

A Distinct Class of Dominant Negative Ras Mutants

CYTOSOLIC GTP-BOUND Ras EFFECTOR DOMAIN MUTANTS THAT INHIBIT Ras SIGNALING AND TRANSFORMATION AND ENHANCE CELL ADHESION*

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Cytosolic GTP-bound Ras has been shown to act as a dominant negative (DN) inhibitor of Ras by sequestering Raf in non-productive cytosolic complexes. Nevertheless, this distinct class of DN mutants has been neither well characterized nor extensively used to analyze Ras signaling. In contrast, DN Ras17N, which functions by blocking Ras guanine nucleotide exchange factors, has been well characterized and is widely used. Cytosolic GTP-bound Ras mutants could be used to inhibit particular Ras effectors by introducing additional mutations (T35S, E37G or Y40C) that permit them to associate selectively with and inhibit Raf, RalGDS, or phosphoinositide 3-kinase, respectively. When the wild-type Ras effector binding region is used, cytosolic Ras should associate with all Ras effectors, even those that are not yet identified, making these DN Ras mutants effective inhibitors of multiple Ras functions. We generated cytosolic GTP-bound H-, N-, and K-Ras, and we assessed their ability to inhibit Ras-induced phenotypes. In fibroblasts, cytosolic H-, N-, and K-Ras inhibited Ras-induced Elk-1 activation and focus formation, induced a flattened cell morphology, and increased adhesion to fibronectin through modulation of a β_1 -subunit-containing integrin, thereby demonstrating that DN activity is not limited to a subset of Ras isoforms. We also generated cytosolic GTP-bound Ras effector domain mutants (EDMs), each of which reduced the ability of cytosolic GTP-bound Ras proteins to inhibit Elk-1 activation and to induce cell flattening, implicating multiple pathways in these phenotypes. In contrast, Ras-induced focus formation, platelet-derived growth factor (PDGF)-, or Ras-induced phospho-Akt levels and cell adhesion to fibronectin were affected by T35S and Y40C EDMs, whereas PDGF- or Ras-induced phospho-Erk levels were affected only by the T35S EDM, implying that a more limited set of Ras-mediated pathways participate in these phenotypes. These data constitute the first extensive characterization of this functionally distinct class of DN Ras inhibitor proteins.

The small GTPase, Ras, is involved in numerous aspects of normal cellular metabolism including proliferation, survival/

apoptosis, differentiation, and adhesion/motility (1). Ras must be localized to the inner leaflet of the plasma membrane where, in its GTP-bound active form, it translocates cytosolic effector proteins such as Raf kinase, Ral guanine nucleotide dissociation stimulator (RalGDS),¹ or phosphoinositide 3-kinase (PI3-K) to the membrane for subsequent activation. Ras is anchored at the membrane as the result of a series of post-translational modifications beginning with the attachment of a farnesyl isoprenoid to a conserved cysteine residue (cysteine 186 in H- and N-Ras; cysteine 185 in K-Ras) located within the "CAAX box," a motif consisting of the carboxyl-terminal four amino acids of Ras. Mutation of the CAAX cysteine therefore prevents both Ras farnesylation and membrane association, leaving the Ras protein unprocessed in the cytosol, where the mutationally active, GTP-bound form can bind effector proteins but without translocating them to the membrane for activation. Thus, cytosolic GTP-bound H-Ras(C186S) is thought to act as a dominant negative (DN) inhibitor of normal Ras signaling (2, 3) by sequestering Ras effectors in nonproductive complexes (2, 4).

This mode of DN action is in contrast to that of the conventional DN Ras mutant (S17N) (5), a distinct class of DN Ras that is well characterized (reviewed in Ref. 2). Ras(S17N) is membrane-localized but GDP-bound. Therefore, Ras(S17N) fails to bind effector proteins but instead binds tightly to guanine nucleotide exchange factors, sequestering them in non-productive complexes and thereby preventing them from activating Ras. The Ras(G15A) mutant is also of this class of DN, although it is considered to be nucleotide-free and therefore even more efficient at sequestering Ras guanine nucleotide exchange factors. Ras(S17N) is widely used as a specific inhibitor of Ras activation and has proven useful in defining the role of Ras in a variety of cellular functions (2).

Despite their potential utility as biochemical tools to study Ras signaling, cytosolic GTP-bound Ras proteins are not well characterized, and literature regarding them is limited. Cytosolic GTP-bound Ras(G12V) was originally shown to inhibit Ras-induced germinal vesicle breakdown in *Xenopus* oocytes (6), implying the existence of a cytosolic factor required for Ras function. Later, Ras(S17N) and cytosolic GTP-bound Ras were shown to inhibit preferentially signaling by wild-type and oncogenic Ras, respectively (3), which is consistent with their different mechanisms of action. We have shown previously (4)

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¹ The abbreviations used are: RalGDS, Ral guanine nucleotide dissociation stimulator; PI3-K, phosphoinositide 3-kinase; DN, dominant negative; PDGF, platelet-derived growth factor; EDMs, effector domain mutants; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; CAAX, cysteine aliphatic aliphatic any amino acid; HA, hemagglutinin; FITC, fluorescein isothiocyanate.

that cytosolic GTP-bound H-Ras(Q61L), generated pharmacologically by treatment with an inhibitor of the Ras-prenylating enzyme farnesyltransferase, forms nonproductive cytosolic complexes with Raf and prevents Raf activation. Similar results were obtained using H-Ras(G12V) rendered cytosolic by mutation of the farnesylated cysteine (7). However, it is not known whether N- or K-Ras can also act as DN inhibitors, whether DN activity is dependent on the Ras-activating mutation (position 12 *versus* 61), or whether Ras effectors other than Raf can be functionally blocked.

One potential advantage of cytosolic GTP-bound Ras is that additional single mutations (*e.g.* at residues 35, 37, and 40) can be introduced within the Ras effector binding domain (residues 32–40) that selectively impair Ras association with specific effector proteins (8), including the most well characterized Ras effectors, the serine/threonine kinase Raf, PI3-K, and RalGDS. Ras effector domain mutants (EDMs) are well characterized and, in their fully processed and membrane-localized forms, have been used extensively to demonstrate that individual Ras effector pathways are sufficient to induce particular Ras-dependent phenotypes (see for example Refs. 8–15). We envisioned that, in a complementary way, the unprocessed and therefore cytosolic GTP-bound Ras EDMs could be used to selectively impair Ras signaling through specific effectors and to show that particular pathways are necessary for Ras-dependent phenotypes.

To characterize the potential of cytosolic GTP-bound Ras EDM proteins to act as DN inhibitors of Ras activity, we generated a series of unprocessed, oncogenically activated H-, N-, and K-Ras4B mutants to study various phenotypes that are altered during Ras-induced transformation, including activation of transcription factors and signaling intermediates and changes in cell morphology and adhesion. Together, our data characterize the function of cytosolic GTP-bound H-, N-, and K-Ras4B proteins and demonstrate that they can be used effectively as DN inhibitors of Ras-dependent signaling, thereby permitting the dissection of Ras signaling pathways in ways different from those of the more conventional Ras(S17N) and Ras(G15A) DN mutants.

MATERIALS AND METHODS

Generation of Cytosolic GTP-bound Ras Constructs—The following unprenylated Ras mutants were generated by PCR by methods described previously (16) using templates containing activating mutations at either position 12 or position 61: H-Ras(G12V/C186S), H-Ras(G12V/T35S/C186S), H-Ras(G12V/E37G/C186S), H-Ras(G12V/Y40C/C186S), H-Ras(Q61L/C186S), H-Ras(Q61L/T35S/C186S), H-Ras(Q61L/E37G/C186S), H-Ras(Q61L/Y40C/C186S), N-Ras(G12D/C186S), N-Ras(G61K/C186S), and K-Ras(G12V/C185S). Mutation of the prenylated cysteine (position 185/186) near the carboxyl terminus was accomplished using mutagenic 3'-PCR primers (University of North Carolina Lineberger Comprehensive Cancer Center, Nucleic Acids Core Facility). Effector domain mutants (EDMs) were generated from templates that also contained effector domain mutations at positions 35, 37, or 40. PCR primers were designed to introduce restriction sites for subcloning of the product into the mammalian expression vectors pZIPneoSV(X)1 (17) or the pZIP-related vector pZBE-HA (G. Clark, National Institutes of Health). Both vectors drive protein expression from a Moloney murine leukemia virus 5'-long terminal repeat promoter and contain an SV40 origin of replication (16). Ras coding sequences in pZBE-HA are fused to the coding sequence for the hemagglutinin epitope (MASSYPYDVPD-YASLGGPSS-). The Ras coding sequence of each construct was confirmed before use by automated DNA sequencing (Automated Sequencing Facility, University of North Carolina).

Transient Transcriptional Transactivation Assay for Elk-1 Activation—NIH 3T3 fibroblasts were maintained humidified at 37 °C in DMEM-H (Invitrogen) with 10% Colorado calf serum (Colorado Serum Co., Denver, CO) and antibiotics. All luciferase assays were performed in NIH 3T3 fibroblasts as described previously (18). Briefly, plasmid DNA was precipitated in the presence of high molecular weight carrier DNA (calf-thymus DNA, Roche Molecular Biochemicals) with 125 mM

calcium phosphate and layered onto cells for 3–5 h. Cells were washed, shocked in 15% glycerol for 3 min, and returned to complete medium for 48 h, washed twice with phosphate-buffered saline (PBS), pH 7.2. Cells were then lysed and assayed for luciferase activity using reagents from the Enhanced Luciferase Assay Kit (PharMingen, San Diego, CA) according to the manufacturer's protocols.

To evaluate the ability of each cytosolic Ras protein to inhibit Elk-1 activation by each membrane-localized Ras protein, NIH 3T3 cells (2×10^5 cells/35-mm dish plated the day before use) were transiently transfected in duplicate with 100 ng of plasmid (pZIP) containing the coding sequence for each membrane-localized GTP-bound Ras protein (to drive Elk-1 activation) along with 1 μ g of vector (pZIP or pZBE-HA) or vector containing the coding sequence of each cytosolic Ras protein to inhibit Elk-1 activation. Each transfection also contained 250 ng of a Gal-Elk-1 reporter plasmid and 2.5 μ g of 5 \times Gal-Luciferase plasmid (19). Raw data from duplicate samples were averaged and expressed as percent luciferase activity compared with vector co-transfected cells. All assays were performed at least three times, and representative data are displayed \pm S.D.

Generation and Characterization of Stable Cell Lines—NIH 3T3 fibroblasts plated at 2.5×10^5 /60-mm dish the day before use were transfected as described above with 100–200 ng of each Ras-expressing construct by calcium phosphate precipitation. After 48 h, cells were passaged into complete medium with selection (750 μ g/ml G418) and fed every other day with fresh selection medium. Colonies appeared after 2 weeks, and 40–70 colonies of each cell line were pooled for use. Protein expression was confirmed by SDS-PAGE and Western analysis or by autoradiography following radiolabeling of cells with [³⁵S]cysteine/methionine and immunoprecipitation using Ras-specific antibodies. Similar expression levels were observed for each protein (data not shown). To confirm that apparent functional differences were not because of unusually unstable proteins, we performed pulse-chase assays on each of the stable cell lines. Our data (not shown) indicate that all cytosolic Ras constructs had a half-life comparable to that of membrane-localized H-Ras(Q61L) (*i.e.* \approx 22 h (20)) and that our results were therefore unlikely to result from variations in protein stability. We also confirmed that all unprenylated Ras proteins remained cytosolic by cell fixation in formaldehyde followed by immunofluorescent staining as described previously (21), using either anti-HA or anti-pan-Ras antibodies visualized with FITC-conjugated secondary antibody on a Zeiss Axioskop fluorescence microscope.

Inhibition of PDGF- or Ras-induced Akt and Erk Activation by Cytosolic GTP-bound Ras—To evaluate the ability of cytosolic Ras proteins to inhibit the phosphorylation of Akt and Erk by growth factor stimulation, NIH 3T3 cells stably expressing cytosolic H-Ras(Q61L/C186S), its corresponding effector domain mutants (T35S, E37G, or Y40C), or vector were plated at 1×10^4 cells/35-mm dish and allowed to adhere overnight. Cells were serum-starved (0.5% serum) overnight and then stimulated with 50 ng/ml platelet-derived growth factor (PDGF; Sigma) for 5 min. Alternatively, adherent cells were transfected as described above with 200 ng of oncogenic H-Ras(Q61L) and serum-starved overnight. In either case, cells were washed with PBS and lysed in 1% Triton X-100, 10% glycerol in 50 mM Tris, pH 7.4, 100 mM NaCl, plus protease inhibitors (10 μ g/ml aprotinin (Sigma), 5 μ g/ml leupeptin, 0.5 mM Pepabloc (Roche Molecular Biochemicals), 20 mM β -glycerophosphate (Sigma), and 10 mM *para*-nitrophenyl phosphate (Sigma)) and a phosphatase inhibitor (1 mM sodium vanadate). Lysates were normalized to total protein, and 5 μ g of total protein/lane was subjected to SDS-PAGE and Western analysis using either anti-phospho(serine 473)-Akt antibody, anti-total Akt antibody, anti-phospho-Erk antibody, or anti-total Erk antibody (all antibodies from Cell Signaling, Beverly, MA). To demonstrate expression of each cytosolic Ras protein, Western analyses using anti-H-Ras antibody (146-3E4, Quality Biotech, Camden, NJ) were also done to show relative expression levels of cytosolic Ras proteins. Proteins were visualized by ECL (SuperSignal, Pierce).

Cell Adhesion Assays—Confluent NIH 3T3 cells in 100-mm dishes were harvested with 4 ml of pre-warmed non-enzymatic cell dissociation solution (Specialty Media, Phillipsburg, NJ). Alternatively, cells were removed from the dish using pre-warmed 0.25% trypsin/EDTA (Invitrogen) and resuspended in 8 ml of DMEM-H containing 9% bovine calf serum. Adhesion results were similar with both methods. Harvested cells were allowed to recover in complete medium for 40 min at 37 °C, pelleted by low speed centrifugation, and resuspended in 8 ml of DMEM-H with 1% bovine serum albumin (BSA, Pentax) but no serum. Cells were washed twice in the same media and manually counted using a hemocytometer. Cells (1×10^4 per well) were loaded in a 96-well plate coated with 1% BSA or 10 ng/ml fibronectin (Enzyme Research, South Bend, IN). Cells were incubated for 40 min at 37 °C and then

washed 5 times with 100 μ l of the above media. Adherent cells were quantitated using an assay that measures luciferase activity as a function of intracellular ATP, a co-substrate for luciferase (ATP-lite, Packard Instrument Co.). Cell numbers were determined by comparing luminescence values of samples to a standard curve plotted as luminescence versus known cell number. Number of cells adhering to BSA was defined as nonspecific and subtracted from number of cells adhering to fibronectin. Adhesion was expressed as the ratio (%) of adherent cells to total cells loaded into the assay (1×10^4).

To determine whether integrin-specific antibodies could block cell adhesion to fibronectin, subconfluent NIH 3T3 cells were harvested with 0.25% trypsin, 1 mM EDTA and allowed to recover at 37 °C for 30 min in serum-containing growth media. Cells were washed three times in DMEM, 1% BSA to remove serum, counted, and incubated with 40 μ g/ml of β_1 integrin antibody (CD29, clone Ha2/5; BD PharMingen) or control antibody (clone G235-1; BD PharMingen) at 37 °C for 30 min. After incubation with the blocking antibody, 1×10^4 cells were loaded into individual wells of a 96-well plate (Falcon) that had been previously coated with 10 ng/ml human fibronectin (Enzyme Research Laboratories, South Bend, IN). Adhesion was allowed to take place for 25 min at 37 °C. Wells were washed with DMEM, 1% BSA five times to remove unbound cells. The remaining adherent cells were quantitated using the ATP-dependent luminescent assay described above. Results were displayed as the percentage of total cells bound.

Flow Cytometry Analysis of Integrin Expression—Subconfluent NIH 3T3 cells stably expressing each cytosolic Ras mutant or vector were harvested with 0.25% trypsin, 1 mM EDTA, added to 10 ml of serum-containing media, and allowed to recover for 40 min at 37 °C. Cells were washed three times in PBS, 1% BSA, resuspended in 2 ml of PBS, 1% BSA, and incubated with β_1 integrin antibody (CD29, clone Ha2/5 BD PharMingen) in parallel with an isotype-matched control antibody (clone G235-1; BD PharMingen) at 1 μ g/ml for 60 min on ice. For detection of β_1 integrin, FITC-conjugated anti-mouse Ig Fab' antibody (BIOSOURCE International, Camarillo, CA) was used at 10 μ g/ml for 30 min on ice in the dark. Cells were washed two times in cold PBS, 1% BSA and resuspended in 500 μ l of the same buffer. Fluorescence was detected by a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Focus Formation Assays—To assess the ability of cytosolic Ras proteins to inhibit the growth of oncogenic H-Ras-induced foci on a monolayer of NIH 3T3 fibroblasts, cells stably expressing cytosolic Ras proteins or vector were transiently transfected by calcium phosphate precipitation (22) with 200 ng of pZIP H-Ras(G12V) or H-Ras(Q61L). Cells were maintained at 37 °C and 10% CO₂ in DMEM-H (Invitrogen) containing 10% Colorado calf serum plus antibiotics and fed every other day. After 2 weeks cells were washed twice in phosphate-buffered saline, pH 7.4, fixed for 10 min in 75% methanol, 25% acetic acid, stained for 1 min with 0.4% crystal violet in 20% ethanol, and washed extensively with water until excess stain was removed. All transfections were done in duplicate.

RESULTS

Cytosolic GTP-bound H-, N-, and K-Ras4B Inhibit Elk-1 Signaling—Cytosolic Ras proteins containing GTPase-inactivating mutations have been shown to act as DN inhibitors of Ras signaling, whereas corresponding versions lacking a GTPase-inactivating mutation do not (3, 4, 6, 7). However, these studies were somewhat limited. First, only H-Ras, but not N- or K-Ras, was evaluated for DN activity. Second, direct comparisons between Ras(G12V) and Ras(Q61L) to determine the respective effects of these activating mutations on the potency of DN activity were not performed. Third, although it was shown that Ras-dependent Raf signaling could be inhibited, the potential of cytosolic GTP-bound Ras to inhibit other Ras effectors, such as PI3-K, was not evaluated. To address these points, we generated a series of H-, N-, and K-Ras mutants that were rendered unprenylated and, therefore, cytosolic by cysteine to serine mutations at position 186 (185 in K-Ras). Each protein also contained an activating mutation at either position 12 or 61. These constructs were H-Ras(G12V/C186S), H-Ras(Q61L/C186S), N-Ras(G12D/C186S), N-Ras(Q61K/C186S), and K-Ras4B(G12V/C186S). In addition, H-Ras(G12V/C186S) and H-Ras(Q61L/C186S) constructs also containing single mutations in the Ras effector domain (residues 32–40) were gener-

ated. Effector domain mutations (EDMs) at positions 35, 37, and 40 were originally identified by their abilities to bind preferentially to and activate the Ras effector Raf, RalGDS, or PI3-K, respectively (8, 11, 23). Therefore, these Ras proteins H-Ras(G12V/T35S/C186S), H-Ras(G12V/E37G/C186S), H-Ras(G12V/Y40C/C186S), H-Ras(Q61L/T35S/C186S), H-Ras(Q61L/E37G/C186S), and H-Ras(Q61L/Y40C/C186S) were designed to preferentially block Ras signaling through particular effector pathways. Although the H-Ras constructs but not the N- or K-Ras constructs contained an amino-terminal hemagglutinin (HA) tag, we have shown previously that there is no difference between tagged and untagged Ras proteins in either signaling or transformation (16).

To determine whether DN activity is specific to H-Ras or a general feature of Ras proteins, we first assessed the ability of cytosolic, activated H-, N-, and K-Ras to inhibit Elk-1 signaling induced by the corresponding membrane-localized forms when co-transfected into NIH 3T3 cells in a transient transcriptional transactivation assay. As shown in Fig. 1, each cytosolic Ras protein was able to inhibit signaling induced by the corresponding membrane-localized Ras protein (45–90% inhibition). Generally, N-Ras mutants inhibited better (85 and 90%) than H-Ras mutants (55 and 70%). Despite equivalent stability, cytosolic K-Ras(G12V) showed the lowest and most variable ability to inhibit its membrane-localized counterpart, suggesting that this Ras isoform may have more limited DN activity than H- or N-Ras DN. As expected, the DN Ras proteins alone showed no activity in this assay.

The data presented in Fig. 1 also show that each cytosolic Ras protein could cross-inhibit Elk-1 signaling induced by other membrane-localized Ras isoforms. In general, each cytosolic GTP-bound N-Ras mutant (G12D or G61K) most strongly inhibited Elk-1 signaling induced by membrane-localized N-Ras, whereas this isoform specificity was not seen with cytosolic GTP-bound H-Ras or K-Ras4B mutants. Moreover, cytosolic N-Ras(G61K) generally had the highest level of DN activity regardless of the identity of the membrane-localized Ras protein being inhibited. Here again, cytosolic K-Ras4B(G12V) displayed the least ability to inhibit Elk-1 activation and produced the most variable results between assays. Overall, these data suggest that DN activity is a general feature of Ras proteins and is not limited to one isoform or to a particular activating mutation. Nevertheless, K-Ras4B(G12V/C185S) showed decreased DN ability compared with H-Ras or N-Ras in this assay.

Cytosolic GTP-bound H-Ras Effector Domain Mutants (EDMs) Inhibit Elk-1 Activation Induced by Activated H-Ras or K-Ras4B—Although it is well established that Elk-1 activation results from Ras signaling through Raf, it is also clear that Elk-1 activation is modulated by Raf-independent inputs (24–26). Furthermore, since all Ras isoforms bind to and activate the three major Ras effectors (Raf, RalGDS, and PI3-K), the DN inhibition of Elk-1 activation by cytosolic Ras proteins shown in Fig. 1 could be mediated by the sequestration of any (or any combination of) Ras effector proteins within the cytosol. Effector domain mutations (EDMs) in Ras proteins that preferentially permit the association of membrane-localized, GTP-bound Ras with specific effectors were originally described in yeast two-hybrid systems using cytosolic Ras (8, 10, 11, 23) and have been widely used to show that distinct effects are mediated by Ras-dependent Raf, RalGDS, and PI3-K signaling pathways. Here we have used a corresponding set of cytosolic GTP-bound H-Ras mutants also containing EDMs (T35S, E37G, and Y40C) to inhibit signaling by endogenous wild-type Ras through Raf, RalGDS, and PI3-K, respectively.

The data presented in Fig. 2A show partial inhibition of

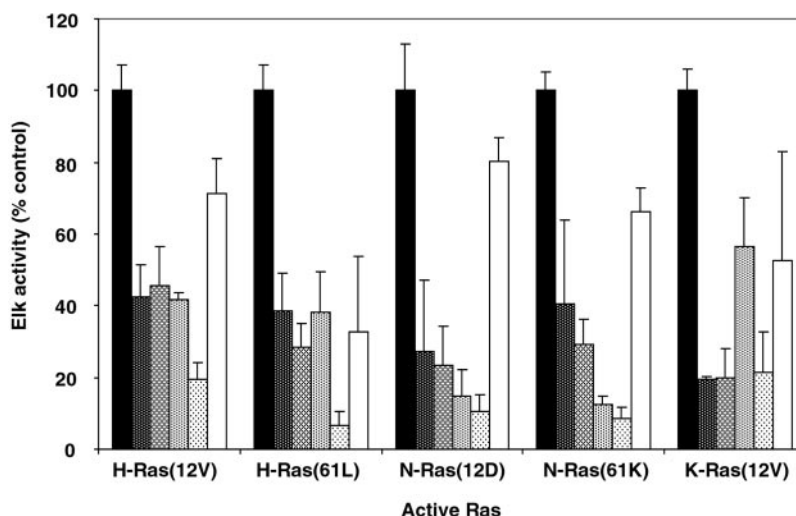


FIG. 1. Cytosolic GTP-bound H-, N-, and K-Ras4B inhibit Elk-1 activation by their membrane-localized counterparts and cross-inhibit Elk-1 activation by other Ras isoforms. Co-transfection of fibroblasts with 100 ng of plasmid coding for each GTP-bound Ras proteins (H-Ras(G12V), H-Ras(Q61L), N-Ras(G12D), N-Ras(G61K) or K-Ras(G12V)) along with 1 μ g of plasmid coding for each cytosolic GTP-bound Ras protein (H-Ras(G12V/C186S), H-Ras(Q61L/C186S), N-Ras(G12D/C186S), N-Ras(G61K/C186S), or K-Ras(G12V/C185S)) in every combination shows that each cytosolic GTP-bound Ras can act as a DN inhibitor of Ras signaling regardless of which Ras protein is inducing Elk-1 activation. Overall, K-Ras(G12V/C185S) produced the most limited inhibitory effect. Data are shown as average luciferase activity \pm S.D. from at least two independent experiments done in duplicate. All data using cytosolic Ras proteins are normalized to luciferase activity generated by GTP-bound Ras in the presence of vector alone, which is defined as 100%. Co-transfection is as follows: vector, *black bars*; H-Ras(G12V/C186S), *dark stippled bars*; H-Ras(Q61L/C186S), *medium stippled bars*; N-Ras(G12D/C186S), *light stippled bars*; N-Ras(G61K/C186S), *dotted bars*; and K-Ras(G12V/C185S), *white bars*.

Elk-1 signaling by each of the effector domain mutants (55–60%), compared with 85–90% inhibition by parental H-Ras(Q61L/C186S). This suggests that all three signaling pathways are necessary for full activation of Elk-1 by H-Ras and that partial DN activity can occur via inhibition of different signaling pathways. Similar results were obtained if farnesylated K-Ras(G12V) (Fig. 2B) or geranylgeranylated K-Ras(G12V/188L) (Fig. 2C) was used to induce Elk-1 activity, which suggests that both H-Ras and K-Ras activate Elk-1 by the same multipathway mechanism (compare Fig. 2, A and B). Because it is well established that activation of Raf induces Elk-1 activation, it is not surprising that H-Ras(Q61L/T35S/C186S), which should preferentially block Raf, inhibits Elk-1 activation. It has also been shown that PI3-K may lead to the activation of Elk-1 (25). Our observation that H-Ras(Q61L/Y40C/C186S) inhibits Elk-1 activation supports this conclusion. Our data also indicate that Ras effectors capable of binding E37G, such as RalGDS or phospholipase C ϵ (27), are involved in Elk-1 activation because the E37G EDM, which should preferentially inhibit these effectors, reduces Elk-1 activation to a similar degree as the T35S and Y40C EDMs.

Although this shows that cytosolic H-Ras can inhibit signaling by membrane-associated, GTP-bound K-Ras, treatment of cells with farnesyltransferase inhibitors not only generates cytosolic H-Ras but also generates alternatively prenylated (geranylgeranylated) K-Ras (28, 29), whose signaling properties may differ from those of the farnesylated form (30). Thus we evaluated the DN activity of H-Ras(Q61L/C186S) on signaling by K-Ras mutated at position 188 to be exclusively geranylgeranylated (K-Ras(G12V/188L)) and whether such inhibition was affected by EDMs within cytosolic H-Ras (Fig. 2C). Similar to results with farnesylated K-Ras(G12V), cytosolic GTP-bound H-Ras strongly inhibited Elk-1 activation (90%) by K-Ras(G12V/188L), supporting the possibility that DN Ras activity may indirectly contribute to farnesyltransferase inhibitor action. In contrast, each H-Ras(Q61L/C186S) EDM produced only partial inhibition (60%), suggesting that Raf, RalGDS, and PI3-K all participate in Elk-1 activation by

K-Ras(G12V/188L), and further suggesting that activated geranylgeranylated K-Ras functions similarly to activated farnesylated K-Ras in this process.

Dominant Negative Ras Mutants Remain Cytosolic—We confirmed that Ras proteins containing mutations of the prenylated cysteine at position 185/186 were cytosolic and did not significantly associate with the membrane by performing immunofluorescence analysis on cells stably expressing each unprenylated protein. Fig. 3 shows that all 185/186 Ras mutants are exclusively cytosolic within the limits of detection in this assay. None shows significant membrane localization, which contrasts sharply with the characteristic membrane fluorescence seen in H-Ras(Q61L)-expressing cells. That the cytosolic fluorescence observed in these cells is not due simply to non-specific staining is confirmed by the lack of cytosolic fluorescence in vector cells. Qualitatively similar levels of fluorescence observed in each cell line are consistent with similar overall protein expression levels.

Cytosolic GTP-bound H-Ras Inhibits Phosphorylation of Akt and Erk Stimulated by PDGF or Oncogenic Ras—Our data suggest that cytosolic GTP-bound Ras proteins inhibit Ras-induced Elk-1 activation through Raf, RalGDS, and PI3-K (Fig. 2). Because PI3-K is an upstream activator of Akt (31, 32) and Raf is an upstream activator of Erk, we asked whether Akt or Erk activation were also affected by cytosolic GTP-bound Ras proteins. To stimulate phosphorylation of Akt or Erk, NIH 3T3 cells stably expressing cytosolic H-Ras(Q61L), corresponding EDMs (T35S, E37G, or Y40C), or vector were either stimulated with PDGF to trigger endogenous Ras signaling or co-transfected with membrane-localized, oncogenic H-Ras(Q61L). Phospho-Akt (P-Akt) and phospho-Erk (P-Erk) levels were assessed by Western analysis. As shown in Fig. 4, cytosolic H-Ras(Q61L) significantly reduced PDGF-stimulated phospho-Akt or phospho-Erk levels compared with vector cells (Fig. 4A). These results demonstrate that cytosolic, active Ras can act as a DN inhibitor of growth factor-stimulated Akt or Erk activation, presumably by inhibiting Ras signaling through PI3-K or Raf, respectively. However, partial inhibition of P-Akt resulted from

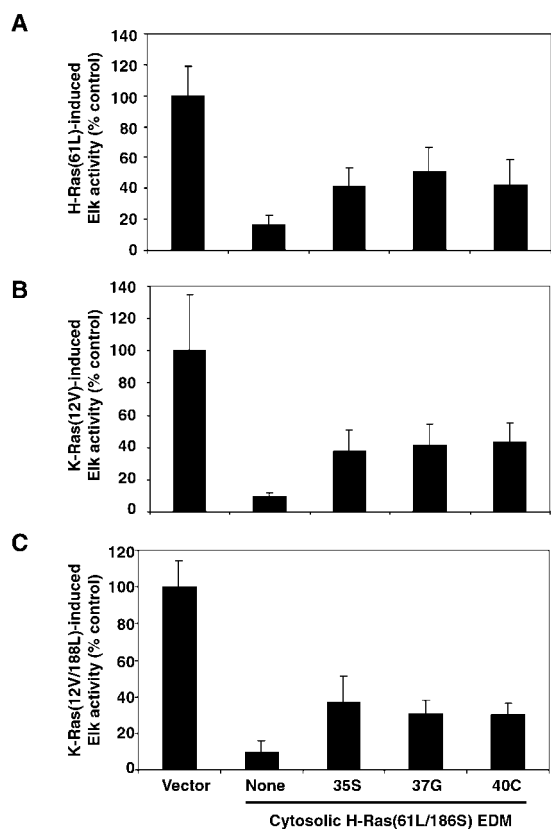


Fig. 2. Cytosolic GTP-bound H-Ras EDMs partially inhibit Elk-1 signaling by H-Ras, farnesylated K-Ras4B, and geranylgeranylated K-Ras4B. NIH 3T3 fibroblasts were transiently co-transfected as described in Fig. 1 with H-Ras(Q61L), farnesylated K-Ras(G12V), or geranylgeranylated K-Ras(G12V/C188L) plus H-Ras(Q61L/C186S), H-Ras(Q61L/T35S/C186S), H-Ras(Q61L/E37G/C186S), or H-Ras(Q61L/Y40C/C186S). In comparison to maximal inhibition of Elk-1 activation by H-Ras(Q61L/C186S), each H-Ras EDM had limited inhibitory ability regardless of which Ras protein was used to activate Elk-1. Data are shown as average luciferase activity \pm S.D. from at least two independent experiments done in duplicate. All data using cytosolic Ras proteins are normalized to luciferase activity generated in the presence of vector alone, which is defined as 100%. Similar results were obtained using H-Ras(G12V/C186S) and corresponding EDMs.

both the T35S and Y40C DN EDMs, whereas only T35S significantly inhibited P-Erk. This suggests that both Raf and PI3-K are involved in the activation of Akt, whereas only Raf is involved in Erk activation. That inhibition of PI3-K-reduced Akt phosphorylation is not surprising given the well established functional connection between these proteins. However, the fact that inhibition was only partial and that inhibition of Raf also resulted in reduced Akt phosphorylation is surprising because it implies an as yet unidentified role for Raf in Akt activation. The inability of the cytosolic E37G EDM to inhibit Akt or Erk activation suggests that the Ras effector RalGDS is not involved in these processes. Similar results were obtained when P-Akt and P-Erk levels were stimulated by co-transfection with active Ras (Fig. 4B), demonstrating that active, cytosolic Ras proteins can act as DN inhibitors of both normal and oncogenic Ras signaling.

Cytosolic GTP-bound Ras Induces Cell Flattening in Stably transfected Cells—Ras is known to participate in numerous aspects of cellular metabolism including changes in the cytoskeleton and adhesion resulting from integrin activation. The most striking feature of cells stably expressing cytosolic GTP-bound Ras proteins was their altered morphology and reduced growth rate. In the data presented in Fig. 5 each cell line shown

is expressing only the Ras protein indicated. Whereas vector cells have a morphology characteristic of normal NIH 3T3 fibroblasts, those expressing only cytosolic GTP-bound H-, N-, or K-Ras had a distinctly flattened appearance characterized by low refractility and more extensive spreading, suggesting that these dominant negative proteins are affecting a process in normal cells that modulates cell adhesion through endogenous signaling proteins. This effect contrasts sharply with the highly refractile, spindle-like transformed morphology of cells expressing dominant active H-Ras(Q61L), suggesting, as expected, that dominant active Ras and dominant negative Ras have opposing effects. Interestingly, cytosolic H-Ras(G12V) did not induce flattening. It is not clear why this is the case because this protein was expressed at similar levels to the other proteins (not shown), was cytosolic (Fig. 3), and inhibited Elk-1 activation as effectively as other cytosolic Ras proteins (Fig. 1). However, its inability to induce cell flattening is consistent with its relative inability to inhibit focus formation (Fig. 7). This inability is unlikely to be specific to H-Ras (*versus* N-Ras or K-Ras4B) because cytosolic H-Ras(Q61L) is able to induce this flattened morphology very efficiently. Also, the possibility that the Q61L mutation confers greater DN potency than the G12V mutation is not supported by the ability of cytosolic N-Ras(G12D) and K-Ras(G12V) to induce a flattened morphology. Finally, cytosolic K-Ras(G12V) can induce morphological changes even though it poorly inhibits Elk-1 activation (Fig. 1) whereas the reverse is true of cytosolic H-Ras(G12V), suggesting that Elk does not play a major role in cell morphology. Moreover, because of the central role played by PI3-K in modulating cell morphology (11), we predicted that inhibition of this pathway by the cytosolic Ras Y40C EDM would revert cells to a normal phenotype, whereas the T35S and E37G EDMs would have a lesser effect. However, none of the cytosolic Ras EDMs produced a flattened morphology, suggesting either that signaling through multiple Ras effectors is necessary for this phenotypic change or that these effects are too weak to be detected in this system. A role for multiple effector pathways in cell flattening is consistent with the relative inability of any of the H-Ras EDMs to fully inhibit Elk-1 activation (Fig. 2). Because simple visual inspection is not quantitative enough to conclude that each pathway has equivalent influence, further analysis is warranted.

Cytosolic GTP-bound Ras Proteins Enhance Cell Adhesion to Fibronectin—Overexpression of GTP-bound H-Ras has been reported to inhibit cell adhesion in Chinese hamster ovary cells by down-regulating integrins (33). Hence, cytosolic GTP-bound H-Ras might be expected to increase cell adhesion by blocking H-Ras signaling to integrins. To determine whether this was the case, we first assessed the adhesion to fibronectin of NIH 3T3 fibroblasts stably expressing each cytosolic GTP-bound Ras protein used in Fig. 1. Consistent with previous studies (33), prenylated GTP-bound H-Ras decreased cell adhesion to fibronectin by over 50% compared with vector alone, as shown in Fig. 6A. GTP-bound R-Ras(38V) served as a positive control; cells expressing it adhered to fibronectin 2-fold higher than control cells transfected with vector alone (34) (Fig. 6, A and B). The cytosolic form of GTP-bound H-Ras increased adhesion to levels comparable with that of active R-Ras, suggesting that DN Ras can counteract a Ras-induced decrease in adhesion in a manner similar to R-Ras. We also show that cytosolic GTP-bound N-Ras and K-Ras were also able to increase adhesion, presumably by blocking endogenous Ras signaling to integrins (Fig. 6A). These data are consistent with a dominant negative mode of action for cytosolic GTP-bound Ras mutants and with the idea that reversal of basal Ras signaling can result

FIG. 3. Dominant negative Ras mutants are cytosolic. Sparsely plated NIH 3T3 fibroblasts stably expressing the indicated DN Ras mutants were fixed in formaldehyde and stained with either anti-Ras or anti-HA antibodies followed by FITC-conjugated secondary antibodies. Cells expressing empty vector or prenylated H-Ras(Q61L) served as negative and positive controls, respectively. Representative examples of stained cells are shown. Significant cytosolic fluorescence seen in DN cell lines compared with vector indicates that DN mutant proteins are expressed at significant levels. The lack of membrane-specific fluorescence compared with prenylated H-Ras(Q61L) demonstrates that DN proteins do not localize to membranes to any significant degree.

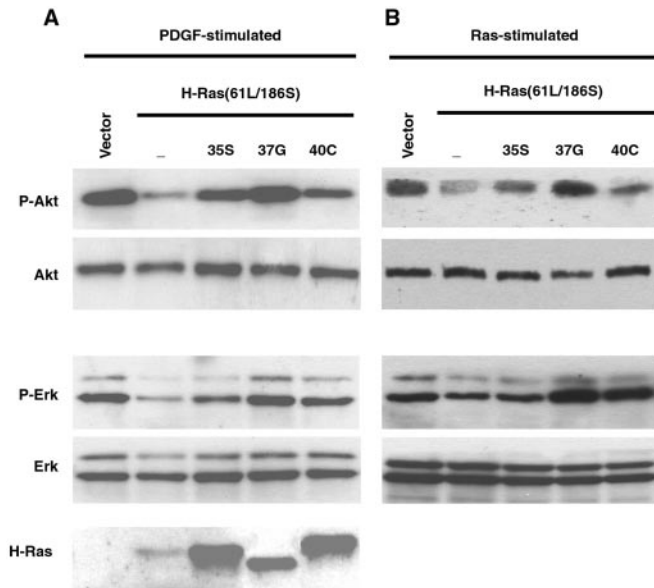
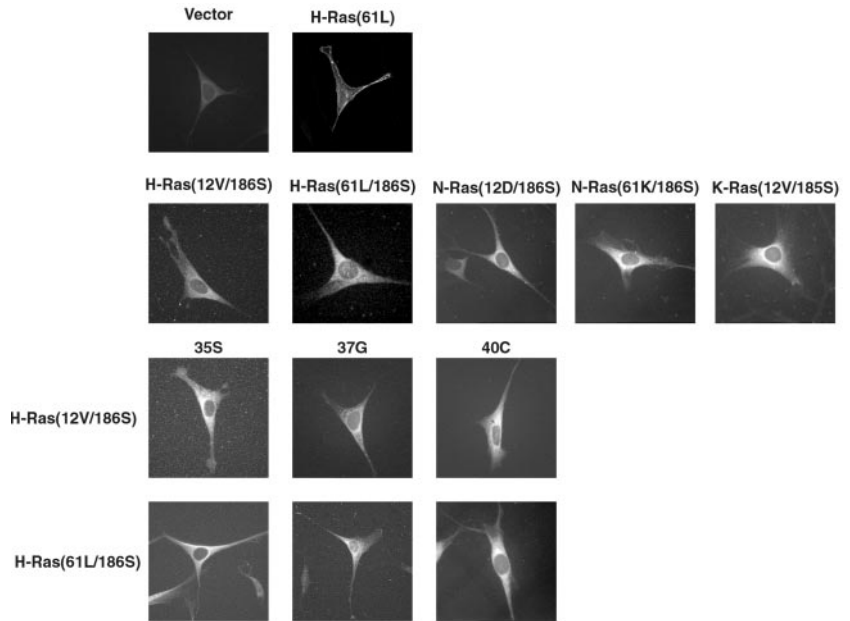


FIG. 4. Cytosolic GTP-bound H-Ras inhibits phosphorylation of Akt and Erk in a Raf- and PI3-K-dependent manner. NIH 3T3 fibroblasts stably expressing cytosolic H-Ras(Q61L), corresponding EDMs, or vector were serum-starved overnight, stimulated with PDGF for 5 min, and lysed in the presence of protease and phosphatase inhibitors (A). Alternatively, the same series of stable cell lines was transfected with fully prenylated, oncogenic Ras, serum-starved overnight, and lysed (B). Lysates containing 10 μ g of total protein were subjected to SDS-PAGE and Western analysis using antibodies against phospho-(Ser-473)-Akt, total Akt, phospho-Erk, total Erk, or H-Ras. Proteins were visualized by ECL. The lower panel in each pair demonstrates that differences in phospho-Akt and -Erk levels were not the result of differences in total Akt or Erk levels.

in enhanced integrin-dependent adhesion to extracellular matrices.

Because H-Ras stimulates at least three downstream effector pathways, we assessed which effector(s) is required for Ras-dependent inhibition of adhesion using cells stably expressing cytosolic H-Ras and corresponding EDMs (T35S, E37G, or Y40C). As shown in Fig. 6B, the T35S and Y40C EDMs showed significant 2-fold increases in cell adhesion to fibronectin. To a lesser extent the E37G EDM also increased adhesion relative to vector. These data suggest that Raf and PI3-K, and to a

lesser extent RalGDS, act downstream of H-Ras to down-regulate the fibronectin adhesion response in NIH 3T3 cells. The cytosolic E37G Ras EDM is able partially to block Elk activation (Fig. 1) demonstrating that this mutant is functional and that its relative inability to block adhesion to fibronectin is significant. That the T35S and Y40C EDMs can increase adhesion as effectively as parental H-Ras(Q61L/C186S) suggests that both pathways are necessary for decreased adhesion and that inhibiting either one is sufficient to block this effect. Alternatively, T35S or Y40C may be more complete blockers of a particular pathway than the parental mutants because their selectivity allows the whole population of that protein to associate with and inhibit a single effector rather than having to simultaneously titrate three (or more) effectors.

Ras is known to affect cell adhesion in part through the modulation of integrins (33) and that adhesion to fibronectin is mediated largely through the $\alpha_5\beta_1$ integrin (35). Therefore, DN Ras may be increasing adhesion by inhibiting the deactivation of a β_1 -containing integrin by endogenous Ras. To support this possibility we assessed the expression level of the β_1 integrin subunit in DN Ras cells to confirm that changes in adhesion did not result from changes in expression of this integrin subunit. First, we demonstrated the ability of anti-integrin antibody to prevent adhesion to fibronectin in cell expressing cytosolic Ras proteins. As shown in Fig. 6C pretreatment of cells with anti- β_1 integrin antibody (*white bars*) reduced adhesion of each cell line compared with corresponding cells pretreated with a control antibody (*black bars*). This demonstrates that adhesion is mediated in part by a β_1 -containing integrin. Residual adhesion after pretreatment with β_1 antibody implies that β_1 -independent mechanisms also participate in this adhesive process. We also analyzed cell surface expression using β_1 -specific antibody quantitated by flow cytometry. The *left peak* in each panel of Fig. 6D corresponds to basal fluorescence using a control antibody, whereas the *right peak* shows β_1 -specific fluorescence. The mean fluorescence intensity of the right peak correlates with β_1 expression. All DN cell lines express the β_1 integrin subunit suggesting that modulation of integrin activation state may be affected by DN Ras.

Cytosolic GTP-bound Ras Proteins Inhibit Focus Formation by Membrane-localized H-Ras—Because cytosolic GTP-bound Ras proteins were able to inhibit Elk-1 and Akt activation, alter the normal morphology of NIH 3T3 fibroblasts, and increase

FIG. 5. Cytosolic GTP-bound Ras induces a flattened morphological phenotype in stably transfected cells. Untransformed NIH 3T3 cells stably expressing each cytosolic GTP-bound Ras protein displayed a morphology distinct from vector-expressing cells, characterized by extensive cell spreading and low refractility indicative of cell flattening. This effect was observed in cells expressing cytosolic H-Ras(Q61L), N-Ras(G12D), N-Ras(G61K), and K-Ras(G12V) but not in cell expressing cytosolic H-Ras(G12V) or its EDMs. In contrast, cells expressing only membrane-localized, GTP-bound H-Ras(Q61L) were highly refractile and spindle-shaped, characteristic of Ras transformation. This suggests that DN Ras (cytosolic) and dominant active Ras (membrane-localized) have opposing effects on cell morphology.

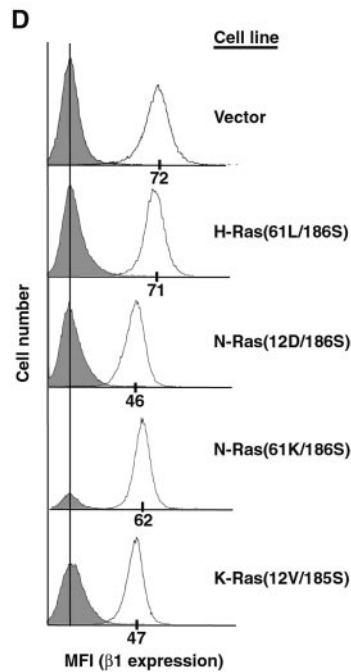
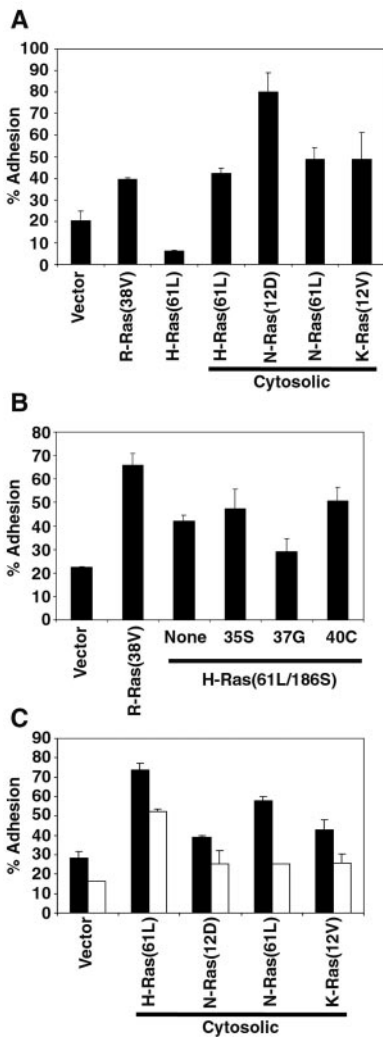
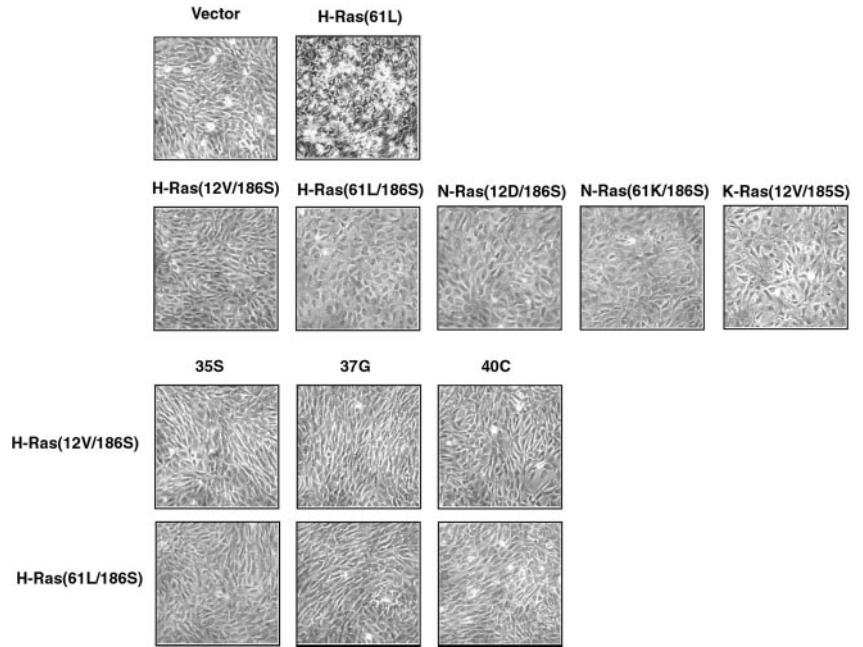
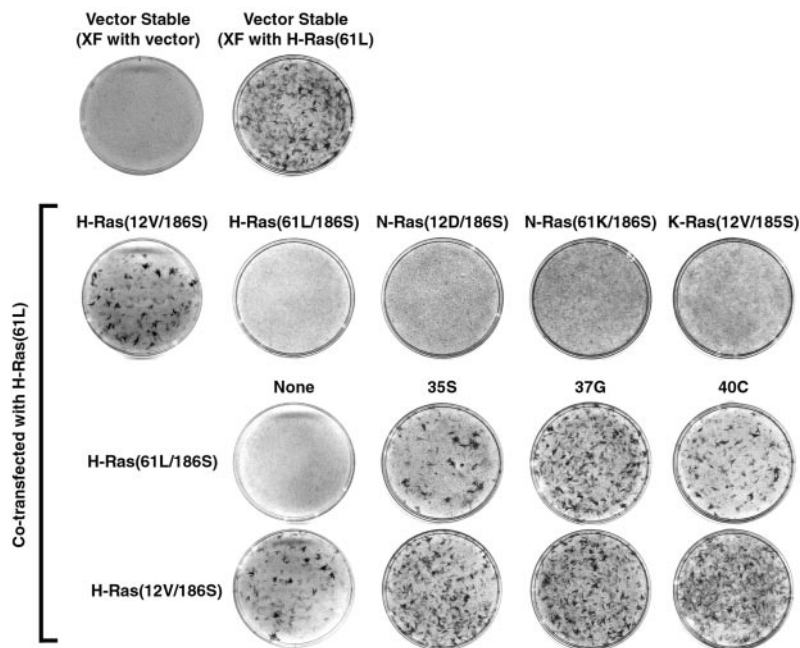


FIG. 6. Cytosolic GTP-bound Ras proteins enhance cell adhesion to fibronectin. NIH 3T3 fibroblasts stably expressing membrane-localized H-Ras(Q61L) or R-Ras(G38V), cytosolic H-Ras(Q61L/C186S), and corresponding EDMs, N-Ras(G12D/C186S), N-Ras(G61K/C186S), K-Ras(G12V/C185S) or vector were non-enzymatically removed from tissue culture plates, washed, counted, and loaded onto a fibronectin-coated 96-well plate for 40 min at 37 °C. After washing, total adherent cells were quantitated using a standard curve based on intracellular ATP content. Specific adhesion was determined by subtracting adhesion to BSA (see “Materials and Methods”). *A*, Vector represents the negative control of cells transfected with empty plasmid. The positive adhesion control is R-Ras (“38V”) is an activating mutation analogous to G12V in H-Ras). *B*, H-Ras with no EDM is the cytosolic GTP-bound form H-Ras(Q61L/C186S). T35S, E37G, and Y40C represent individual point mutations in the H-Ras effector loop in the context of cytosolic GTP-bound H-Ras(Q61L/C186S). *C*, cytosolic Ras-induced increase in cell adhesion to fibronectin is inhibited by preincubation with anti- β_1 antibody (white bars) compared with preincubation with control antibody (black bars) showing that β_1 is involved in the adhesive process. *D*, surface expression levels of the β_1 integrin subunit were evaluated by fluorescence labeling with anti- β_1 antibody and quantitated by flow cytometry. Gray peak, control antibody; white peak, β_1 -integrin antibody. Mean fluorescence intensity (MFI) correlates with β_1 expression. In all panels error bars depict S.E. of duplicate means from representative experiments. Each experiment was performed at least three times.

adhesion to fibronectin, it was of interest to next determine their effectiveness in preventing the more complex process of Ras-induced transformation, which involves each of these elements. NIH 3T3 fibroblasts stably expressing cytosolic GTP-

bound Ras proteins were transfected with constitutively active, GTP-bound H-Ras to induce focus formation. Only H-Ras(Q61L)-transfected cells are shown in Fig. 7; identical results were also obtained when foci were induced with

FIG. 7. Cytosolic GTP-bound Ras proteins inhibit focus formation by membrane-localized H-Ras. NIH 3T3 fibroblasts stably expressing each cytosolic GTP-bound Ras protein or vector were transfected with 200 ng of pZIP H-Ras(Q61L) to induce foci. Vector cells were also transfected with empty pZIP vector as a negative control. All plates are labeled with the identity of the stably expressed protein. All plates, except where indicated, were transfected with H-Ras(Q61L). Stained cells are shown after 2 weeks of growth under standard conditions. All cytosolic GTP-bound Ras proteins inhibited focus formation in comparison to vector cells transfected with H-Ras(Q61L), although cytosolic H-Ras(G12V) showed the most limited inhibition. Raf and PI3-K but not RalGDS are implicated in focus formation by the observed reduction in inhibition in cells expressing the T35S or Y40C EDMs of cytosolic H-Ras(Q61L), respectively. Similar results were obtained using H-Ras(G12V) to induce foci. All focus assays were performed in duplicate with identical results. *XF*, transfected.



H-Ras(G12V). All cells co-expressing active H-Ras and the active cytosolic Ras proteins produced fewer foci than did cells co-transfected with H-Ras and empty vector. Interestingly, cells expressing cytosolic H-Ras(G12V) showed the least inhibition of focus formation, whereas cytosolic H-Ras(Q61L), N-Ras(G12D), N-Ras(G61K), and K-Ras(G12V) all completely blocked focus formation. The reduced ability of cytosolic H-Ras(G12V) and its corresponding EDMs to inhibit focus formation is consistent with its reduced ability to induce morphological changes in NIH 3T3s (Fig. 4), although the reason for this is not clear because all cytosolic H-Ras(G12V) proteins are expressed at comparable levels to the other Ras proteins. Cytosolic EDMs T35S and Y40C but not E37G showed reduced inhibition, suggesting that the Raf and PI3-K but not the RalGDS effector pathways are involved in the induction of foci by H-Ras. The general ability of all Ras isoforms to inhibit focus formation is consistent with our observation that all isoforms also inhibit Elk-1 activation and alter the morphology of NIH 3T3 cells. It is also consistent with our observation that NIH 3T3 cells stably expressing cytosolic GTP-bound Ras proteins generally proliferated more slowly than vector-transfected cells (not shown). To demonstrate that cells stably expressing cytosolic GTP-bound Ras could be successfully transfected, we transfected each line with Raf(22W), a Ras-independent form of the c-Raf kinase that is able, by itself, to induce foci in NIH 3T3 fibroblasts (36). Raf(22W) induced foci in all cell lines (not shown) demonstrating that these cells could be transfected and that the lack of H-Ras(Q61L)- and H-Ras(G12V)-induced foci shown in Fig. 7 is the result of inhibition of Ras-induced focus formation by cytosolic GTP-bound Ras proteins.

DISCUSSION

Here we present the first extensive characterization of cytosolic GTP-bound Ras proteins and of their function as dominant negative (DN) inhibitors of Ras signaling. In contrast to normal, fully processed Ras, the cytosolic forms of GTP-bound Ras associate with effectors but fail to translocate them to the plasma membrane, thus sequestering them in non-productive complexes and blocking signaling. We have also used these proteins, and corresponding versions containing Ras effector binding domain mutations, to study the role of Ras effector pathways in various aspects of Ras signaling and transforma-

tion. The Ras EDMs T35S, E37G, and Y40C preferentially retain the ability to associate with Raf, RalGDS, and PI3-K, respectively.

We and others (4, 7) have shown previously that cytosolic GTP-bound H-Ras inhibits activation of the Ras effector Raf. Extending that observation, we show here for the first time that cytosolic GTP-bound N-Ras and K-Ras can also act as DN, that the potency of inhibition is independent of the activating mutation (position 12 *versus* position 61) in the cytosolic protein, and that signaling through another canonical Ras effector, PI3-K, can also be inhibited. Specifically, cytosolic GTP-bound H-, N-, and K-Ras4B can inhibit the activation of Elk-1 by any membrane-localized GTP-bound Ras in transient transcriptional transactivation reporter assays, regardless of the activating mutation. Maximal inhibition of Elk-1 by cytosolic GTP-bound H-Ras results from simultaneous inhibition of the Raf, RalGDS, and PI3-K pathways, because single effector domain mutations (T35S, E37G, and Y40C) in the cytosolic protein each yielded only partial inhibition. Cytosolic H-Ras inhibited phosphorylation of Akt and Erk, whereas introduction of additional mutations at T35S or Y40C limited this inhibition and E37G prevented it completely. In stably expressing NIH 3T3 fibroblasts, cytosolic Ras proteins were expressed at similar levels, remained cytosolic, and in the absence of EDMs, induced cellular flattening compared with vector-expressing cells. Consistent with this, we show that cytosolic GTP-bound Ras proteins increase NIH 3T3 cell adhesion to fibronectin and that a β_1 -subunit-containing integrin may be involved in this process. Finally, we show that transformation as measured by focus formation is inhibited by cytosolic GTP-bound H-, N-, and K-Ras and that this effect requires inhibition of Raf and PI3-K but not RalGDS.

Defining the roles of Ras and its effector pathways in either wild-type or oncogenic Ras signaling has been accomplished primarily through two approaches. The first involves expressing in cells membrane-localized, constitutively GTP-bound Ras proteins or their EDMs, which selectively permit Ras to signal through particular effector proteins, and showing that this is sufficient for the induction of a corresponding phenotype, such as altered cell morphology, adhesion, or motility, increased proliferation, or reduced apoptosis (8–15, 23). A second, com-

plementary approach involves small chemical inhibitors of several kinases downstream of Ras to block Ras-induced aspects of transformation, showing that either Ras or particular Ras effectors are necessary to induce a given a phenotype. Cytosolic GTP-bound Ras proteins are similar in function to chemical inhibitors but should also inhibit effectors even if those effectors are not well characterized. For example, in addition to Raf, RalGDS, and PI3-K, many other proteins, such as phospholipase C ϵ (27), AF-6 (37), Rin1 (38), p120Ras-GAP (39), and NF-1 (40), for which chemical inhibitors are not available, also interact with Ras and may also be effectors of Ras function. Moreover, collectively Raf, RalGDS, and PI3-K are insufficient to reconstitute all aspects of Ras transformation, implicating other additional effectors in Ras signaling. Because they contain an intact Ras effector binding domain, cytosolic GTP-bound Ras DN mutants should effectively inhibit all Ras effectors, even those that are as yet unknown. In this way cytosolic Ras DN mutants would be more effective inhibitors of Ras-mediated signaling than chemical inhibitors directed against specific proteins downstream of Ras.

Our observation that cytosolic H-, N-, and K-Ras could each significantly inhibit Elk-1 activation demonstrates that each Ras isoform could act as a dominant negative inhibitor of Ras signaling and that the identity of the activating mutation (12 *versus* 61) had no effect on this ability. Furthermore, our data show that DN activity is independent of the identity of the membrane-localized Ras that is used to induce Elk-1 activation, consistent with the fact that these proteins act through direct association with effector proteins. However, cytosolic GTP-bound K-Ras showed the least inhibition. This finding is inconsistent with a previous report (41) showing that K-Ras is a more potent activator of the canonical Ras/Raf/MEK/Erk/Elk-1 cascade than is H-Ras, suggesting that cytosolic K-Ras should sequester Raf and inhibit Elk-1 more effectively than cytosolic H-Ras. Neither the expression level nor the stability of cytosolic K-Ras was below that of H- or N-Ras constructs. It is possible that the relative inability of cytosolic K-Ras to inhibit Ras-induced Elk-1 activation is due to differences in affinity of K-Ras *versus* H-Ras for effectors. Such differences might be unmasked only in the cytosolic proteins, by loss of proximity to different effector pools due to membrane localization of the normal fully processed Ras proteins in (H-Ras) or out (K-Ras) of lipid rafts (42).

Partial inhibition of H-Ras-induced Elk-1 activity by each cytosolic GTP-bound Ras EDM suggests that each of these Ras effector pathways is necessary for full Elk-1 activity. We obtained similar results regardless of whether Elk-1 activation was stimulated by H-Ras, farnesylated K-Ras(G12V), or K-Ras(G12V) mutated in the carboxyl-terminal CAAX motif to be exclusively geranylgeranylated. Although H- and K-Ras have been reported to preferentially utilize either PI3-K or Raf, respectively (41, 43), these results suggest that K-Ras, like H-Ras, also requires multiple effector pathways for full activation of Elk-1. That inhibition of Raf with the cytosolic GTP-bound Ras T35S EDM reduces Elk-1 activity was not surprising because the functional connection between Raf and Elk-1 via the Raf/MEK/Erk/Elk-1 pathway is well characterized. Similarly, inhibition of PI3-K with the cytosolic GTP-bound Ras Y40C EDM resulted in lower Elk-1 activity, consistent with the work of others (25, 44) who showed that inhibition of PI3-K with LY294002 or wortmannin could block both Raf and Erk activation, possibly by blocking PI3-K-mediated effects on p21-activated kinase (26). Also, both wortmannin and a dominant negative p85 regulatory subunit of PI3-K (Δ p85) have been shown to block A-Raf and Erk activation (24). However, activation of Elk-1 by PI3-K is not observed in all contexts. For

example, in tumors in nude mice caused by NIH 3T3 cells expressing the membrane-localized, GTP-bound H-Ras Y40C EDM (12), no increased Erk activation was observed, suggesting that PI3-K is not upstream of Erk. Although our data support a role for PI3-K in Elk-1 activation, we cannot rule out the possibility that inhibition of Akt by the T35S EDM results from residual association with PI3-K. Perhaps more importantly, the presumed ability of parental unprenylated Ras proteins to bind and inhibit all cytosolic Ras effectors suggests that at least some of the various effects we have observed may result from inhibition of effectors other than Raf, RalGDS, or PI3-K and may even involve as yet unidentified proteins.

Our observation that the cytosolic GTP-bound H-Ras E37G EDM partially blocks Elk-1 activation suggests that RalGDS is also upstream of Elk. However, the preponderance of literature suggests that Ras-induced RalGDS signaling does not result in Erk activation. For example, it has been shown (9) that neither RalGDS nor the membrane-localized, GTP-bound H-Ras E37G EDM, which preferentially signals through RalGDS, activated Erk in NIH 3T3 fibroblasts, the same cell type used in our studies. Other evidence exists as well (12, 14, 15, 45). However, the effector binding specificity of the Ras E37G EDM may not be complete. For example, although Ras(E37G) associates primarily with RalGDS (8) or phospholipase C ϵ (27), it has been shown to retain some Raf-binding ability (12). Furthermore, RalGDS and oncogenic Ras(E37G) were not functionally interchangeable in a muscle differentiation assay (46), suggesting other downstream targets of Ras(E37G). Thus, more than one Ras effector pathway may be partially inhibited by cytosolic GTP-bound Ras(E37G).

Although cytosolic GTP-bound Ras strongly inhibits PDGF- and Ras-induced phosphorylation of Akt and Erk presumably by sequestering PI3-K and Raf, respectively, the corresponding Y40C EDM, which selectively impairs the PI3-K pathway, had a limited effect on phospho-Akt levels. These results suggest that another signal(s) is required for full Akt activation. Our data show that such a pathway may involve Raf, because blocking Raf with the cytosolic GTP-bound Ras T35S EDM inhibits Akt to approximately the same degree as does blocking PI3-K with the corresponding Y40C EDM. Several studies (24–26)² have demonstrated modulation of Raf activity by PI3-K, primarily by showing that blocking PI3-K activity with LY294002 or wortmannin inhibits Raf-dependent phenotypes. It has also been shown that Akt directly phosphorylates Raf resulting in inhibition of Raf kinase activity (48–50). However, our observation that the cytosolic T35S EDM also partially inhibits P-Akt suggests that the reverse is also true, that Raf contributes to PI3-K activity. It has been shown (51) that Raf is not a direct activator of PI3-K. However, Raf activation of PI3-K may be indirect. A recent report (52) demonstrates that Raf-induced transformation of NIH 3T3 cells requires an interleukin 1 autocrine loop, suggesting that blocking Raf may cause a decrease in interleukin 1-induced stimulation of PI3-K activity by the interleukin-1 receptor, thereby resulting in the reduced Akt activation that we observed. The dependence of Elk-1 activity on PI3-K may also be explained in this way, and it will be interesting to distinguish between direct and autocrine effects of Raf inhibition on Akt activation.

Ras is known to modulate cell adhesion. Oncogenic H-Ras-induced signaling through Erk correlates with a decrease in integrin activation (33), suggesting that one potential role for GTP-bound Ras mutants in human cancer is to decrease cell adhesion, thereby contributing to cell survival and prolifera-

² Q. T. Lambert, J. M. Lambert, J. M. Trzaskos, A. C. Tabaka, J.-S. Yan, L. M. Graves, and C. J. Der, unpublished results.

tion in the absence of substratum and to metastasis. In contrast, the Ras-related protein R-Ras increases cell adhesion to fibronectin (34) and collagen (53). Our results show that cytosolic GTP-bound H-, N- and K-Ras can increase the adhesion of NIH 3T3 fibroblasts to fibronectin to levels at least as high as those obtained using cells stably expressing GTP-bound R-Ras, possibly by inhibiting Ras-induced inactivation of integrins via the Erk kinase pathway (33). If integrins are involved in the increase in adhesion we observed, our results also suggest that inactivating integrins and reducing adhesion to the extracellular matrix are normal functions of endogenous wild-type Ras. Previous studies (33, 34, 54–56) utilized mutationally active Ras to modulate integrin activation making it difficult to evaluate the physiological relevance of these observations.

Our data also show that the cytosolic T35S and Y40C EDMs increase adhesion to the same degree as parental, cytosolic H-Ras, whereas the E37G EDM has a reduced effect. These results suggest that a Ras-induced decrease in cell adhesion is primarily dependent on Raf and PI3-K and to a lesser but significant extent on RalGDS. This is consistent with a study (33) showing that suppression of Ras-induced integrin activation correlated with activation of the Erk pathway, and with our observation that Elk-1 activation is dependent on multiple effector pathways including Raf and PI3-K. Because none of the cytosolic Ras EDMs (T35S, E37G, and Y40C) were able to induce cell flattening, whereas all were able to increase adhesion to fibronectin, it seems likely that the morphological changes we observed are not the result of inhibition of integrin deactivation alone but also involve other Ras-dependent events.

Cellular transformation, as demonstrated by the formation of Ras-induced foci in an NIH 3T3 cell monolayer, is a complex process that involves reduced contact inhibition of growth and alterations in cell morphology. Induction of foci by oncogenic H-Ras was completely inhibited by cytosolic GTP-bound H-, N-, and K-Ras, further confirming the general ability of Ras proteins to act as DN inhibitors regardless of activating mutation. The inability of cytosolic H-Ras(G12V) to inhibit focus formation relative to the other cytosolic Ras proteins was consistent with its inability to induce apparent cell flattening but was unexpected given its comparable level of expression, cytosolic localization, and ability to strongly inhibit Elk-1 activation. In contrast, cytosolic K-Ras gave the opposite results, producing only limited Elk-1 inhibition while strongly inhibiting focus formation. The explanation for this differential inhibition is unclear. That cytosolic K-Ras is less able than other cytosolic Ras proteins to inhibit Elk-1 activation, but is equally capable of inhibiting focus formation, supports the conclusion that multiple Ras effectors are required for maximum Ras-induced transformation. Further, the relative failure of cytosolic GTP-bound K-Ras to inhibit Elk-1 activation does not preclude its inhibiting other Ras effectors that are required for transformation. Alternatively, even weak inhibition of Elk-1 by cytosolic K-Ras may reduce Elk-1 activity below a threshold level necessary for transformation. That parental cytosolic H-Ras but none of the corresponding EDMs could produce complete inhibition of focus formation also supports the requirement for multiple Ras effectors in Ras-induced transformation.

The relative inability of cytosolic H-Ras(G12V) and its EDMs to fully inhibit foci or induce cell flattening compared with corresponding H-Ras(Q61L) proteins may be the result of previously observed functional differences between these activating mutations. For example, although both Ras(G12V) and Ras(Q61L) bind with similar affinities to the Ras effector Raf (57, 58), the G12V mutation reduces Ras affinity for Ras-GAP compared with wild-type, whereas the Q61L mutation has the

opposite effect, increasing affinity by almost 50-fold (59). Because Ras-GAP not only modulates Ras activity but may itself be an effector of Ras function, differential inhibition of Ras-GAP by cytosolic H-Ras(G12V) or H-Ras(Q61L) may account for the functional differences we observed. That both cytosolic Ras mutants inhibit Elk activation equally well suggests that any presumed effector function of Ras-GAP is not involved in this process.

Partial inhibition of Ras-induced foci by the cytosolic Ras T35S or Y40C EDMs demonstrates that Raf and PI3-K, but not RalGDS, are necessary for full Ras-induced focus formation. This is consistent with previous observations that membrane-localized, GTP-bound Ras T35S (Raf signaling) and E37G (RalGDS signaling) EDMs were partially impaired or fully impaired, respectively, in generating foci (8, 10), suggesting that signaling through Raf or RalGDS alone is insufficient for maximal Ras-induced morphological transformation. However, signaling through RalGDS is capable of synergistically enhancing foci produced by Ras T35S EDM (Raf signaling) (8), suggesting that RalGDS is necessary but not sufficient for maximal focus formation. Although our observation that the cytosolic GTP-bound Ras E37G EDM completely fails to inhibit Ras-induced foci confirms the insufficiency of RalGDS for this phenotype, it is inconsistent with the assertion that RalGDS is necessary for maximal Ras-induced focus formation. The unexpected observation that inhibition of PI3-K by LY294002 blocked Raf-induced focus formation² supports our observation that both Raf and PI3-K are necessary for maximal Ras-induced focus formation. Whether these Ras effectors exert their transforming effects through parallel pathways or sequentially in the same pathway as suggested by our data remains to be resolved.

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