rho functions in the formation of actin stress fibers (34) and that Rac controls formation of filopodia and lamellipodia, which represent the events in membrane ruffling (29, 35). Because these proteins are activated in the transformation process induced by Ras, these findings suggested that Rho and Rac proteins may be the downstream effectors of change in cellular adhesion which occurs as a result of Ras transformation. Further studies using dominant negative Rho and Rac have demonstrated that these proteins control actin stress fiber formation and membrane ruffling, respectively, in Ras-transformed cells (19, 26, 33). Inhibition of Rho and/or Rac pathways can inhibit transformation by Ras, suggesting that these proteins regulate pathways as essential for Ras transformation. Moreover, Cdc42Hs and Rac have been shown to be required for the activation of the stress-activated or Jun kinase (9, 28, 31), and RhoA has been shown to be necessary for activation of the serum response factor by serum and other agents that act through the pertussis-sensitive G-protein pathway (17).

The approach used in the experiments reported here has been to increase Rsu-1 expression in several different cell types and then to analyze the biochemical pathways downstream of Ras to detect differences between the Rsu-1 transfectants and the control cells. These studies used NIH 3T3 cells and the pheochromocytoma cell line PC12, and the results indicate

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that the biochemical changes induced by Rsu-1 are very similar in the two cell lines. Rsu-1 expression increases the level of SOS, GTP-bound Ras, and Erk-2 signal transduction downstream of Ras. However, the levels of Ras-specific GTPase-activating protein (RasGAP) and the GAP-associated protein, p190, increase in Rsu-1 transfectants. Because p190 has RhoGAP activity (37) and the association with GAP activates this activity (27), the status of pathways regulated by G proteins downstream of Ras was examined. The activation of Jun kinase was tested in the Rsu-1 transfectant cells. The results shown here indicate that Rsu-1 expression inhibits Jun kinase. Therefore, it appears that the block to Ras transformation induced by Rsu-1 may involve the inhibition of pathways downstream of Ras controlled by the small G proteins Rac and Rho.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells and NIH 3T3 transfectants were maintained in Dulbecco modified Eagle medium (DMEM) (Gibco BRL) with 10% fetal bovine serum (FBS), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 50 mg of streptomycin per ml, and 50 U of penicillin per ml. PC12 cells, obtained from the American Type Culture Collection, and PC12 transfectants were grown in DMEM with 10% charcoal-stripped horse serum (Biofluid), 5% dialyzed FBS (Gibco BRL), 10 mM HEPES (pH 7.4), 50 mg of streptomycin per ml, and 50 U of penicillin per ml. Cos1 cells, obtained from the American Type Culture Collection, were grown in DMEM with 10% FBS, 50 mg of streptomycin per ml, and 50 U of penicillin per ml.

Plasmid construction and transfection. p3v54 was generated by directionally inserting a 1.3-kb fragment encoding the entire open reading frame and 3′ untranslated region of the *rsu-1* cDNA into the pMAMneo vector (Clontech Laboratories Inc., Palo Alto, Calif.). The *rsu-1* fragment was isolated from p3v1-1a (11), a pBluescript-derived plasmid, by digestion with *Sma*I and *Sal*I. It was gel purified and ligated into vector pMAMneo between a blunt-ended *Nhe*I site and an *Xho*I site. The resulting plasmid, p3v54, contained Rsu-1 under the control of a dexamethasone-inducible mouse mammary tumor virus long terminal repeat promoter. To obtain stable transfectants, p3v54 was transfected into PC12 cells by electroporation using a Gibco BRL electroporation apparatus. Briefly, 5×10^6 cells per ml in serum free DMEM were electroporated with 20 μg of plasmid DNA (pMAMneo or p3v54), incubated on ice for 10 min, resuspended in DMEM supplemented with 4.5 g of glucose per liter, 15% charcoal-stripped horse serum, and 5% FBS, and plated on tissue culture dishes. After 48 h, the medium was replaced with medium containing 500 μg of G418 (Gibco BRL) per ml for the selection of positive clones. After 30 days of selection, single colonies were isolated and analyzed by Northern (RNA) blotting and Western blotting (immunoblotting) analyses. Screening of 25 clones revealed two clones inducible by dexamethasone, PC12-20 and PC12-26, which were retained for further analysis. NIH 3T3 cells were transfected by calcium phosphate precipitation as previously described (11). For transient transfections, Cos1 cells were seeded at a density of 2×10^5 in 60-mm-diameter dishes the day before transfection. The cells were transfected by lipofection using DOTAP reagent (Boehringer Mannheim) with 20 μg of plasmid DNA for 24 h. The constructs used were p3v26, which contained *rsu-1* cDNA under the control of a simian virus 40 promoter, and p521, the vector control. At 72 h posttransfection, the transfected cells were lysed and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Northern blotting. Eighty percent confluent cell cultures, with or without induction by 1 mM dexamethasone for 48 h, were lysed with guanidine thiocyanate, and total RNA was purified on cesium chloride gradients. Ten micrograms of total RNA was loaded on a 1% agarose gel containing morpholinepropanesulfonic acid (MOPS; pH 7) and formaldehyde (2%) and run at 50 V for 5 h. The gel was transferred to nylon membrane (Genescreen; DuPont-NEN, Boston, Mass.), cross-linked, and hybridized with a mouse *rsu-1*-specific probe in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–1× Denhardt's solution–100 μg of sonicated salmon sperm DNA per ml–0.1% SDS at 42°C for 24 h. The blots were washed for 5 min at room temperature in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS and then 1 h at 55°C in 0.1% SSC–0.5% SDS with three buffer changes. The *rsu-1* probes were labeled with [^{32}P]dCTP (DuPont-NEN) to a specific activity of $>5 \times 10^8$ cpm/μg by random priming.

Antibodies. Anti-Rsu-1 polyclonal antibody was obtained by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein containing the COOH-terminal 45 amino acids of Rsu-1 (44). Monoclonal antibodies recognizing Erk-2, Shc, and phosphotyrosine (4G10) were purchased from Upstate Biotechnology Inc., Lake Placid, N.Y. Polyclonal antibodies against Erk-2, Raf-1, Mek1 and -2, p120 GAP, Rho, and Rac and the monoclonal antibody against p120 GAP and Ras (Y13-259) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.). Monoclonal antibodies against p190 RhoGAP, Raf-1, SOS, and Grb2 were purchased from Transduction Laboratories, Lexington, Ky. Monoclonal antibody PY20, against phosphotyrosine, was obtained from ICN (Irvine, Calif.). Monoclonal antibody against vimentin (vim11) was purchased from Sigma (St. Louis, Mo.), and monoclonal antibody against Jun kinase was from Pharmingen.

Immunoprecipitation. The cells grown to 80% confluence were chilled on ice, washed with phosphate-buffered saline (PBS), scraped, and lysed for 10 min on ice with lysis buffer containing 1% Nonidet P-40 (NP-40) or 1% Triton X-100 (Sigma), 0.5% sodium deoxycholate (DOC; Calbiochem, La Jolla, Calif.), 150 mM sodium chloride, 20 mM Tris-HCl (pH 7.5), 2.5 μg of aprotinin per ml, 2 μg of leupeptin per ml, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and 1 mM sodium vanadate. After protein concentration determination by the Bradford protein assay (Bio-Rad), specific amounts of lysates were incubated with the antibodies for 2 h on ice, and the immunoprecipitates were collected by using agarose-conjugated recombinant protein G (Gibco BRL). After four washes with lysis buffer, the beads were resuspended in 1× SDS sample buffer containing 100 mM dithiothreitol (DTT) and boiled for 5 min. Following centrifugation to pellet the beads, the proteins in the supernatant fluids were separated by SDS-PAGE.

Western blotting. Lysates (50 μg) were separated by SDS-PAGE, transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.), and blocked overnight in 1× blocking reagent, 3% bovine serum albumin (BSA) (Boehringer Mannheim) in Tris-buffered saline. The filters were reacted with primary antibodies at a concentration of 1 μg/ml, washed in Tris-buffered saline containing 0.4% Tween 20 (United States Biochemical Corp., Cleveland, Ohio), and incubated with the specific horseradish peroxidase-conjugated secondary antibody (Amersham Life Science Inc., Arlington Heights, Ill.). The filters were developed by chemiluminescence, using the Boehringer Mannheim system and Kodak X-Omat film. To detect p33^{rsu-1}, filters were reacted with 10 μg of anti-Rsu-1 immunoglobulin G (IgG) and then alkaline phosphatase-conjugated anti-rabbit IgG. Detection was accomplished with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT-BCIP).

Expression and purification of recombinant Ki-Ras. Ki-*ras-2*(4B) was introduced into the expression vector pD30 (25), replacing Ha-*ras* and expressed under the control of an inducible λ p_L promoter. The new plasmid, called pRV11, was transformed into *Escherichia coli* N-4380/1 by electroporation. The production of the protein was induced by temperature shift from 35 to 42°C for 2 h; after induction, the cultures were harvested, lysed in a buffer containing 8 M urea and 20 mM Tris (pH 7.5), and sonicated. After clarification, the lysates were loaded on a Sephadex G-100 size column, and 0.5-ml fractions were collected. All of the fractions were analyzed by Western blotting and Coomassie blue staining. Fractions containing the highest level of Ras were pooled and subjected to a second round of chromatography. Seventy percent pure fractions were used in the experiments.

Ras GTPase-activating activity assay. The GTPase-activating activity assay was performed on cell lysates by using slight modifications of previously described methods. Briefly, confluent cultures of the transfectant cell lines were lysed in a lysis buffer containing 0.5% NP-40, 180 mM NaCl, 25 mM Tris-HCl (pH 7.5), inhibitors for 10 min on ice and then clarified by centrifugation. Ten micrograms of lysate was incubated in a total volume of 50 μl of lysis buffer at 30°C for 30 min in the presence of 1 ng of purified recombinant Ki-Ras preloaded with [α-^{32}P]GTP by incubation in a buffer containing 50 mM Tris HCl (pH 7.5), 80 mM NaCl, and 5 mM $MgCl_2$ in the presence of 50 μCi of [α-^{32}P]GTP (DuPont-NEN) for 30 min at 30°C. The reactions were stopped by addition of 300 μl of cold lysis buffer, and Ki-Ras was immunoprecipitated with 2.5 μg of the rat monoclonal antibody Y13-259 and goat anti-rat precoated agarose-conjugated recombinant protein G for 1 h at 4°C. After several washes with lysis buffer, the immunoprecipitates were resuspended in 10 μl of 1% SDS–10 mM EDTA, heated at 65°C for 10 min, and loaded on a polyethyleneimine (PEI)-cellulose thin-layer chromatography plate (Aldrich Chemical Company, Inc.) for separation using 1.3 M $LiCl_2$. The plates were then subjected to autoradiography and scanned by an AMBIS system (Automated Microbiology System Inc., San Diego, Calif.).

The GAP GTPase-activating activity assay was performed as previously described, with minor changes. Briefly, confluent cultures of the transfectant cell lines were lysed in a lysis buffer containing 1% Triton X-100, 0.5% deoxycholate, 50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, and 1 mg of BSA per ml for 10 min on ice and clarified by centrifugation. Then 700 μg of lysate was immunoprecipitated with 2 μg of a specific anti-p120 GAP polyclonal antibody and agarose-conjugated recombinant protein G. The immunoprecipitates were washed four times with a buffer containing 0.1% Triton X-100, 50 mM HEPES (pH 7.4), 0.5 M NaCl, and 5 mM $MgCl_2$ and once with a GTPase buffer containing 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 2 mM DTT, 0.1% NP-40, and 500 μg of BSA per ml. The immunoprecipitates were resuspended in 100 μl of GTPase buffer and incubated with 0.5 ng of purified recombinant Ki-Ras, preloaded with [α-^{32}P]GTP as previously described, for 30 min at 37°C. The reactions were stopped by adding 250 μl of lysis buffer, and Ki-Ras was immunoprecipitated with Y13-259 antibody and analyzed by thin-layer chromatography as previously described.

Analysis of GTP-Ras in vivo. To analyze the levels of GTP-Ras in the transfectants, confluent cell cultures were labeled for 16 h in phosphate-free DMEM (Gibco BRL) supplemented with 5% FBS in the presence of 0.5 mCi of $^{32}P_i$ (DuPont-NEN). The medium was removed, and the cell layers were washed with cold PBS and lysed in NP-40–DOC lysis buffer. Ras was immunoprecipitated

with 2.5 μg of Y13-259 and goat anti-rat precoated recombinant protein G-agarose. After several washes with lysis buffer, the immunoprecipitates were resuspended in 1% SDS–10 mM EDTA, heated for 10 min at 65°C, and separated by thin-layer chromatography on PEI plates. The plates were autoradiographed and quantitated with an AMBIS system.

Mitogen-activated protein kinase (MAPK) assay. NIH 3T3 transfectants were maintained in medium containing 1% FBS for 16 h prior to stimulation with 500 ng of tetradecanoyl phorbol acetate (TPA; Calbiochem) per ml. PC12 transfectants were maintained in regular medium before stimulation with 500 ng of TPA per ml or 50 ng of recombinant epidermal growth factor (EGF; Gibco BRL) per ml. For Erk-2 phosphorylation gel shift assay, 50 μg of NP-40–DOC lysates from stimulated or unstimulated cells was resolved by SDS-PAGE on a 10% Tris glycine gel, transferred to nitrocellulose, and reacted by Western blotting analysis with a specific anti-Erk-2 monoclonal antibody and detected by alkaline phosphatase-conjugated secondary antibody and NBT-BCIP (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). For myelin basic protein (MBP) phosphorylation assay, 100 μg of NP-40–DOC lysates from stimulated or unstimulated cells was immunoprecipitated with a specific polyclonal antibody that recognize both Erk-1 and Erk-2. The immunoprecipitates were washed twice with lysis buffer and twice with a kinase buffer containing 30 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 1 mM DTT. The immunoprecipitates were resuspended in 30 μl of kinase buffer containing 5 μg of MBP and 10 μCi of [γ-^{32}P]ATP (DuPont-NEN) and incubated for 30 min at 30°C. The reactions were stopped by the addition of 2× sample buffer and resolved by SDS-PAGE. After transfer to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.), the filters were subjected to autoradiography and quantitated with an AMBIS system.

Jun kinase assay. The Jun kinase assay was performed in two different ways. In Rsu-1 stable transfectants and vector control cell (PC12) clones, Jun kinase activity was measured from crude lysate of cells as described previously (8). Cells were lysed in buffer (25 mM HEPES [pH 7.5], 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 20 mM β-glycerol phosphate, 1 mM vanadate, 0.1% Triton X-100, 1 mM AEBSF, 20 μg of aprotinin per ml, 20 μg of leupeptin per ml), nuclei were removed, and the protein content of the lysate was determined. Five micrograms of GST–c-Jun fusion protein bound to glutathione-Sepharose beads was incubated with 200 μg of lysate at 4°C for 3 h with continuous agitation. GST–c-Jun beads were recovered by centrifugation, washed three times in PBS–1% NP-40–2 mM vanadate), one time in 100 mM Tris (pH 7.5)–0.5 M LiCl, and one time in kinase buffer (12.5 mM MOPS [pH 7.5], 12.5 mM β-glycerol phosphate, 7.5 mM $MgCl_2$, 0.5 mM ethylene glycol-bis(B-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA), 0.5 mM NaF, 0.5 mM vanadate), and resuspended in 30 μl of kinase buffer containing 1 μCi of [γ-^{32}P]ATP, 20 mM unlabeled ATP, and 3.3 mM DTT for 30 min at 30°C. Beads were heated in sample buffer prior to electrophoresis on SDS–10% polyacrylamide gels. The protein was transferred to a nitrocellulose filter, and the filter was used for autoradiography and quantitation by AMBIS screening. Immunoprecipitates of Jun kinase were prepared and used for immune kinase assay with GST–c-Jun as a target. Jun kinase was precipitated from PC12 cell transfectants by using a Jun kinase monoclonal antibody and protein G-Sepharose. Jun kinase activity from cells transiently transfected with Rsu-1 or a control expression plasmid and a plasmid encoding hemagglutinin (HA)-tagged Jun kinase was recovered by immunoprecipitation with anti-HA antibody (clone 12CA5; Boehringer Mannheim) and protein G-Sepharose (9). The Jun kinase immunoprecipitates were incubated with GST–c-Jun in kinase buffer for 30 min at 30°C with continuous agitation. The beads were recovered, washed, and resuspended in sample buffer for SDS-PAGE. Proteins were transferred to nitrocellulose filters, which were used for autoradiography and quantitation with an AMBIS system, and reacted with anti-HA and anti-GST antibodies to determine the amount of protein in each sample.

Immunofluorescence. Cells were grown to 80% confluence on chambered coverglass (Nunc Inc., Naperville, Ill.). For actin staining, the medium was aspirated and the cells were washed with cold PBS, fixed in a 3.7% solution of formaldehyde-PBS for 10 min at room temperature, and permeabilized by incubation for 10 min in 0.2% Triton X-100 in PBS. Fluorescein isothiocyanate-phalloidin (Sigma) was then added to the cells at a concentration of 333 nM in PBS, and the cells were incubated for 45 min at room temperature. After several washes in PBS, the coverslips were mounted on a slide with 50% glycerol–PBS and observed and photographed with a Zeiss Axiophot with an automatic exposure system on Kodak TMAX film (ASA 400). For vimentin staining, the cells were fixed in 100% methanol maintained at −20°C for 5 min on ice, washed in cold PBS, and incubated with a dilution 1:400 of the primary antibody in PBS for 1 h at room temperature. After several washes in PBS, the cells were reacted with a rhodamine-conjugated rabbit anti-mouse antibody (Kirkegaard & Perry) at a 1:100 dilution. The slides were washed and observed as described above.

Binding of GST-Rsu-1 to Raf-1. The Rsu-1 open reading frame was introduced into the GST fusion protein vector GEX-2T to create Rsu-1 2T-4. In addition, two other GST–Rsu-1 fusion proteins were prepared: Rsu-1 2T-term2, containing a premature termination codon introduced at amino acid 212, which gave rise to a truncated Rsu-1 protein, and Rsu-1 2T-4Δ, in which amino acids 236 to 277 of Rsu-1 were introduced in frame with GST, giving rise to a carboxy-terminal Rsu-1 fusion protein. The GST fusion proteins were prepared in bacteria and purified by using glutathione-Sepharose (Pharmacia). For binding to Raf-1, 10 μg of each fusion protein or the GST control protein bound to glutathione-Sepharose beads was incubated with histidine-tagged Raf-1 protein purified from insect cells infected with a Raf-1 vector. Raf-1 baculovirus-infected insect cell lysates, provided by Ulf Rapp, were lysed under denaturing conditions, and the Raf-1 protein was purified by nickel chromatography. Column fractions containing Raf-1 were identified and characterized by staining and Western blotting using anti-Raf-1 antibody. Fractions were dialyzed against 0.1% Triton X-100 in 20 mM Tris (pH 8). Prior to binding, GST–Rsu-1 proteins were washed twice in a buffer consisting of 0.25% Triton X-100, 20 mM Tris (pH 8), and 25 mM NaCl; binding to Raf-1 was performed in that buffer at 4°C for 45 min on a rocker. The GST fusion proteins were washed twice in a buffer consisting of 0.5% Triton X-100 20 mM Tris (pH 8), and 25 mM NaCl prior to heating in gel loading buffer. Following SDS-PAGE and transfer to nitrocellulose filters, the presence of Raf-1 was detected by using a monoclonal antibody to Raf-1.

RESULTS

Construction of Rsu-1 transfectant cell lines. Although p33^{rsu-1} is expressed ubiquitously in cells in culture, transfection of cells with vectors containing *rsu-1* under the control of a heterologous promoter allowed the isolation of cell lines expressing higher levels of p33^{rsu-1} which were resistant to transformation by v-Ras (11). To determine the mechanism of suppression of Ras transformation, Rsu-1 transfectant cell lines were compared with control cell lines to detect biological and biochemical differences in Ras signaling pathways. The original Rsu-1 transfectant cell lines contained *rsu-1* under the control of a mouse metallothionein-1 promoter, which was not completely regulatable in NIH 3T3 fibroblasts and resulted in constitutive transcription of vector-encoded *rsu-1* RNA. Because the transfectants constitutively expressing a higher level of p33^{rsu-1} were slowly growing cell lines, it was difficult to maintain long-term cultures of these cells. Therefore, additional Rsu-1 transfectant cell lines were isolated and, along with one original Rsu-1 transfectant (11), were used for comparison with control cell lines. Additional NIH 3T3 cell lines were constructed by using a vector, p3v54, which was derived from pMAMneo and contained *rsu-1* under the control of a dexamethasone-inducible mouse mammary tumor virus promoter. Following screening of p3v54 NIH 3T3 transfectant cell lines by Northern and Western blotting, a cell line, NIH 3T3 clone q, which had an increased level of Rsu-1 expression compared with the control NIH 3T3 cells, was selected (Fig. 1). This cell line was retained and used for additional experiments along with a vector control cell line, MAM, and one of the original NIH 3T3 transfectant, 8-1, containing *rsu-1* under the control of a metallothionein-1 promoter (11).

Rsu-1 transfectants in the pheochromocytoma cell line PC12 were isolated following electroporation of p3v54 DNA into PC12 cells and selection in G418. As seen in Fig. 1, two PC12 p3v54 transfectants which exhibited regulatable expression of *rsu-1* RNA in response to dexamethasone were obtained. Western blotting of lysates from dexamethasone-induced cells revealed that the level of p33^{rsu-1} was higher than in the control cell line following induction (Fig. 1). These cell lines, referred to as PC12-20 and PC12-26, were retained for further experimentation along with a PC12 pMAMneo vector control, PC12-a.

There is an increase in the level of GTP-bound Ras and an increase in SOS protein level in Rsu-1 transfectant cell lines. Rsu-1 transfectants were examined for changes in Ras and the proteins associated with Ras signal transduction pathways. Lysates of Rsu-1 transfectant cell lines and control cell lines were isolated from 90% confluent cultures and used for the determination of levels of specific proteins. An NIH 3T3 cell transfectant, clone q, and the control cells were exposed to dexamethasone for 24 h prior to lysis. The NIH 3T3 cell line 8-1 was untreated. All of the PC12 cell lines were exposed to dexamethasone for 36 h prior to lysis to induce expression of

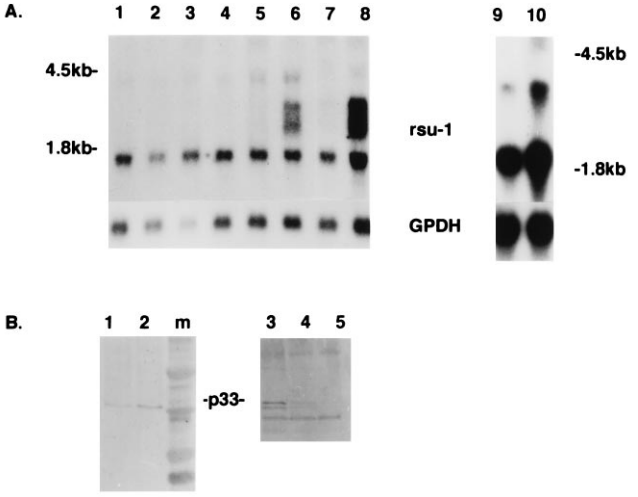

FIG. 1. Expression of Rsu-1 RNA and protein in NIH 3T3 and PC12 transfectant cell lines. (A) Northern blots of 10 μg of total RNA from transfectant cell lines hybridized to the mouse *rsu-1* probe and then stripped and hybridized to a probe for glyceraldehyde-3-phosphate dehydrogenase (GPDH). Lanes 1 and 2, PC12 transfectant a; lanes 3 and 4, PC12 transfectant 13; lanes 5 and 6, PC12 transfectant 20; lanes 7 and 8, PC12 transfectant 26; lane 9, NIH 3T3 cell MAM vector control; lane 10, NIH 3T3 cell clone q. Lanes 1, 3, 5, and 7 contain RNA from untreated cells; lanes 2, 4, 6, 8, 9, and 10 contain RNA from cells exposed to dexamethasone for 48 h prior to RNA extraction. (B) Western blots of lysates from dexamethasone-treated cells reacted with anti-Rsu-1 IgG and alkaline phosphatase-conjugated anti-rabbit IgG and detected with NBT-BCIP. Lane 1, NIH 3T3; lane 2, clone q; lane m, size markers; lane 3, PC12 clone 26; lane 4, PC12 clone 20; lane 5, PC12 clone a.

p33$^{rsu\text{-}1}$. The level of p21^{ras} as determined by immunoprecipitation and Western blotting changed little from cell line to cell line (data not shown). The levels of Grb2, Shc, and SOS, the Ras guanine nucleotide dissociation stimulator, were determined by Western blotting of 50 μg of Rsu-1 transfectant and control cell lysates. The results seen in Fig. 2A indicate that there was an increase in the level of SOS protein in the Rsu-1 transfectants compared with the control cell lines, while the levels of Grb2 and Shc were relatively constant. However, there was an increase in the level of SOS protein in the Rsu-1 transfectants compared with the control cell lines, and there was a slight retardation in the migration of SOS. The levels of small G proteins Rac and Rho were also determined by Western blotting of NIH 3T3 and PC12 cell transfectant lysates. The results shown in Fig. 2A indicate that while Rho levels were similar in vector control cell lines and Rsu-1 transfectants, the Rac protein level was higher in the PC12 Rsu-1 transfectants. This difference is not as visible in the NIH 3T3 cell background. No differences in the level of RNA encoding these proteins were detected in the transfectant cell lines compared with control cells.

In addition, the levels of GTP-bound Ras in the NIH 3T3 Rsu-1 transfectant cell lines and the control NIH 3T3 cells were compared. Ras was immunoprecipitated from subconfluent cells labeled with ^{32}P for 16 h in the presence of serum. Ras-bound GTP and GDP were resolved by thin-layer chromatography, and the results were quantitated. The graph in Fig. 2B represents the mean of three determinations and indicates that the NIH 3T3 cell Rsu-1 transfectants had a higher level of GTP-bound Ras than the vector control cell line.

Changes in Ras-GAP and GAP-associated p190 in response to increased expression of Rsu-1. RasGAP is an important regulator of Ras function and may serve to link Ras signaling to other G-protein signaling pathways. Stimulation of cells via extracellular receptors and increases in the level of GTP-bound Ras have been shown to result in translocation of p120 RasGAP to the plasma membrane and to increase the association of p120 GAP with p190. GAP-associated p190 has homology to RhoGAP and exhibits GAP activity for Rho, Rac, and cdc42Hs (37). To determine whether there were detectable changes in p120 GAP or p190 in the NIH 3T3 Rsu-1 transfectants compared with the control cell line, the levels and activities of the proteins were assayed. By direct Western blotting, the levels of p120 GAP were similar among all NIH 3T3 cell lines examined. However, the level of p190 was slightly higher in both of the Rsu-1 transfectants than in the vector controls (Fig. 3A). The level of p190 associated with p120 RasGAP and the level of p190 tyrosine phosphorylation were assayed in the same cells following precipitation of cell lysates with anti RasGAP antibody and blotting with anti-p190 antibody or antibody directed against phosphotyrosine (Fig. 3B). The levels of p190 associated with GAP and p190 tyrosine phosphorylation were equivalent in Rsu-1 transfectants and the control cells.

In a similar manner, the level of p120 RasGAP was determined in PC12 Rsu-1 transfectant cell lines. The results in Fig. 3A show direct Western blotting of lysates and indicate that the level of p120 GAP was higher in the Rsu-1 transfectants (clones 20 and 26) than in the PC12 vector control (clone a). The levels of p120 GAP increased in response to dexametha-

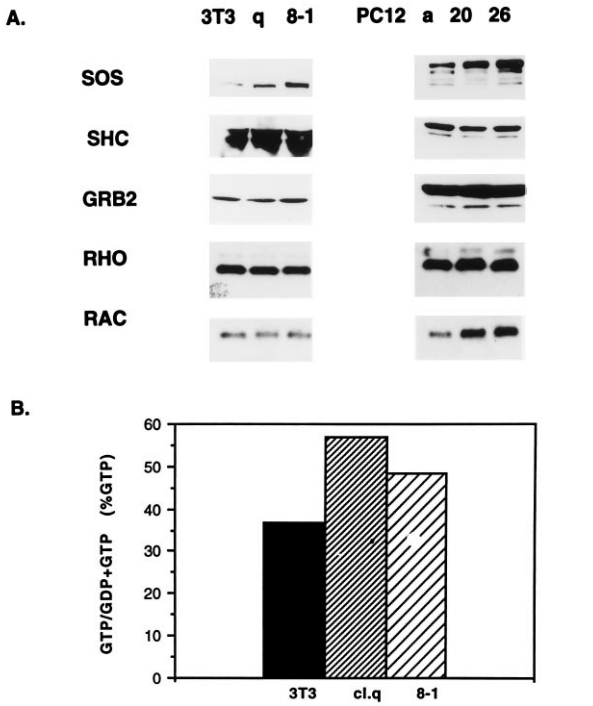

FIG. 2. Expression of proteins involved in Ras signal transduction in Rsu-1 transfectant cell lines. (A) Cell lysates were examined by Western blotting using antibodies for SOS, Grb2, Shc, Rac, and Rho followed by horseradish peroxidase-linked secondary antibodies and developed with chemiluminescent substrates. NIH 3T3 and clone q cells were exposed to dexamethasone for 24 h prior to lysis. PC12 clone a, 20, and 26 cells were exposed to dexamethasone for 36 h prior to lysis. The same filter was used for reactivity to SOS, Grb2, and Shc antibodies. (B) The level of GTP-bound Ras in Rsu-1 transfectants. Ras Y13-259 immunoprecipitates from ^{32}P-labeled NIH 3T3, clone q, and 8-1 cells were analyzed by PEI-phosphocellulose chromatography to determine the amounts of GTP and GDP bound to Ras. Following quantitation of GTP and GDP, the percent GTP-bound Ras was determined. The results represent the mean of three separate determinations.

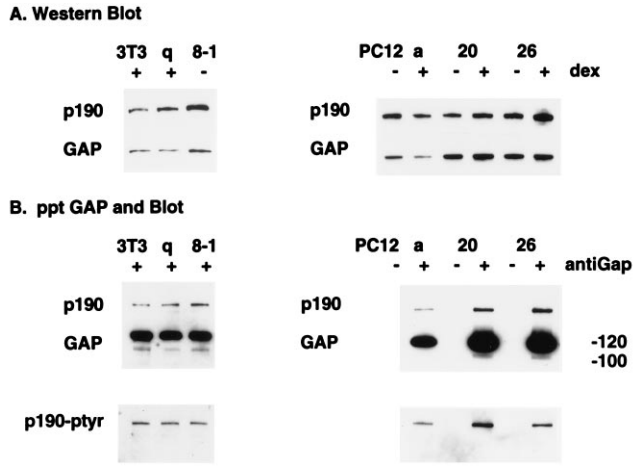

FIG. 3. Expression of p120 RasGAP and GAP-associated p190 in Rsu-1 transfectants and vector control cells. (A) Lysates of cells were used for direct Western blotting with anti-GAP and anti-p190 antibodies. NIH 3T3 and clone q cells were exposed to dexamethasone (dex) for 24 h prior to lysis; the 8-1 cell line was untreated. PC12 clones a, 20, and 26 were exposed to dexamethasone (+) for 36 h or were untreated (−). Secondary antibodies linked to peroxidase and chemiluminescent substrate were used for detection. (B) Immunoprecipitation and Western blotting of lysates. NIH 3T3 and clone q cells were exposed to dexamethasone for 24 h; 8-1 cells were untreated. PC12 clones a, 20, and 26 were exposed to dexamethasone for 36 h. Lysates were immunoprecipitated with anti-GAP polyclonal IgG, recovered with protein G-agarose, and transferred to polyvindylidene difluoride filters, which were reacted with a combination of anti-p120 GAP and anti-p190 antibodies. Peroxidase-conjugated secondary antibodies were used with chemiluminescent substrate for detection. Blots were stripped of antibody and reacted with antiphosphotyrosine (p190-ptyr) antibody. Peroxidase-conjugated secondary antibody and chemiluminescent substrate were used.

sone in the Rsu-1 transfectants but not in the control cell lines (Fig. 3A). Anti-RasGAP immunoprecipitation of lysates from dexamethasone-induced PC12 clones a, 20, and 26 followed by blotting with antibody against p190, p120 GAP, or phosphotyrosine revealed an increase in p120 GAP, GAP-associated p190, and p190 phosphorylation in both Rsu-1 transfectants compared with the control cell line (Fig. 3B). In addition, this method detected a protein of approximately 100 kDa which reacted with anti-GAP antibody. It is likely that this band represents p100 GAP, which suggests that induction of Rsu-1 expression in the Rsu-1 transfectants results in an increase in p100 GAP as well as p120 GAP.

RasGAP activity decreased in Rsu-1 transfectants compared with vector control cells. Assay of the RasGAP activity in lysates of NIH 3T3 and PC12 Rsu-1 transfectants by using purified GTP-bound Ras as a substrate indicated that in spite of an increase in the level of p120 RasGAP, there was less detectable GTPase-activating activity in the Rsu-1 transfectant cell lysates than in lysates of control cells (Fig. 4). Using a detergent lysis protocol similar to that used for Western blotting and immunoprecipitation experiments, the level of Ras GTPase-activating activity in soluble cell lysates was measured. The results are shown in Fig. 4A as the percentage of Ras remaining GTP bound following incubation with lysates from NIH 3T3 and PC12 Rsu-1 cell transfectants. The data indicated that the Rsu-1 transfectants in both cell backgrounds contained a lower level of Ras GTPase-activating activity than the control cell lines. Dexamethasone induction of vector-encoded *rsu-1* transcription resulted in an increase in the amount of Ras remaining GTP bound following exposure to lysates of the Rsu-1 transfectants compared with vector control cells. To determine if there was a reduction in p120 GAP GTPase-activating activity specifically, GAP immunoprecipitates from Rsu-1 and control cells were tested. The results of the PEI-phosphocellulose chromatography of GTP and GDP are shown in Fig. 4C. p120 RasGAP immunoprecipitates from the NIH 3T3 Rsu-1 transfectant, clone q, contained less RasGAP activity than p120 GAP precipitates from the vector control cells, 3T3-1. The antibody control immunoprecipitates from both cell lines were devoid of GTPase-activating activity.

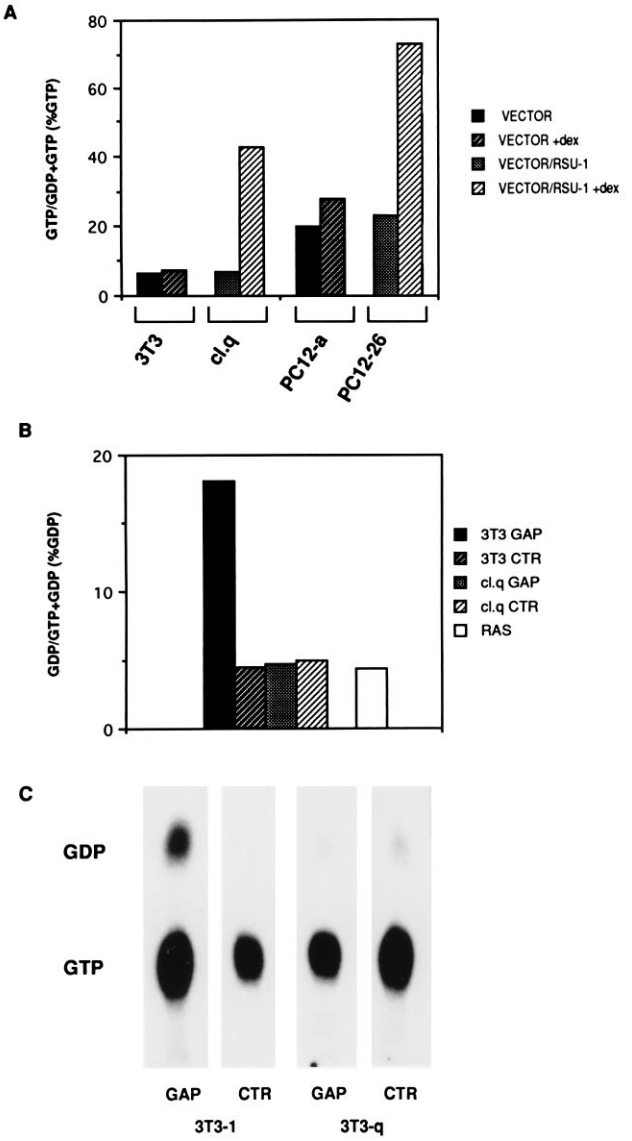

FIG. 4. RasGAP activity decreased in Rsu-1 transfectants compared with vector control cells. (A) Ras GTPase-activating activity was measured in lysates of Rsu-1 transfectants and vector control cell lines. Cells were exposed to dexamethasone (dex) for 24 h prior to harvest or were untreated as indicated. Ten micrograms of cell lysate was incubated with [α-^{32}P]GTP-bound Ras, Ras was recovered by immunoprecipitation, and GTP and GDP were separated by PEI-phosphocellulose chromatography. Following quantitation of the plates, the percentage of GTP-bound Ras was calculated. cl.q, clone q. (B) Ras GTPase-activating activity in p120 GAP immunoprecipitates from Rsu-1 transfectants. Graphic representation of the results presented in panel C is shown. (C) Lysates from NIH 3T3 and clone q cells, exposed to dexamethasone for 24 h prior to lysis, were immunoprecipitated with anti-GAP or control (CTR) antibody. Precipitates were incubated with α-^{32}P-bound Ras, and GTP and GDP were separated as described above. The results of the PEI-phosphocellulose chromatography are shown, and the results of quantitation are shown in panel B.

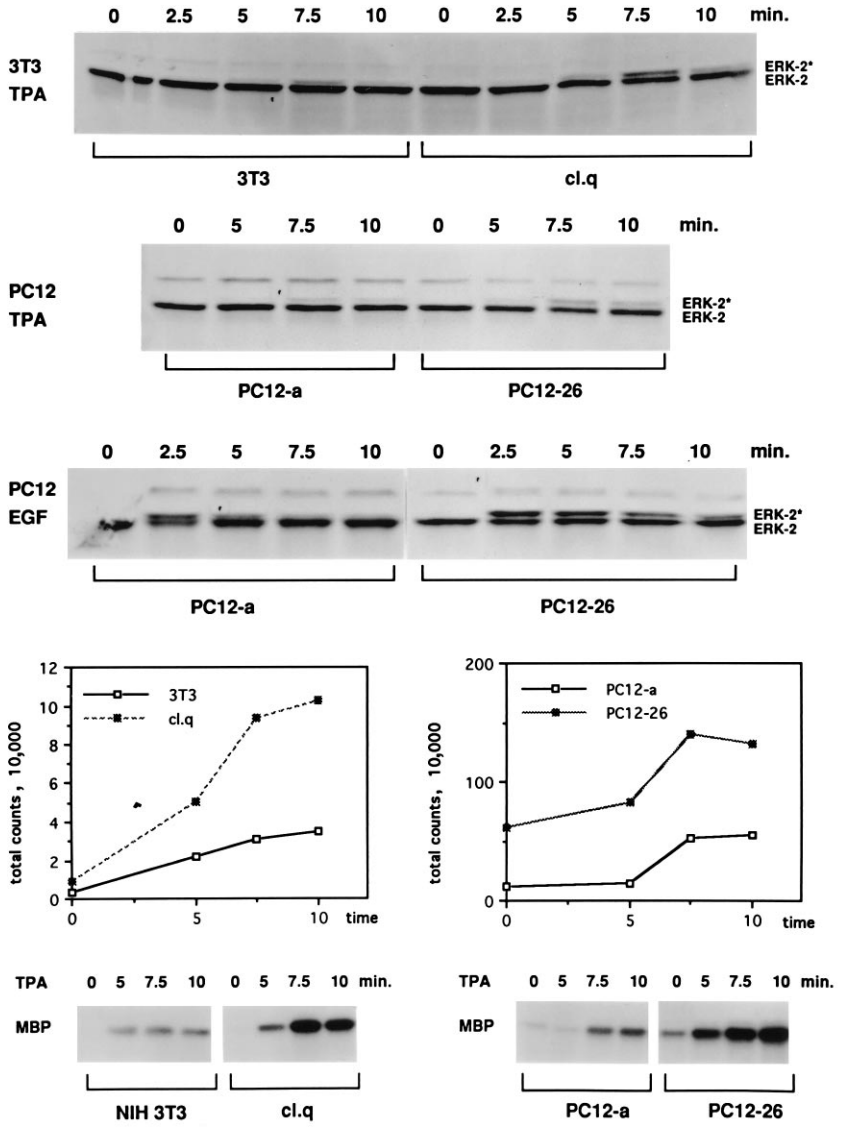

FIG. 5. Activation of Erk-2 in response to TPA and EGF in Rsu-1 transfectants. (Top panels) Western blot of Erk-2 following stimulation of NIH 3T3 and clone q cells with TPA and of PC12 clones a and 26 with TPA or EGF. Cells were lysed at time intervals after the addition of TPA or EGF. Cell lysates were Western blotted. Blots were reacted with anti-Erk-2 antibody, alkaline phosphatase-conjugated secondary antibody, and NBT-BCIP substrate. (Bottom panels) Phosphorylation of MBP by Erk-2 immunoprecipitates from TPA-treated NIH 3T3 and clone q cells and from TPA-treated PC12 clones a and 26. Cells were lysed at time intervals after stimulation with TPA. Erk-2 was immunoprecipitated and used to phosphorylate MBP. MBP phosphorylation was quantitated by electrophoresis, transfer to a filter, autoradiography, and AMBIS scanning. Graphic representation of quantitated data is presented above the autoradiograms.

Quantitation of the chromatographic results from Fig. 4C is shown graphically in Fig. 4B as the percentage of GTP-bound Ras converted to a GDP-bound form following incubation with the immunoprecipitates. It is clear that only the GAP immunoprecipitate from control NIH 3T3 cells effectively converted GTP-bound Ras to the GDP-bound state. This result indicated that the decrease in the Ras GTPase-activating activity in the Rsu-1 transfectants could be attributed to a decrease in p120 RasGAP activity.

There is an increase in signaling from Ras to MAPK in Rsu-1 transfectant cell lines. Numerous studies have shown that activated Ras induces Erk-2 phosphorylation primarily via the activation of Raf and MEK (41). Therefore, the integrity of the signaling pathways downstream of Ras in Rsu-1 transfectants was examined by analyzing the activation of Erk-2 in response to TPA by gel shift and by immune kinase phosphorylation of MBP substrate. By gel shift analysis, NIH 3T3 cells treated with TPA showed weak activation of Erk-2 within 7.5 min, which returned to the baseline level by 10 min poststimulation. In the NIH 3T3 Rsu-1 transfectant, clone q, this response was stronger, appeared earlier, and was sustained longer (Fig. 5, top). Quantitation of the TPA time course by Erk-2 phosphorylation of MBP revealed that the level of Erk activation increased to severalfold the level seen upon TPA stimulation of control NIH 3T3 cells (Fig. 5, bottom). Similar results were seen when a PC12 Rsu-1 transfectant cell line, clone 26, was compared with the control cell line, clone a, following stimulation with TPA or EGF (Fig. 5). This effect was especially strong in EGF-treated cells. In the absence of stimulation, the PC12 Rsu-1 transfectants had an elevated basal level of MBP kinase activity which was induced to a higher level and sustained for a longer time following TPA

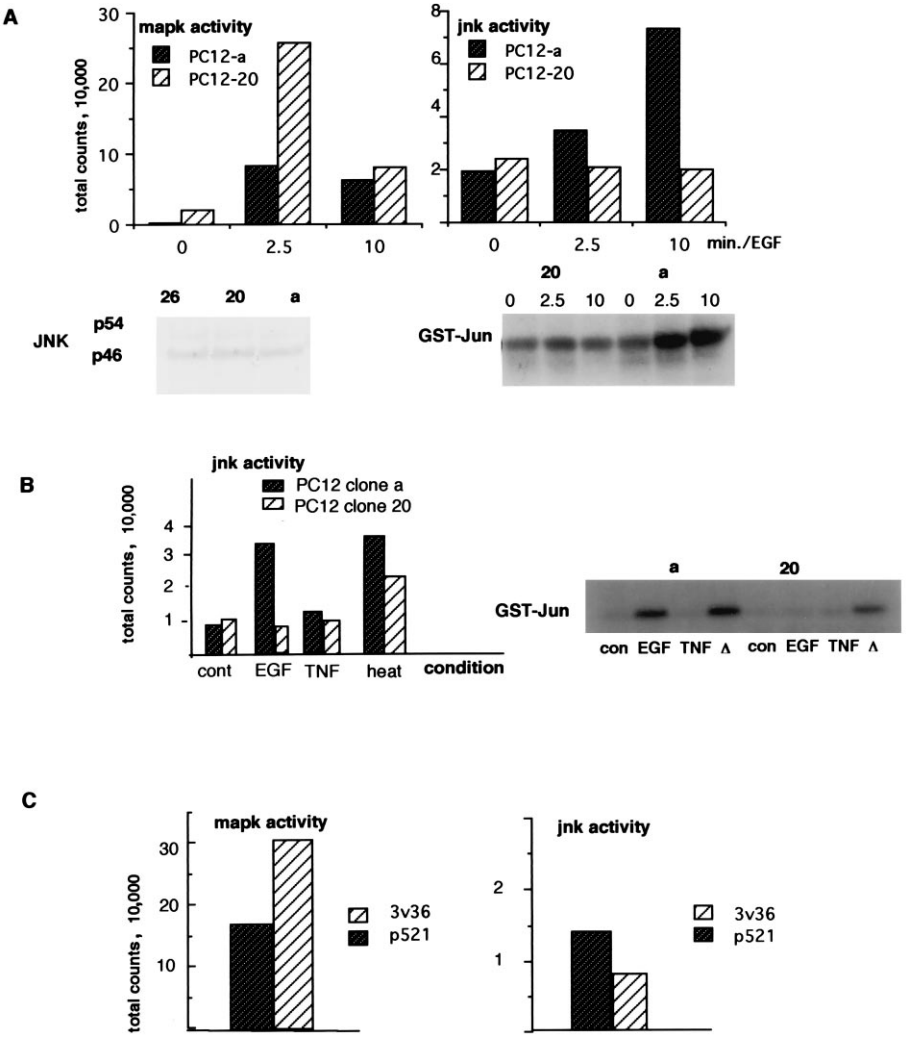

FIG. 6. c-Jun amino-terminal kinase activity in Rsu-1 transfectants. Rsu-1 transfectants and control cells were assayed for Jun kinase activity by using GST–c-Jun as a substrate. (A) PC12 cell clones a and 20 were stimulated with EGF for 0, 2.5, or 10 min. Lysates harvested at each time point were divided into aliquots. (Top left) An aliquot was used to measure Erk-2 immunoprecipitate phosphorylation of MBP. (Top right) One aliquot was mixed with GST–c-Jun bound to Sepharose beads, and GST–c-Jun phosphorylation was measured as described in Materials and Methods. The results represent the mean from two separate experiments. (Bottom right) Primary GST–c-Jun phosphorylation data from one of the experiments used are shown. (Bottom left) Western blot of Jun kinase from lysates of PC12 cell transfectants exposed to dexamethasone for 36 h. (B) PC12 cell clones a and 20 were untreated or stimulated with EGF or tumor necrosis factor alpha for 15 min or exposed to heat (42°C for 30 min). Lysates were harvested, and Jun kinase was immunoprecipitated with a Jun kinase-specific monoclonal antibody. The Jun kinase immunoprecipitates were mixed with GST–c-Jun bound to Sepharose beads in reaction buffer with [γ-^{32}P]ATP, and GST–c-Jun phosphorylation was measured. cont, untreated cells; EGF, EGF-treated cells; TNF, tumor necrosis factor alpha-treated cells; Δ, cells exposed to 42°C. (C) (Left) Lysates of Cos1 cells transiently cotransfected with Rsu-1 expression vector or a control vector and a plasmid encoding HA-tagged Erk-2 kinase. Cells were lysed 48 h after transfection; HA-tagged Erk-2 kinase was recovered with anti-HA antibody and protein G-agarose beads. The recovered Erk-2 was assayed with MBP as a substrate. (Right) Cos1 cells were transiently cotransfected with Rsu-1 expression vector or a control vector and a plasmid encoding HA-tagged Jun kinase. Cells were lysed 48 h after transfection; HA-tagged Jun kinase was recovered with anti-HA antibody and protein G-agarose beads. The recovered Jun kinase was assayed with GST–c-Jun as a substrate. The data represent an average of four experiments; the Rsu-1 p3v26 transfectant Jun kinase activity ranged from 46 to 67% of the p521 transfectant Jun kinase activity.

addition than in the vector control transfectants. These results indicate that the signaling pathway leading to Erk-2 is intact, and superactivatable, in these Rsu-1 transfectants.

Changes in Jun kinase in Rsu-1 transfectants. Because the pathway from Ras to Erk-2 appeared to be intact, and because of the detectable increase in p190, a molecule with RhoGAP activity, the integrity of pathways downstream of Ras involving other G proteins was examined in the Rsu-1 transfectants. Disruption of signaling by Rho, Rac, and Cdc42Hs can inhibit the ability of Ras to transform NIH 3T3 cells (19, 33). Therefore, signal transduction requiring functional Cdc42Hs and Rac was analyzed. Initial experiments examined the stimulation of Jun kinase activity in Rsu-1 transfectants and in control cells. The amount of Jun kinase activity was measured in cytoplasmic extracts of cells by incubation of the extracts with target GST–c-Jun, the amino-terminal 79 amino acids of c-Jun fused to GST. This assay has been demonstrated to measure authentic Jun kinase activity in other cell lines (8). Erk-2 activation was also measured from the same cell lysates by phosphorylation of MBP by Erk-2 immunoprecipitates. To test Jun kinase activation, PC12 clones a and 20 were exposed to dexamethasone for 36 h prior to stimulation with EGF. Cells were lysed at 2.5 and 10 min after addition of EGF, and Jun kinase and Erk-2 activities were assayed. The Jun kinase activity was not stimulated in the Rsu-1 transfectants, but in the control cell line there was a severalfold increase in Jun kinase activity

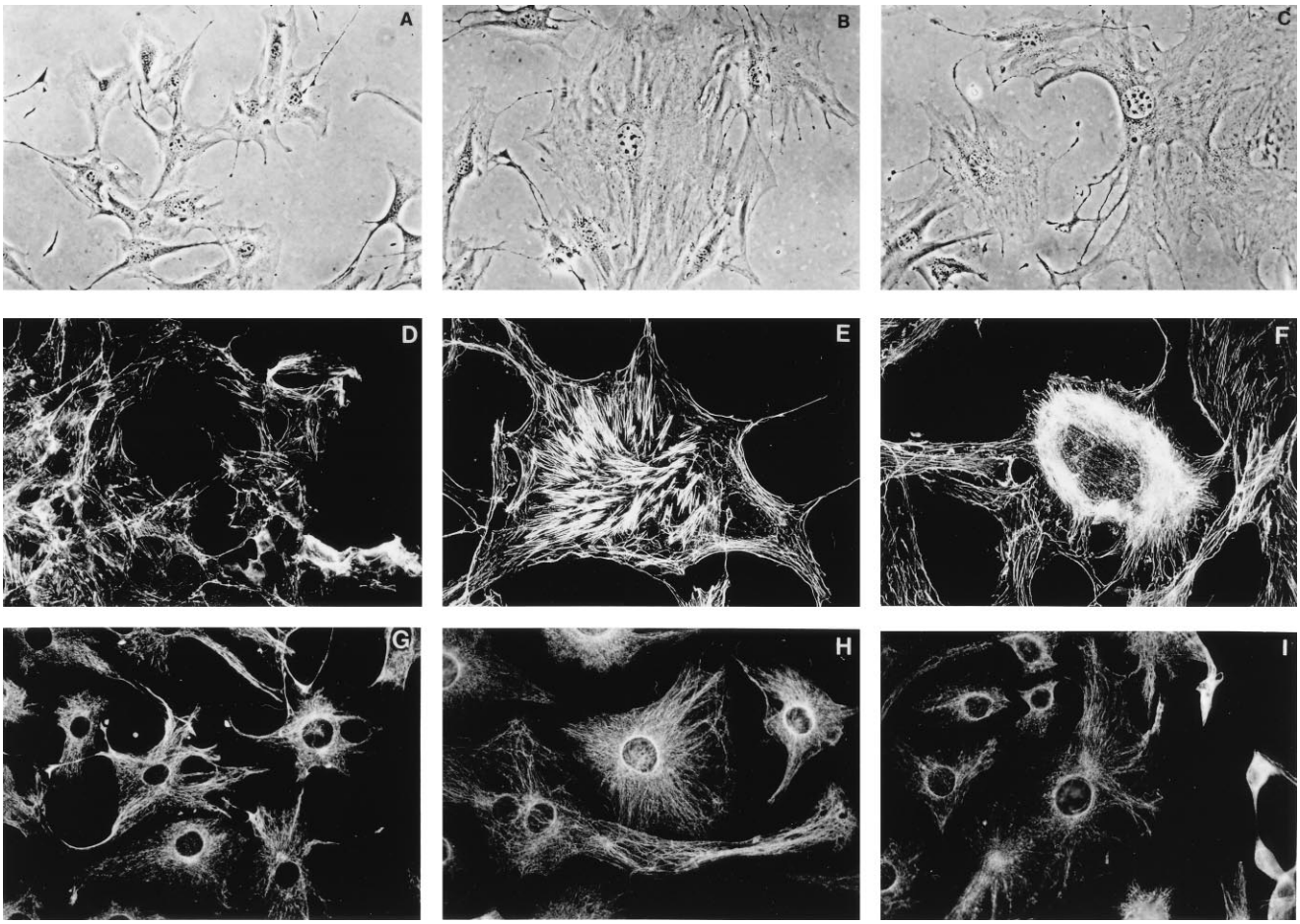

FIG. 7. Morphology and actin cytoskeleton of Rsu-1 transfectants. Shown are phase-contrast microscopy of Rsu-1 transfectants and control cells (magnification, ×156), phalloidin staining of Rsu-1 transfectants and control cells (magnification, ×312), and immunofluorescence of vimentin in Rsu-1 transfectants and control cells (magnification, ×312). Phase-contrast microscopy: (A) NIH 3T3 cells; (B) clone q cells; (C) clone q cells. Phalloidin staining: (D) NIH 3T3 cells; (E) clone q cells; (F) clone q cells. Vimentin immunofluorescence: (G) NIH 3T3 cells; (H) clone q cells; (I) clone q cells.

following growth factor addition (Fig. 6A). As seen previously, the Erk-2 activity was stimulated to a greater degree in the Rsu-1 transfectants than in the vector control cells (Fig. 6A). Western blotting of lysates from the PC12 transfectant cell lines induced with dexamethasone for 36 h revealed that there was no difference in the level of p46 or p54 Jun kinase in Rsu-1 transfectants compared with the vector control transfectant (Fig. 6A, bottom left). Therefore, the change was not due to the level of Jun kinase in the cells, and while the Erk-2 signaling pathway was intact in the cells, the Jun kinase pathway was selectively inhibited. Activation of Jun kinase in response to other stimulators was tested by using Jun kinase immunoprecipitates to phosphorylate the GST–c-Jun target protein. The results shown in Fig. 6B demonstrate that exposure to heat (42°C for 30 min) resulted in Jun kinase activation in the Rsu-1 transfectant, while again growth factor failed to activate the enzyme. This result indicated a block to Jun kinase activation possibly specific to a Ras-dependent pathway.

In addition to testing activation of Jun kinase in Rsu-1 stable transfectant cell lines, p33 Rsu-1 was transiently expressed in Cos cells along with HA-tagged Jun kinase or HA-tagged Erk-2. This allowed us to test the effect of Rsu-1 expression on Jun kinase and MAPK directly. Again, Rsu-1 expression enhanced the activity of Erk-2 (Fig. 6C), and in agreement with the results obtained from the stable Rsu-1 transfectants, Rsu-1 expression inhibited the Jun kinase activity in the Cos1 cells compared with vector control transfectants (Fig. 6C).

Morphology and actin cytoskeleton of Rsu-1 transfectant cell lines. The NIH 3T3 cell Rsu-1 transfectants, 8-1 and clone q, displayed a very flat, highly enlarged cell morphology (Fig. 7A to C). This observation in combination with the effect of Rsu-1 on functions regulated by Rac and Cdc42Hs suggested that Rsu-1 expression might alter cellular adhesion. NIH 3T3 cells and clone q were plated on coverslips and stained with phalloidin to illustrate the appearance of the actin cytoskeleton. As seen in Fig. 7E and F, the Rsu-1 transfectant cell line, clone q, exhibited an altered actin cytoskeleton in comparison with control cells (Fig. 7D). There was evidence of a decrease in organization and an increase in accumulation of altered stress fibers in the transfectant but not the control cell line. In addition, some evidence of nuclear ruffling was seen in the clone q cells as well (Fig. 7F). Vimentin staining of the same cells in parallel indicated that the intermediate filaments in the cell appeared mostly intact (Fig. 7G to I). Hence, there is some degree of specificity in the alteration of the cytoskeleton as a result of Rsu-1 expression.

Binding of GST–Rsu-1 to Raf-1. The amino-terminal two-thirds of Rsu-1 consists of a series of leucine base repeats, each 23 amino acids in length (11). These repeats are homologous to the repeats found in yeast adenylyl cyclase in the region

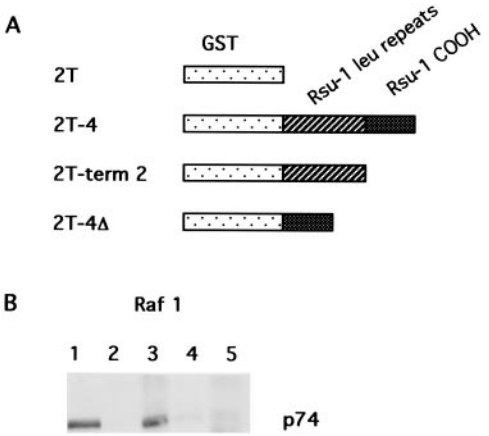

FIG. 8. Binding of GST–Rsu-1 to Raf-1. (A) GST fusion proteins encoding full-length or specific regions of Rsu-1 were prepared. Structures of the GST–Rsu-1 fusion proteins are shown. (B) GST–Rsu-1 fusion proteins were purified by binding to glutathione-Sepharose beads. The fusion proteins were incubated with Raf-1 protein purified from Raf-1 baculovirus-infected insect cell lysates. Following washing to remove unbound Raf-1, the fusion protein beads were resuspended in gel buffer, and the bound protein was separated by SDS-PAGE and transferred to a nitrocellulose filter. Raf-1 bound to the fusion proteins was detected by exposure of the filter to Raf-1 monoclonal antibody and detection by chemiluminescence. Lane 1, purified Raf-1 (40% of the amount used in the binding reaction for each GST fusion protein); lane 2, Raf-1 bound to GEX 2T; lane 3, Raf-1 bound to GEX 2T-4; lane 4, Raf-1 bound to GEX 2T-term 2; lane 5, Raf-1 bound to GEX 2T-4Δ.

through which Ras activates adenylyl cyclase in *S. cerevisiae* (5, 13). With the use of a yeast two-hybrid system, interaction between Ras and the leucine repeats has been demonstrated. However, Ras did not bind to the leucine repeats with the same affinity as it bound to Raf-1 (45). Presumably it is for this reason that direct association between Ras and the Rsu-1 leucine repeats in vitro has not been demonstrated. However, because Rsu-1 expression increased the activation of signal transduction pathways downstream of Raf-1, binding of Rsu-1 to Raf-1 was tested. Recombinant Raf-1 produced in baculovirus was incubated with a series of GST fusion proteins encoding the entire Rsu-1 open reading frame (2T-4), only the amino-terminal leucine repeats (2T-term 2), or only the carboxy terminus of Rsu-1 (2T-4Δ). As shown in Fig. 8B, Raf-1 bound to the full-length Rsu-1 fusion protein (lane 3) and to a much lesser degree to the leucine repeats or the carboxy-terminal fusion proteins (lanes 4 and 5). There was no binding to GST alone (lane 2). These results indicated that full-length Raf-1 is capable of binding to full-length Rsu-1. Several other proteins have been tested for GST–Rsu-1 binding without success. Therefore, these results suggest that Rsu-1 may regulate Raf-1 function, possibly through the formation of a complex with Ras and Raf-1.

DISCUSSION

The *rsp-1*/*rsu-1* cDNA was cloned on the basis of its ability to suppress transformation by v-Ras. In in vitro assays the suppressor activity is specific for transformation by Ras in that an increase in Rsu-1 expression does not inhibit transformation by the v-*src*, v-*raf*, or v-*mos* oncogene (11). This finding suggested that Rsu-1 inhibited at a specific point downstream of Ras, and the results reported here describe the effect of Rsu-1 expression on signal transduction pathways downstream of Ras. A comparison of Rsu-1 transfectants and control cell lines revealed that the Rsu-1 transfectants exhibited an increase in the level of SOS, the Ras GTP-GDP exchange protein (3). While an increase in the amount of p120 RasGAP was detected in the Rsu-1 transfectants, there was a decrease in the Ras GTPase-activating activity. Therefore, the increase in the level of GTP-bound Ras in Rsu-1 transfectants was probably a result of an increase in nucleotide exchange, as well as decreased GAP activity. The inhibition of GAP activity is somewhat unique. In T cells, GAP activity appears to regulate the level of GTP-bound Ras (10), while in many other cells, SOS appears to be a major regulator. Observed along with the increase in activation of Ras was an increase in signaling downstream of Ras. Upon stimulation with TPA, or EGF in the case of PC12 cells, there was an increase in the activation of Erk-2. Both PC12 and NIH 3T3 Rsu-1 transfectants showed an increase in Raf-1 and MEK in vitro kinase activity compared with control cells (data not shown). This may result from an increase in Ras–Raf-1 association, reflecting the ability of Rsu-1 to associate with Raf-1 in vitro. Therefore, in contrast to the Ras suppressor, Rev-1/Rap-1, and Ras-binding proteins, Rin and RalGDS, which inhibit the activation of Raf-1 (7, 16, 20), Rsu-1 expression renders cells resistant to Ras transformation in spite of activation of this downstream signaling pathway.

The explanation for the resistance to Ras transformation in the Rsu-1 transfectant cell lines may lie in the changes in RasGAP and its associated molecules, primarily p190 (37, 38). In NIH 3T3 cells, the level of p120 RasGAP does not differ significantly between Rsu-1 transfectants and control cells. However, there is an increase in the level of p190 and the level of p190 associated with GAP. In PC12 Rsu-1 transfectants, in which the level of Rsu-1 was more regulatable, the GAP and p190 changes were more pronounced. The increase in p190 associated with GAP suggested that there might be a concomitant increase in GAP activity for the small G proteins Rac and Rho in the cells, since p190 has been shown to possess a GAP activity for those molecules (37). Rho and Rac appear to function downstream of Ras in transformation and are required for transformation by Ras (19, 26, 33). Because the GTPase RhoA is involved in the control of actin stress fiber formation (34), the likely consequence of an increase in RhoGAP activity in the cells would be changes in the actin cytoskeleton. In fact, changes of that nature are readily apparent in the Rsu-1 transfectants; the Rsu-1 transfectants show a disorganized actin stress fiber network with an apparent increased accumulation of actin. These results support the conclusions of others that the Rho-associated changes in the cytoskeleton are necessary for transformation and that their inhibition is sufficient to prevent transformation (19, 26). A previous report indicated that the p190 RhoGAP activity increased upon association with the N-terminal region of GAP, causing cytoskeletal changes (27), and changes in the cytoskeleton associated with elevated levels of p190 have been observed as a consequence of differentiation (4).

Recent studies have indicated that inhibition of Rac and cdc42Hs can inhibit the activation of Jun kinase, thereby directly linking this pathway to changes required for Ras transformation (9, 19, 26, 28, 31). In addition, introduction of p190 into cells inhibited the activation of Jun kinase (9). The analysis of Jun kinase activity in the Rsu-1 transfectants indicated that Jun kinase activity is reduced in Rsu-1 transfectants compared with vector control cells, although Erk-2 activity was increased in the same cells. The inhibition of Jun kinase activation was confined to pathways dependent on Ras for activation; the heat activation of Jun kinase was functional in the Rsu-1 transfectants. Therefore, it appears that Rsu-1 inhibits Ras transformation by inhibition of specific downstream signaling pathways, i.e., the Jun kinase pathway, possibly as a result

of increased p190 GAP activity for Rac or cdc42Hs. Interestly, Minden et al. demonstrated that expression of DNRac1(N17) did not completely inhibit transformation by v-*src*- or v-*src*-induced activation of Jun kinase, while DNRas(N17) completely eliminated both activities (28). This result may explain the inability of Rsu-1 expression to prevent v-*src* transformation (11). Because previous studies have shown that Erk kinase pathways downstream of Ras are also required for transformation, the conclusion from this study is that both Erk and Jun kinase pathway activation is necessary for the induction of the transformed phenotype by Ras. Moreover, increased activation of Erk-2 does not supplant the need for Jun kinase in the events leading to transformation by Ras in fibroblasts.

The Rsu-1 Ras suppressor protein contains a series of 23-amino-acid leucine-rich amphipathic repeats similar to the repeats found in yeast adenylyl cyclase in the region through which Ras activates adenylyl cyclase in *S. cerevisiae* (5, 13). It has been proposed that these leucine-based repeats are involved in protein-protein interaction, and interaction between adenylyl cyclase leucine repeats and Ras has been demonstrated with a yeast two-hybrid system (45). The demonstration that Rsu-1 could bind to Raf-1 in vitro suggests a mechanism of Rsu-1 activation of Raf-1 and kinase pathways to Erk-2. Rsu-1 may promote Ras and Raf-1 interaction by increasing affinity between the molecules. In yeast cells, expression of 14-3-3 protein can activate Raf-1 (14, 18). An Rsu-1-promoted increase in the interaction of Ras and Raf-1 may inhibit Ras interaction with RasGAP, RalGDS, MEK kinase, or other molecules regulating signal transduction and cytoskeletal organization. A recent study has indicated that Ras can interact directly with Jun kinase (1), and it is possible that Rsu-1 inhibits that interaction. Further studies will examine the association of Ras with specific effectors as a result of increased Rsu-1 expression. Also, recent demonstrations that Ras-Raf interaction involves two regions of each molecule (2, 12) suggest that there is more than one mechanism by which binding can be altered. Additional studies using more sensitive techniques may be necessary to determine if Rsu-1 can bind Ras directly, and the use of Rsu-1 mutants should elucidate its role in regulating Ras-Raf interactions.

Changes in signaling to molecules which regulate the cytoskeleton may explain alterations seen in the cell lines transfected with Rsu-1 causing a flatter and enlarged appearance compared with control cells. This phenotypic change has been noted in NIH 3T3 fibroblasts as shown in Fig. 7. In addition, the morphological change was noted in an Rsu-1 transfectant of the U251 glioblastoma cell line when grown in soft agar (44). Control U251 cells which did not express detectable quantities of the Rsu-1 protein prior to transfection with Rsu-1 expression vector formed loose spreading colonies. In contrast, the Rsu-1 transfectants formed tightly adherent colonies. Increased cellular adhesion (39) and changes in expression of cytoskeletal proteins have inhibited transformation by Ras (15, 32, 39). In addition, the tumor suppressor genes APC and NF-2 appear to function in cellular adhesion inducing cytoskeletal changes (36, 40, 42). The possibility that Rsu-1 expression alters signal transduction pathways which affect cytoskeleton is especially intriguing because Rsu-1 transfection into the U251 glioblastoma cell line prevented tumor formation (44).

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