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Heterogeneous Amino Acids in Ras and Rap1A Specifying Sensitivity to GAP Proteins

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Guanosine triphosphatase (GTPase) activity of Ras is increased by interaction with Ras-GAP (GTPase-activating protein) or with the GAP-related domain of the type 1 neurofibromatosis protein (NF1-GRD), but Ras is not affected by interaction with cytoplasmic and membrane forms of Rap-GAP; Rap1A, whose effector function can suppress transformation by Ras, is sensitive to both forms of Rap-GAP and resistant to Ras-GAP and NF1-GRD. A series of chimeric proteins composed of portions of Ras and Rap were constructed; some were sensitive to Ras-GAP but resistant to NF1-GRD, and others were sensitive to cytoplasmic Rap-GAP but resistant to membrane Rap-GAP. Sensitivity of chimeras to Ras-GAP and cytoplasmic Rap-GAP was mediated by amino acids that are carboxyl-terminal to the effector region. Residues 61 to 65 of Ras conferred Ras-GAP sensitivity, but a larger number of Rap1A residues were required for sensitivity to cytoplasmic Rap-GAP. Chimeras carrying the Ras effector region that were sensitive only to Ras-GAP or only to cytoplasmic Rap-GAP transformed NIH 3T3 cells poorly. Thus, distinct amino acids of Ras and Rap1A mediate sensitivity to each of the proteins with GAP activity, and transforming potential of Ras and sensitivity of Ras to Ras-GAP are at least partially independent properties.

THE RAS-RELATED GENES ENCODE A superfamily of proteins that are approximately 21 kD (p21), bind guanine nucleotides, and have intrinsic GTPase activity (*1*). The Ras protein can induce cellular transformation and is active when GTP is bound and inactive when GDP is bound. The intrinsic GTPase activities of Ras, Rap (also called p21 Krev-1 and smg-p21A), (*2–4*), and Rho can be accelerated by the distinct cellular proteins Ras-GAP, Rap-GAP, and Rho-GAP, respectively (*5–7*). The physiological function of the p21 and GAP proteins remains incompletely defined, but overexpression of Rap can suppress transformation by Ras.

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At least some members of the Ras superfamily can be regulated by more than one protein with GAP-like activity. Two distinct Rap-GAP molecules have been described (*6, 8*) and designated cytoplasmic and membrane Rap-GAP, respectively, although some membrane Rap-GAP is in the cytosol (*9*). Interaction of Ras with Ras-GAP or with the GAP-related domain of the type 1 neurofibromatosis protein (NF1-GRD) increases its GTPase activity in vitro and inhibits its biological function in vivo in yeast (*10, 11*). GTP-bound Ras is resistant to both forms of Rap-GAP, and GTP-bound Rap is resistant to NF1-GRD and to Ras-GAP although it interacts physically with Ras-GAP (*3, 12*).

Experimentally induced overexpression of Ras-GAP can inhibit Ras activity in mammalian cells (*13*) and decrease the proportion of GTP-bound Ras (*14*). However, it is not known whether Ras is regulated under physiological conditions by Ras-GAP, NF1,

or both proteins. The interaction between Ras and Ras-GAP is mediated at least in part via the Ras effector region (*15, 16*). The correlation between the biological activity of certain effector domain mutants and their interaction with Ras-GAP suggests that this interaction resembles that presumed to occur between Ras and its target. Ras-GAP itself might therefore represent a target of Ras function (*17, 18*; analogous arguments can also be made for NF1).

Studies of chimeric genes formed from *ras* and *rap*1A have been undertaken to localize the regions of their encoded proteins that mediate their respective effector functions (*19*). We have now used proteins encoded by such chimeras to examine the interaction of Ras and Rap1A with Ras-GAP, NF1-GRD, and the two forms of Rap-GAP.

The ras^H and *rap*1A genes encode colinear proteins of 189 and 184 amino acids, respectively, with *rap*1A encoding two additional residues between ras^H codons 120 to 136 and seven fewer residues between ras^H codons 161 to 186. The transforming function of Ras is mediated by divergent Ras-specific amino acids between residues 21 and 55, and the amino acids required for Rap to suppress Ras transformation have been localized to an even smaller span of Rap-specific amino acids within this region.

The catalytic region of Ras-GAP was used to probe the in vitro sensitivity to Ras-GAP of GTP-bound chimeric proteins (Fig. 1A) (*14*). The chimeric proteins could be divided into three groups: those with a Ras-GAP sensitivity similar to that of Ras, those that were resistant to Ras-GAP, and those with intermediate sensitivity. A chimera designated Ras(61–189) (*20*), in which amino acids 1 to 60 were encoded by *rap* and amino acids 61 to 189 were encoded by *ras*, was about as sensitive as authentic Ras to Ras-GAP, indicating that sensitivity of the chimera to Ras-GAP mapped to Ras amino acids located outside the effector region. This result also implies that amino acids 1 to 60 of Rap can substitute functionally for the analogous amino acids of Ras, although these amino acids include those that specify the opposite effector functions of Ras and Rap. Thus, Ras effector activity and sensitivity to Ras-GAP can be partially independent characteristics.

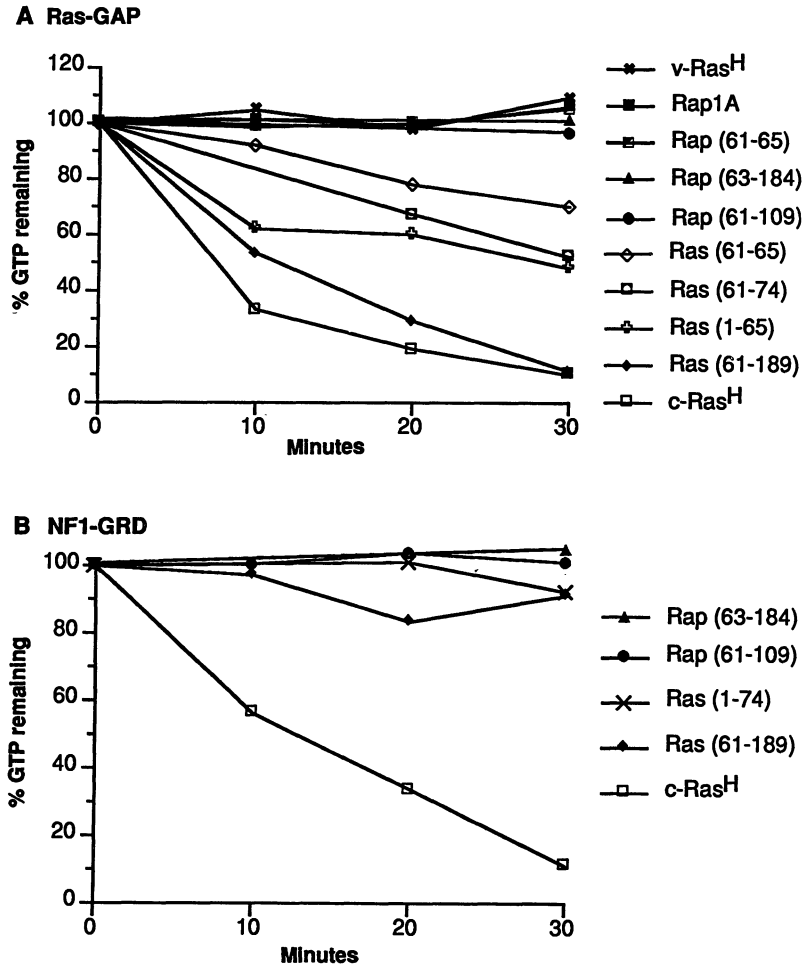

Fig. 1. Stimulation of GTPase activity of chimeric proteins (*29*) with Ras-GAP (**A**) and NF1-GRD (**B**). Chimeric proteins were expressed in *Escherichia coli* and purified as described (*30*). The reaction mixture for binding nucleotide and protein, which consisted of (in 50 μl) 0.1 M sodium phosphate (pH 6.8), 1 mM EDTA, sodium cholate (0.0025%), 5 mM $MgCl_2$, bovine serum albumin (BSA; 0.5 mg/ml), 0.5 mM DTT, and 1 μg of chimeric protein was incubated at 37°C for 15 min, and 1 μl of [γ-^{32}P]GTP (ICN, 1500 Ci/mM) was added. The mixture was then incubated at 30°C for 15 min. Five microliters of this mixture was incubated with purified Ras-GAP or NF1-GRD (*14*), in 50 μl of a buffer containing 20 mM Hepes (pH 7.5), 1 mM $MgCl_2$, BSA (0.5 mg/ml), and 1 mM DTT for 10, 20, or 30 min at room temperature. The reaction was stopped by the addition of 0.5 ml of ice-cold 20 mM tris (pH 7.5), 1 mM $MgCl_2$, and the product was trapped and washed on BA-85 filters (Schleicher & Schuell) and counted in a scintillation counter. Data shown are from a single experiment for the proteins sensitive to Ras-GAP. All proteins were tested multiple times. In most experiments with Ras(1–65), Ras(1–74), and Ras(61–65), the amount of GTP remaining after 30 min was closer to that shown for Ras and Ras(61–189) than in the experiment shown. The activation of GTPase activity of Ras and Ras(61–189) was reproducibly similar.

The importance of amino acids 61 to 65 of Ras was also reflected in the Ras-GAP resistance of a chimeric protein in which the only Rap amino acids were residues 61 to 65, Rap(61–65). Other chimeric proteins carrying these Rap residues, such as Rap(61–109), were also resistant to Ras-GAP. Furthermore, deletion of amino acids 58 to 63 from Ras caused resistance to Ras-GAP in vitro (*21*).

The chimera Rap(63–184) was resistant to Ras-GAP under our assay conditions, which are probably less sensitive than those which have detected a small degree of GTPase acceleration by Ras-GAP for a mutant Ras carrying Glu^{61} (*12, 22*). Since this chimera differs from Ras(1–65), which is sensitive to Ras-GAP, only at amino acids 63 to 65, its resistance suggests that these three amino acids of Rap can render a protein resistant to Ras-GAP.

Amino acid 61 is important for sensitivity of Ras to Ras-GAP. Mutation of Gln^{61} to Thr (which is the normal amino acid at residue 61 in Rap) in Ras or Ras(1–65) produces proteins, Ras-T^{61} and Ras(1–65)T, respectively, that are resistant to Ras-GAP. Other substitution mutants of residue 61 also render the protein resistant to Ras-GAP (*16,*

18). On the basis of crystallographic analysis of Ras, residues 61 and 63 are suggested to be the main activating group for in-line attack of water in initiating GTP hydrolysis (*23*). Substitution of Lys for Glu^{63} can partially activate the transforming activity of Ras (*24*), presumably by making Ras resistant to Ras-GAP.

In the crystal structure of Ras protein, there are two loops with flexible conformations. One is the effector loop (also called loop 2 or switch 1), which spans amino acids 26 to 36; its conformation varies depending upon whether Ras is bound to GTP or GDP (*25*). The other loop (called loop 4 or switch 2) is composed of residues 59 to 65. Loop 4 can assume several conformations, only one of which appears to be competent for GTP hydrolysis. The two loops are located close to each other on the exterior of the protein, where they should be readily accessible to interaction with other macromolecules.

Previous analysis of *ras* point mutants has emphasized that the effector region is necessary for Ras-GAP sensitivity. Our results indicate that the interaction between the Rap effector region and Ras-GAP is sufficiently similar to that between Ras and Ras-GAP so that the Rap effector, although it antagonizes Ras effector function, can substitute functionally for the Ras effector region in the in vitro Ras-GAP assay. Because residues 59, 60, and 62 in loop 4 are identical in Ras and Rap, the data indicating that residues 61 to 65 determine if a chimeric protein will be sensitive to Ras-GAP imply that the loop 4 region is the major determinant of Ras-GAP sensitivity, if there is also appropriate interaction with the effector region. Thus, it is likely that Ras-GAP interacts with loops 2 and 4. Placement of loop 4 in the permissive conformation may represent the rate-limiting step in hydrolysis of GTP by Ras (*23*). Our results suggest that Ras-GAP may function in part by promoting stabilization of loop 4 in this conformation.

Each chimera that was sensitive to Ras-GAP was resistant to affinity-purified recombinant NF1-GRD, although authentic Ras was sensitive to NF1-GRD (Fig. 1B) (*11*). Chimeric proteins that had been resistant to Ras-GAP were also resistant to NF1-GRD.

The resistance of the Ras(1–74) and Ras(61–189) chimeric proteins to NF1-GRD, both of which had been sensitive to Ras-GAP, implies that amino acids of Ras NH_2-terminal to residue 61 and COOH-terminal to residue 74 are required for sensitivity to NF1-GRD. Thus, many more amino acids of Ras are required to confer sensitivity to NF1-GRD than are required for sensitivity to Ras-GAP.

Partially purified membrane Rap-GAP was used to examine the sensitivity of chimeric proteins to this form of Rap-GAP (Fig. 2B) (*8*). As expected, the intrinsic GTPase activity of Rap was accelerated by this preparation, while that of Ras was resistant. None of the chimeric proteins tested were sensitive to membrane Rap-GAP, including Rap(63–184), Rap(61–109), Rap(61–74), and Rap(61–65), which were all resistant to Ras-GAP and NF1-GRD. Similar results were obtained with membrane Rap-GAP expressed from cDNA in insect cells infected with a baculovirus vector (*9, 26*). These findings suggested that some Rap amino acids NH_2-terminal to residue 61 are required for sensitivity to membrane Rap-GAP.

We assessed the sensitivity of chimeric proteins to cytosolic Rap-GAP from a cytosolic extract of NIH 3T3 cells (Fig. 2A). Only those chimeras that were resistant to Ras-GAP and NF1-GRD were examined because the cytosolic extract also contained Ras-GAP. The possible contribution of membrane Rap-GAP was not considered because NIH 3T3 cells do not appear to contain this form of Rap-GAP (*9*), and all chimeras were resistant to this form of Rap-GAP. Authentic Rap was sensitive to the extract, as were Rap(63–184) and Rap(61–109). Thus, the sensitivity or resistance of the cytosolic extract appeared to be independent of whether the first 60 amino acids were derived from Ras or Rap, as had been true for sensitivity to Ras-GAP. The results also suggest there may be structural similarity between the regions of Ras-GAP and cytosolic Rap-GAP that interact with the effector domains of Ras and Rap. Because the ability of Rap to suppress transformation by Ras maps to the first 60 amino acids of Rap, the effector function of Rap and its sensitivity to cytoplasmic Rap-GAP are partially independent properties.

Rap(61–74) was resistant to the extract containing cytosolic Rap-GAP, which indicates that when *ras* encodes the first 60 amino acids, the region of Rap that confers sensitivity to cytoplasmic Rap-GAP is larger

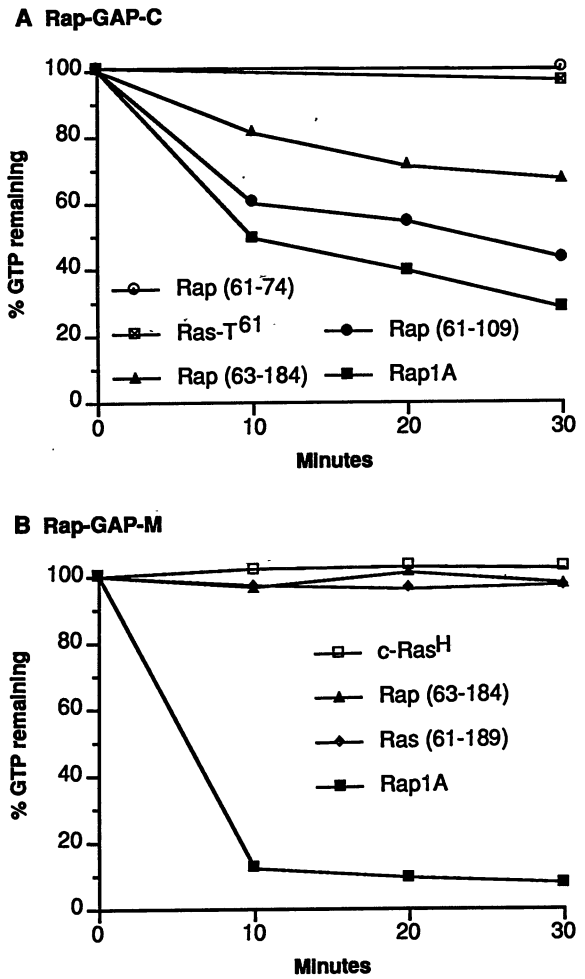

Fig. 2. Stimulation of chimeric proteins with cytoplasmic Rap-GAP [Rap-GAP-C, (**A**)] and membrane Rap-GAP [Rap-GAP-M, (**B**)]. Purification of membrane Rap-GAP has been described (*8*). To prepare cytoplasmic Rap-GAP, NIH 3T3 cells were scraped from culture plates and washed in PBS. The cells were homogenized in buffer containing 20 mM Hepes (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT. After centrifugation at 100,000*g* for 1 hour, the cytoplasmic fraction was adjusted to 1.5 mg of protein per milliliter and stored at −70°C. The assay for membrane Rap-GAP stimulation was performed as described in Fig. 1. For the cytoplasmic Rap-GAP assay, chimeric proteins were labeled under similar conditions but with [α-^{32}P]GTP and stimulated with the cytoplasmic Rap-GAP extract in buffer containing 20 mM tris-HCl (pH 8.0), 0.1 mM NaCl, 5 mM $MgCl_2$, and NP40 (0.5%). The reaction was then immunoprecipitated with either monoclonal antibody to Ras (Y13-238) (*31*), Ras antibody to the COOH-terminal portion of Ras (HA-034, NCI Repository, Bethesda, Maryland), or a rabbit polyclonal antibody to Rap, and protein A–Sepharose. The Sepharose beads were washed and the bound nucleotides were solubilized and subjected to chromatography on polyethyleneimine (PEI) cellulose plates with 1.3 M LiCl. The radioactivity of GDP and GTP was quantitated on an AMBIS Radioanalytic Imaging System. Each protein was tested several times. Data are shown from a single experiment in which chimeric proteins were tested for sensitivity to cytoplasmic Rap-GAP. In other experiments, the increase in GTPase activity caused by cytoplasmic extract was similar for Rap1A, Rap(61–109), and Rap(63–184).

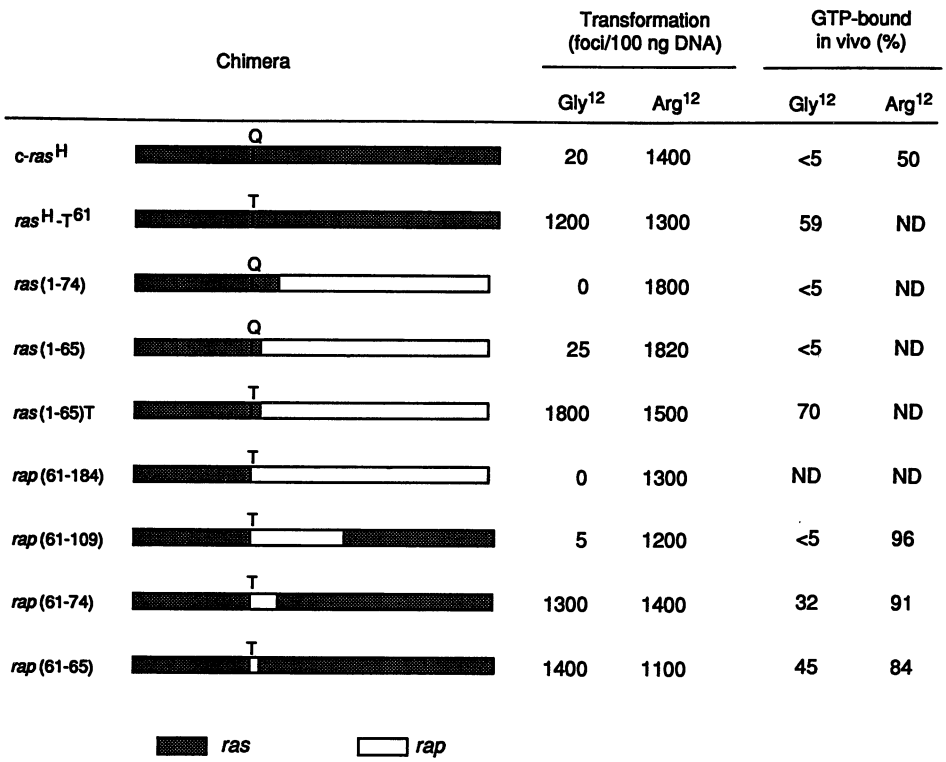

Fig. 3. Transforming activities of chimeric *ras-rap* genes and in vivo GTP binding to their encoded proteins. Residue 61, which is Gln (Q) in ras and Thr (T) in rap, is shown for each chimera; residue 61 is the authentic amino acid encoded by each gene, except for ras^{H}-T and *ras*(1–65)T, in which Glu^{61} is replaced with Thr. The number of foci formed by mouse NIH 3T3 cells transfected with each gene was determined as described (*30*). The percentage of chimeric protein complexed with GTP in vivo was calculated from the ratio of GTP- to GDP-bound protein as described with minor modification (*32, 33*).

than the region of Ras that confers sensitivity to Ras-GAP. The Thr encoded at residue 61 by *rap* is not necessary for sensitivity to cytoplasmic Rap-GAP since Rap(63–184) was sensitive to the extract (although it was resistant to the other three molecules with GAP activity). Residue 61 in Rap may therefore be less critical to the intrinsic GTPase activity of Rap (*12*) and the sensitivity of Rap to cytoplasmic Rap-GAP than is this residue to the intrinsic GTPase of Ras and the sensitivity of Ras to Ras-GAP. Assuming that a continuous segment of Rap is required for sensitivity to cytoplasmic Rap-GAP, resistance of the extract to Rap(61–74) and Ras(1–65)T implies that sensitivity to Rap-GAP requires Rap amino acids 66 to 74 and at least some of residues 63 to 65 and some of residues 74 to 109. These distinctions from Ras and Ras-GAP suggest there may be subtle, but important, differences in the mechanisms of intrinsic GTP hydrolysis by Ras and Rap or of activation of GTPase activity by Ras-GAP and cytoplasmic Rap-GAP.

Having found that sensitivity to Ras-GAP and cytoplasmic Rap-GAP was specified by amino acids COOH-terminal to the effector domain, we sought to determine if in vitro sensitivity of the chimeric proteins to these GAP proteins might correlate with the biological activity of the chimeras. We therefore tested whether expression of representative chimeric genes could induce focal transformation of NIH 3T3 cells. Each chimera had the potential to cause transformation, because amino acids 1 to 60, which include the effector region, were encoded by *ras*. To confirm this potential, the transforming activity of each chimera was first tested after replacing the normal Gly^{12} by Arg, which rendered the chimeras resistant to all GAP proteins (*26*) and is an activating mutation in Ras; these mutant chimeras transformed NIH 3T3 cells with high efficiency (Fig. 3). The biological activity of the chimeras was then examined in the context of the normal Gly^{12} (Fig. 3).

The Rap amino acids encoded by four chimeras began at Thr^{61}: *rap*(61–65), *rap*(61–74), *rap*(61–109), and *rap*(61–184). The two chimeric genes with the smaller number of *rap* codons, *rap*(61–65) and *rap*(61–74), transformed NIH 3T3 cells efficiently, whereas the other two chimeras had low biological activity. Thus, Rap(61–65) and Rap(61–74), which were resistant to all four GAP proteins, readily caused transformation, and Ras(61–109) which was sensitive only to cytoplasmic Rap-GAP had lower biological activity (Fig. 1 and 2). Rap(61–184) protein was not tested for its GAP sensitivity, but it would be expected to be similar to Rap(61–109).

Two other chimeras, *ras*(1–65) and *ras*(1–74), behaved similarly to authentic c-*ras*. These chimeras transformed cells inefficiently (Fig. 3), which correlated with their encoded proteins being sensitive to Ras-GAP, although they were resistant to NF1-GRD and the Rap-GAP proteins (Fig. 3). If these genes were modified to encode Thr in place of Gln^{61}, they transformed NIH 3T3 cells efficiently (Fig. 3) and the encoded proteins were resistant to Ras-GAP (*26*).

The biological activity of authentic ras is usually correlated with the proportion of the encoded Ras protein that is bound to GTP in vivo (*14, 27*). This correlation also held for the chimeras; GTP represented less than 5% of the nucleotide bound to the protein encoded by chimeric genes that transformed cells poorly, whereas it accounted for more than 30% of the nucleotide bound to the protein encoded by chimeras that efficiently transformed NIH 3T3 cells (Fig. 3). Thus, the protein encoded by each chimera that transformed cells inefficiently was sensitive in vitro to one of the GAP proteins, but the proteins encoded by chimeras that transformed cells efficiently were resistant to all GAP proteins. The results suggest that endogenous amounts of Ras-GAP and cytoplasmic Rap-GAP are sufficient to regulate Ras and Rap, respectively, in NIH 3T3 cells. The intermediate sensitivity of the Ras(1–65) and Ras(1–74) to Ras-GAP in vitro (Fig. 1) appears to be sufficient to allow their regulation by Ras-GAP in vivo.

Our data suggest that regulation by NF1 may not be required in NIH 3T3 cells to maintain Ras predominantly in the inactive, GDP-bound form. The apparent capacity of Ras-GAP alone to regulate Ras function in NIH 3T3 cells, although they express NF1 (*28*), may be relevant to the lack of phenotypic changes in fibroblasts and most other cell types in patients with type 1 neurofibromatosis.

Note added in proof: Properties of chimeras formed between Ras and Rap have been reported (*28a*) leading to similar conclusions regarding the amino acids that specify sensitivity to Ras-GAP and cytoplasmic Rap-GAP.

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29. Chimeric genes were made from the plasmids Krev-1 (a synthetic *rap*1A gene), v-*ras*-AT, pK39, and pK40, which have been described (*19*). Each of these genes contain unique Hind III (at codon 5), Pst I (at codon 60), and Xho I 3′ from the stop codon sites. A c-ras^H gene with a Pst I site at codon 60 was made by site-directed mutagenesis. Each chimera was constructed by first making the appropriate Pst I to Xho I fragment that would encode all amino acids COOH-terminal to residue 60. Plasmids pK39 and pK40 were used for *rap*(61–184) and *rap*(61–109), respectively. The fragments containing this Pst I to Xho I segment of *ras*(1–74), *ras*(1–65), and *ras*(1–65)T were synthesized from Krev-1 by means of the polymerase chain reaction (PCR). The partially homologous oligonucleotide used as the 5′ primer contained the desired coding sequence from codons 60 to 80 and the 3′ primer was oligonucleotide complementary to the 3′ noncoding region including the Xho I site. The fragments containing this segment of *rap*(61–74) and *rap*(61–65) were made from c-ras^H as above (oligonucleotide sequences are available on request). The Pst I and Xho I fragments containing these chimeric COOH-terminal regions were used to replace the COOH-terminal region of c-ras^H, v-*ras*-AT in a retroviral vector, pBW1423 as described (*30*). Chimeric genes encoding Ras(61–189), Ras(61–74), and Ras(61–65) were constructed by replacing the region encoding the COOH-terminal region of Rap1A with the Pst I to Xho I fragments encoding Ras, Ras(1–74) and Ras(1–65), respectively. To express chimeric proteins in bacteria, the Hind III to Xho I fragments encoding the amino acids from 5 through the COOH-terminus and the 3′ noncoding region were inserted into the Hind III and Sal I sites of bacterial expression vector 166A3 (*34*), which encodes amino acids 1 to 4 of Ras and contains a λ pl promoter.
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33. The cells were washed with 2 ml of phosphate-free Dulbecco's minimal essential medium (DMEM; Gibco) on [^{32}P]orthophosphate (0.5 mCi/ml; Amersham, PBS11A) in phosphate-free DMEM supplemented with dialyzed fetal calf serum (10%). After 12 hours, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and disrupted in 0.8 ml of lysis buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, NP40 (0.5%), aprotinin (1%)]. The nuclei were removed by centrifugation for 5 min in 1.5-ml plastic tubes. Monoclonal antibody Y13-238 (*31*) was added to the supernatant of lysates from cells transfected with constructs encoding v-Ras, c-Ras, Rap(61–109), Rap(61–74), and Rap(61–65), and a rabbit polyclonal antibody to Rap was added to lysates from cells transfected with constructs encoding Ras(1–74), Ras(1–65), and Ras(1–65). Fifty microliters of protein A–Sepharose (40%) were then added, and the mixtures were rotated at 4°C for 1 hour. The precipitate was washed three times with lysis buffer and resuspended in 15 μl of 10 mM tris-HCl (pH 7.5), SDS (1%), 20 mM EDTA, 10 μM GTP, and 10 μM GDP. The suspension was heated at 65°C for 5 min and centrifuged briefly. Five microliters of the supernatant were spotted on a polyethyleneimine (PEI) cellulose thin-layer plate, and developed in 1.3 M LiCl. The guanine nucleotides were visualized by exposure to x-ray film for 2 to 5 days. The radioactivity of GTP and GDP was quantified with an AMBIS Radioanalytic Image System. The percentage of nucleotide bound that was GTP was calculated on the basis of the radioactivity. All guanine nucleotides were assumed to be labeled uniformly.
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Cloning and Expression of the cDNA for Human γ-Glutamyl Carboxylase

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The cDNA for human γ-glutamyl carboxylase, which accomplishes the post-translational modification required for the activity of all of the vitamin K–dependent proteins, was cloned. The enzyme is a 758-residue integral membrane protein and appears to have three transmembrane domains near its amino terminus. The hydrophilic COOH-terminal half of the carboxylase has 19.3 percent identity with soybean seed lipoxygenase. Expression of the cloned cDNA resulted in an increase in carboxylase activity in microsomes of transfected cells compared to mock-transfected cells.

THE POST-TRANSLATIONAL MODIFICATION of glutamic acid to γ-carboxyglutamic acid accomplished by γ-glutamyl carboxylase is essential for the activity of all of the "vitamin K–dependent" proteins. These include some of the blood coagulation and anti-coagulation proteins as well as bone gla (γ carboxyglutamic acid) protein and bone matrix protein (*1*). Carboxylase is an integral membrane protein that was recently purified to near homogeneity (*2*). We now report the cloning, sequencing, and expression of the cDNA for the carboxylase. Knowledge of this sequence allows a better understanding of the mechanism of carboxylation, and availability of the expressed enzyme may allow production of fully functional carboxylated blood coagulation proteins in large amounts from cell culture.

The primary sequence of nine different tryptic peptides from the purified bovine γ-glutamyl carboxylase was obtained (Fig. 1). The longest contiguous amino acid sequence determined was 37 residues; part of this sequence was used for polymerase chain reaction (PCR) amplification of an 86-nucleotide (nt) fragment of carboxylase cDNA. The 55-nt sequence between the two PCR primers (Fig. 1) was used to generate a unique probe for screening a bovine liver cDNA library (*3*). This screening yielded three different partial clones, one of which, λ ZAP bGC1.6, contained the longest carboxylase cDNA insert and included six of the tryptic fragments of carboxylase. The entire λ ZAP bGC1.6 insert was used to screen a human liver cDNA library (*3*). More than 15 positive clones were identified; however, all of the cDNA inserts were small and were located in the 3′ end of λ ZAP bGC1.6. Subsequently, an Eco RI–Bgl II fragment [about 280 base pairs (bp)] from the 5′-end of λ ZAP bGC1.6 was used to screen a human erythroleukemia (HEL) cDNA library in λ gt10 (*4*). One cDNA clone, λ gt10.hGC, which contained the

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