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Mutational Analysis of a *ras* Catalytic Domain

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We used linker insertion-deletion mutagenesis to study the catalytic domain of the Harvey murine sarcoma virus v-*ras*^{H} transforming protein, which is closely related to the cellular *ras*^{H} protein. The mutants displayed a wide range of in vitro biological activity, from those that induced focal transformation of NIH 3T3 cells with approximately the same efficiency as the wild-type v-*ras*^{H} gene to those that failed to induce any detectable morphologic changes. Correlation of transforming activity with the location of the mutations enabled us to identify three nonoverlapping segments within the catalytic domain that were dispensable for transformation and six other segments that were required for transformation. Segments that were necessary for guanosine nucleotide (GDP) binding corresponded to three of the segments that were essential for transformation; two of the three segments share strong sequence homology with other purine nucleotide-binding proteins. Loss of GDP binding was associated with apparent instability of the protein. Lesions in two of the three other required regions significantly reduced GDP binding, while small lesions in the last required region did not impair GDP binding or membrane localization. We speculate that this latter region interacts with the putative cellular target of *ras*. The results suggest that transforming *ras* proteins require membrane localization, guanosine nucleotide binding, and an additional undefined function that may represent interaction with their target.

Proteins encoded by members of the *ras* gene family have been shown to serve essential functions in *Saccharomyces cerevisiae* and mammalian cells (8, 16, 29). These genes also have the capacity to induce morphologic transformation of NIH 3T3 cells either by overproduction of their normal protein product or by certain missense or other mutations of their protein-coding sequences (3, 4, 10, 33, 39, 46). *ras* genes with activating missense mutations have been identified in a wide variety of human and animal tumors (reviewed in reference 25).

It has not yet been determined how *ras* proteins carry out their normal functions or the pathway by which they induce cellular transformation. However, several characteristic features of these proteins that may be relevant to their physiological or pathological activities have been identified. These proteins bind guanosine nucleotides (GDP and GTP) and possess a GTPase activity analogous to that of the regulatory G proteins, with which they share some sequence homology (12, 22, 23, 27, 34, 38, 40). Activated versions of many *ras* proteins are associated with a significantly reduced GTPase activity. Recent experiments in *S. cerevisiae* have indicated that *ras* proteins are required for yeast adenylate cyclase activity, but stimulation by *ras* proteins of mammalian adenylate cyclase has not yet been demonstrated (1, 2, 41). The posttranslational binding of lipid to *ras* proteins and the migration of the molecule from its site of synthesis in the cytosol to the plasma membrane appear to be closely linked functions that are required for transformation (36, 43).

The *ras* proteins can be divided into at least three functional domains. The extreme carboxy terminus is required for lipid binding and membrane localization of the protein (43, 44). A region that lies just upstream from the C terminus is highly divergent among different *ras* genes (reviewed in reference 32); this heterogeneous region, which in mammalian *ras* proteins is about 20 amino acids, is not essential for transformation (45). The N-terminal 160 amino acids, many of which are conserved among *ras* proteins, presumably represent the catalytic domain of the protein. Mutations that enhance the transforming activity of these proteins (and reduce the GTPase activity) have been mapped to two regions: amino acids 12 to 13 and 59 to 63 (reviewed in references 13 and 35). However, the precise relevance of guanosine nucleotide binding to the biological activity of the protein, the regions of the protein that are required for this binding, and the presence or absence of other functions within this domain have not yet been determined.

In the present study we carried out a mutational analysis of the catalytic domain of the Harvey murine sarcoma virus v-*ras*^{H} oncogene. The protein encoded by v-*ras*^{H} is identical to that encoded by the normal human or rodent c-*ras*^{H} gene, except that the viral gene encodes two independently activating mutations, Arg-12 (for Gly) and Thr-59 (for Ala) (9, 47). We identified several segments in this domain that are dispensable for morphologic transformation and other segments that appear to be essential for transformation; some, but not all, of these essential regions affect guanosine nucleotide binding.

MATERIALS AND METHODS

v-*ras*^{H} mutants. We have previously described the technique used to generate in-frame v-*ras*^{H} mutations by deletion and linker insertion mutagenesis (43). In summary, the mutations are constructed by the combination of two sequenced parts (N-terminal front ends and C-terminal tail ends) of v-*ras*^{H} through a *Bcl*I oligonucleotide linker. This linker results in the addition of three novel amino acids within the protein at the site of the deletion, as shown in Table 1 for each mutant. Two eucaryotic vectors and one procaryotic vector were used to express the mutant genes in NIH 3T3 cells and *Escherichia coli*, respectively. One eucaryotic vector contains the Harvey murine sarcoma virus

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TABLE 1. Biological and biochemical activities of the mutant *ras* proteins[a]

Mutant no.	Amino acid structure[b]	Domain[c]	NIH 3T3 cells		Bacterial *ras* protein GDP binding[d]
			Foci[e]	*ras* protein	
pBW1160	1-189	Wild type	1.0	Yes	1.0
pBW1198	1-13 ADQ 14-189	1	0.001	No	0.01
pBW1199	1-21 PDQ 37-189	2	0.001	Yes	0.30
pBW1200	1-21 PDQ 30-189	2	0.001	Yes	1.10
pBW1232	1-27 SDQ 30-189	2	0.001	Yes	1.25
pBW1407	1-34 L 36 R38-189	2	0.001	Yes	0.60
pBW1301	1-41 DQ 44-189	2	0.001	Yes	ND
pBW1231	1-48 LIR 54-189	3	0.001	No	0.02
pBW1303	1-63 SDQ 73-189	A	0.8	Yes[f]	ND
pBW968[g]	1-68 ADQ 77-189	A	0.2	Yes[f]	0.45
pBW1201	1-71 TDQ 77-189	A	0.15	Yes[f]	ND
pBW1202	1-71 TDQ 83-189	4	0.001	No	1.30
pBW1265	1-89 SDQ 87-189[h]		1.0	Yes	ND
pBW1266	1-89 SDQ 98-189	4	0.001	No	0.02
pBW1267	1-92 LIR 96-189	B	1.2	Yes	ND
pBW1000[g]	1-92 LIR 104-189	B	0.5	Yes[i]	ND
pBW1224	1-96 LIR 104-189	B	0.8	Yes[i,j]	0.49
pBW1248	1-101 PDQ 109-189	B	0.85	Yes[i,j]	0.31
pBW1259	1-101 PDQ 112-189	5	0.001	No	ND
pBW1243	1-106 ADQ 106-189	B	0.90	Yes	0.12
pBW1244	1-106 ADQ 112-189	B, 5	0.07	No	0.01
pBW1196	1-110 LIR 104-189[h]		0.8	Yes	0.99
pBW1197	1-110 LIR 112-189	5	0.001	No	0.01
pBW1233	1-112 LIR 104-189[h]		0.65	Yes	ND
pBW1234	1-112 LIR 113-189	5	0.001	No	ND
pBW1263	1-119 PDQ 119-189[h]		0.6	Yes	0.14
pBW1220	1-119 PDQ 126-189	5, C	0.06	Yes[i]	0.01
pBW1235	1-119 PDQ 130-189	5	0.001	No	0.01
pBW1238	1-123 LIR 132-189	C	0.95	Yes[i]	0.03
pBW674[g]	1-128 PDQ 130-189	C	0.30	Yes[i]	ND
pBW677[g]	1-128 PDQ 139-189	C	0.95	Yes[i]	ND
pBW1239	1-130 PDQ 139-189	C	0.90	Yes[i]	0.18
pBW1240	1-130 PDQ 146-189	6	0.001	No	0.01
pBW1241	1-155 LIR 154-189[h]		0.10	Yes[i,j]	ND
pBW1236	1-165 PDQ 184-189	D	0.63	Yes	ND
pBW1260	1-185 LIS	8	0.001	Yes	ND

[a] The last three mutants in the table have been reported previously in the thymidine kinase-based vector (43, 45). They are included here (in the neomycin-based vector) for comparative purposes.

[b] The amino acid structure indicates the v-*ras*^{H} amino acids in front (N terminal) and tail (C terminal) ends, respectively; the letters indicate the three amino acids encoded by the oligonucleotide linker joining the front and tail ends. Mutant BW1301 encodes only two novel amino acids because residue 41 is fortuitously proline in the wild type and in the mutant. Mutant pBW1407 encodes two novel amino acids because residue 36 is isoleucine in the wild type and in the mutant.

[c] The domains are defined in Fig. 1. Essential regions are designated with a number, nonessential regions are shown with a letter.

[d] Relative GDP-binding activity. The wild-type protein, used as a standard in each assay, bound 0.7 to 1.0 M/M *ras* protein in different assays. ND, Not done.

[e] Foci are given as the relative efficiency of focus formation on NIH 3T3 cells compared with that of the wild type. In the neomycin-based vector, the wild type yields 700 to 1,200 foci per 0.2 μg in different assays. The wild-type thymidine kinase-gene in the based vector yields 200 to 300 foci per 0.2 μg.

[f] Reacted positively with *ras* antibody Y13-238, negatively with Y13-259. Yes without a superscript indicates the protein reacted with both antibodies.

[g] The focus-forming activity of these mutants was tested in the thymidine kinase-based vector. The focus-forming activity of the other mutants was tested in the neomycin-based vector.

[h] These mutants contain duplicated *ras* amino acids.

[i] Reacted positively with *ras* antibody Y13-259, negatively with Y13-238.

[j] Only the nonphosphorylated form of p21 was detected.

transforming region (the viral long terminal repeat plus v-*ras*^{H}) and a linker thymidine kinase gene (43). The eucaryotic vector used for most of the mutants was developed by C. Jhappan, G. Vande Woude, and T. Robins (submitted for publication). In this vector, the v-*ras*^{H} gene (the 2.1-kilobase *Bam*HI-*Eco*RI fragment) is located upstream from the simian virus 40 sequences and *neo*^{R} gene of pSVneo (37); these two genes are flanked by the Moloney murine leukemia virus long terminal repeat. The procaryotic vector (pJCL-30), which places the *ras* mutants under control of the lambda p_L promoter and initiates *ras* synthesis from its authentic initiation AUG, has been described previously (20). The *E. coli* cells that harbor the expression vector contain a temperature-sensitive allele of the lambda repressor gene *c*I857.

DNA-mediated gene transfer. The NIH 3T3 cells and DNA transfection procedure have been previously described (43). Cells were grown in Dulbecco modified minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml). G418 (0.5 mg/ml) was added when resistance to this antibiotic was used to select mutants.

Characterization of *ras* proteins. For immunoprecipitation, cultures transfected with Harvey murine sarcoma virus mutants were metabolically labeled with [^{35}S]methionine (250 μCi/ml) in methionine-free medium. Extracts of whole cells were prepared and precipitated as previously described with a *ras* monoclonal antibody, either Y13-238 or Y13-259 (11, 30). For cell fractionation, hypotonic swelling of the

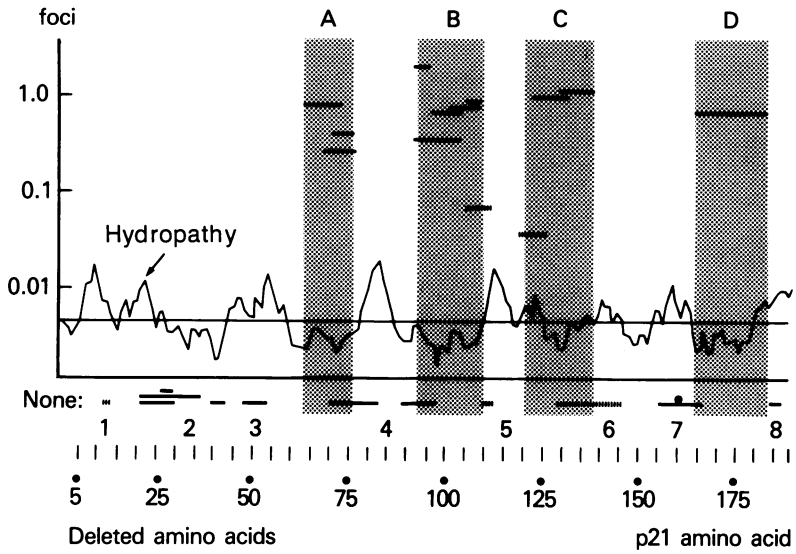

FIG. 1. Transforming activity of representative *ras* mutants and hydropathic index of the *ras* protein. The main horizontal axis represents the amino acids (1 to 189) in the *ras* protein; the vertical axis represents the relative NIH 3T3 focus-forming activity of the mutants. Each mutant is represented by a horizontal line (solid for those mutant proteins that bind GDP, interrupted for those mutants that do not bind detectable levels of GDP); this line corresponds to the location and extent of the deletion. Segments designated A to D represent sequences that are not essential for transformation. Regions designated 1 to 8 contain sequences that are essential for transformation. Data for mutants in A to C and 1 to 6 are taken from Table 1; data for mutants in D, 7, and 8, which are included for comparative purposes, are from reference 42. The two transformation-competent mutants with undetectable GDP-binding activities whose deleted sequences border essential region 5 are pBW1244 and pBW1220. The hydropathic index has been plotted by the method of Kyte and Doolittle (19); hydrophobic regions are above the axis of the midpoint line, hydrophilic regions are below.

cells was followed by homogenization and low-speed centrifugation to remove nuclei. The supernatant was then fractionated into a pellet particulate fraction containing the plasma membranes and a supernatant cytosol fraction as described previously (7) and subjected to immunoprecipitation. This procedure separates the cytosol-associated pro-p21 from the membrane-associated mature p21. Purified bacterially synthesized *ras* proteins were assayed with antibody Y13-259 by electroblotting.

GDP-binding, GTPase, and autophosphorylation activities. Purification of mutant protein and determination of GDP-binding activity by a filter binding assay were carried out as described previously (23). The ability of the proteins to release $^{32}P_i$ from [γ-^{32}P]GTP was measured on purified proteins (24). Autophosphorylation was determined by incubating the purified proteins with [γ-^{32}P]GTP under the GDP binding assay conditions. The extent of phosphorylation of p21 was determined by autoradiography after sodium dodecyl sulfate-gel electrophoresis.

RESULTS

Construction and expression of mutants. The v-*ras*^{H} gene encodes a primary protein product that is 189 amino acids in length. Using linker insertion mutagenesis, we constructed a series of in-frame deletions of various lengths. The mutations were located between amino acids 13 and 146 of v-*ras*^{H} (Table 1). Each mutated v-*ras*^{H} gene encoded three novel amino acids at the site of the deletion because of the oligonucleotide linker used in constructing the mutants. We also made some insertion mutants that did not have any deleted amino acids and a few duplication mutants that served principally as positive controls for certain deletion mutants that were found to be transformation defective (td) when their capacity to induce focal transformation of NIH 3T3 cells was analyzed. The eucaryotic vector into which all the td mutant v-*ras*^{H} genes and most of the transformation-competent genes were placed contained a linked neomycin resistance gene, which encodes a selectable phenotype in NIH 3T3 cells (resistance to the antibiotic G418) that is independent of morphologic transformation (5). This marker enabled us (i) to verify that differences in the efficiency of focal transformation by the mutants did not result from variations in the efficiency with which the cells took up the mutant DNAs, (ii) to characterize the mutants for other biological parameters of transformation after selection with this transformation-independent marker, and (iii) to study in NIH 3T3 cells the *ras* proteins encoded by td mutants. Representative mutants were also placed in a procaryotic expression vector to analyze the GDP-binding, GTPase, and autophosphorylating activities of purified mutant *ras* proteins produced in bacteria.

Transforming activity of the mutants. We first tested the capacity of these mutant v-*ras*^{H} genes to induce focal transformation of NIH 3T3 cells by DNA-mediated gene transfer (transfection) with a eucaryotic expression vector in which transcription of the mutant gene was promoted by a retroviral long terminal repeat. A wide range of focus-forming activity was observed for different mutants (Table 1; results of representative mutants are shown in Fig. 1). Some mutants were transformation competent and induced foci with an efficiency similar to that of the wild-type v-*ras*^{H} gene; the transforming capacity of other competent mutants was significantly reduced; and some mutants were td (any mutant whose transforming activity was more than 3 orders of magnitude lower than that of the wild-type gene will score as td in this assay). The foci induced by mutants possessing a transforming efficiency less than 20% that of the wild-type

gene were generally detected later and remained smaller than those of mutants that induced foci with an efficiency similar to that of the wild-type gene.

Given the evolutionary conservation of most of the v-*ras*^{H} gene, we expected that most deletions would abolish the transforming activity of the gene. Indeed, six different regions between amino acids 13 and 146 were apparently essential for transformation (labeled 1 to 6 in Fig. 1), since lesions in each region rendered the genes td. The precise boundaries of these essential regions are not defined because the td phenotype represents loss of a function, and we did not determine which of the variant amino acids in each mutant were responsible for the td phenotype.

To our surprise, we also noted three different segments between amino acids 63 and 139 where deletions did not abolish the transforming activity of the gene (labeled A to C in Fig. 1). Each nonessential segment was quite large; A, B, and C were at least 13 (residues 64 to 76), 16 (93 to 108), and 19 (120 to 138) amino acids long, respectively. Segment D in Fig. 1 corresponds to the majority of the heterogeneous region; the dispensable nature of this region for transformation has been shown previously (44), and it will not be discussed further.

Relatively unimpaired transforming activities were noted for deletions involving most of the amino acids within segments A (residues 64 to 72) and C (123 to 138) or any amino acids in segment B (93 to 108). However, significantly reduced transforming efficiencies were noted for mutants with deletion of codons at the 3′ end of segment A (amino acids 74 to 76) or the 5′ end of C (120 to 123).

Figure 1 also plots the hydropathic index of the wild-type protein, according to the program of Kyte and Doolittle (19). For soluble globular proteins, interior portions generally map to the hydrophobic side of the axis of the midpoint line, while exterior portions are usually found on the hydrophilic side. Each of the nonessential segments corresponds to hydrophilic regions, suggesting that they are located on the exterior of the protein. The required regions tend to fall on the hydrophobic side, with the notable exception of the hydrophilic region 2.

The ability of cells to grow in low concentrations of serum and to form anchorage-independent colonies in semisolid media are two biological properties associated with cellular transformation. These parameters can be used most effectively as indices of transforming activity when transfected cells have been selected by a marker that is transformation independent. The capacity of representative mutants to grow in low serum (0.5%) or to form colonies to 0.35% agar was therefore tested after the mutants had been selected by resistance to G418, which is transformation independent. These two biological activities correlated quite closely with the focus-forming efficiency of the mutants (Fig. 2); mutants that induced focal transformation with high efficiency grew better in low serum and formed larger colonies in agar.

Biochemical properties of the mutant proteins. We characterized the mutant *ras* proteins to identify parameters that might correlate with their biological activity. The proteins were studied both in the NIH 3T3 cells and after producing them in bacteria.

Purified bacterially derived *ras* proteins were used to determine the in vitro GDP-binding activities of the mutants (Table 1; Fig. 1). Some mutants bound the nucleotide with the high efficiency of the wild-type protein, several displayed low levels of binding activity, and others failed to bind detectable levels of GDP (any mutant that bound less than 1% as much GDP as the wild-type protein registered as negative in this assay). The capacity of the proteins to be autophosphorylated by GTP (34) correlated closely with their GDP-binding activity (data not shown).

All GDP-negative mutants were td, except for two (pBW1244 and pBW1220), and their biological activity was markedly impaired (Table 1; Fig. 2). The mutations in the GDP-negative genes were distributed among three of the six essential regions (1, 5, and 6 [Fig. 1]) identified in the NIH 3T3 transformation studies. Mutants from essential region 2 retained high binding activity, but the few mutants from essential regions 3 and 4 had low (2% of wild type) binding activity. Conversely, all mutants that transformed the cells with at least one-half the efficiency of the wild-type gene bound significant levels of GDP, although one of these mutants (pBW1238) bound the nucleotide only 4% as efficiently as did the wild-type protein. These results suggest that GDP-binding activity is necessary, but not sufficient, for efficient *ras*-mediated transformation and that considerable reduction in the binding activity can occur without significantly impairing the efficiency of focal transformation.

At least some activating mutations in *ras* (including those in v-*ras*^{H}) have been shown to be associated with a reduction in the GTPase activity of the protein (12, 23, 27, 28). We therefore considered the possibility that the reduced or absent transforming activity noted for some mutants might have resulted from restoration of the GTPase activity, particularly those mutants that bound GDP normally but displayed reduced or absent focus-forming activity on the NIH 3T3 cells. However, no mutant was found whose GTPase activity was higher than that of wild-type v-*ras*^{H}-encoded protein, although in each assay the GTPase activity of the normal (Gly-12, Ala-59) protein was at least 10 times higher than that of the wild-type viral protein (data not shown).

Immunoreactivity of the proteins. In addition to analyzing the bacterially derived *ras* proteins, we also characterized the *ras* proteins in NIH 3T3 cells stably transfected with the mutants. In single-dimensional polyacrylamide gel electrophoresis, the mature wild-type v-*ras*^{H} protein in NIH 3T3 cells is seen principally as a doublet that can be immunoprecipitated by several anti-*ras* antibodies, including monoclonal antibodies Y13-238 and Y13-259. The upper band of the doublet represents a phosphorylated form of the protein in the lower band (34). This phosphorylation of Thr-59 is believed to result in vivo from autophosphorylation. The cells were metabolically labeled with methionine and analyzed by polyacrylamide gel electrophoresis for the mutant *ras* protein by precipitation with antibodies Y13-238 and Y13-259 (Table 1; Fig. 3). The proteins encoded by td mutants were studied in the NIH 3T3 cells after G418 selection. For transformation-competent mutants, cells selected by morphologic transformation and G418 resistance gave qualitatively similar results, but the G418-resistant cells contained less *ras* protein, in agreement with the expectation that selection for focus formation represents selection for high p21 expression.

When the td mutants were analyzed, those from essential region 2 were found to contain readily detectable mutant protein (mutant 1199 in Fig. 3D), as had been found previously for td mutants with lesions at the C terminus (mutant 1260 in Fig. 3C). However, the cells with td mutants from the other five essential regions described here did not contain detectable levels of mutant protein when tested with both antibodies (data not shown). Since the bacterially derived proteins encoded by these td mutants bound antibody Y13-259 as well as did wild-type protein (data not shown), we tentatively conclude the mutant *ras* proteins not detected

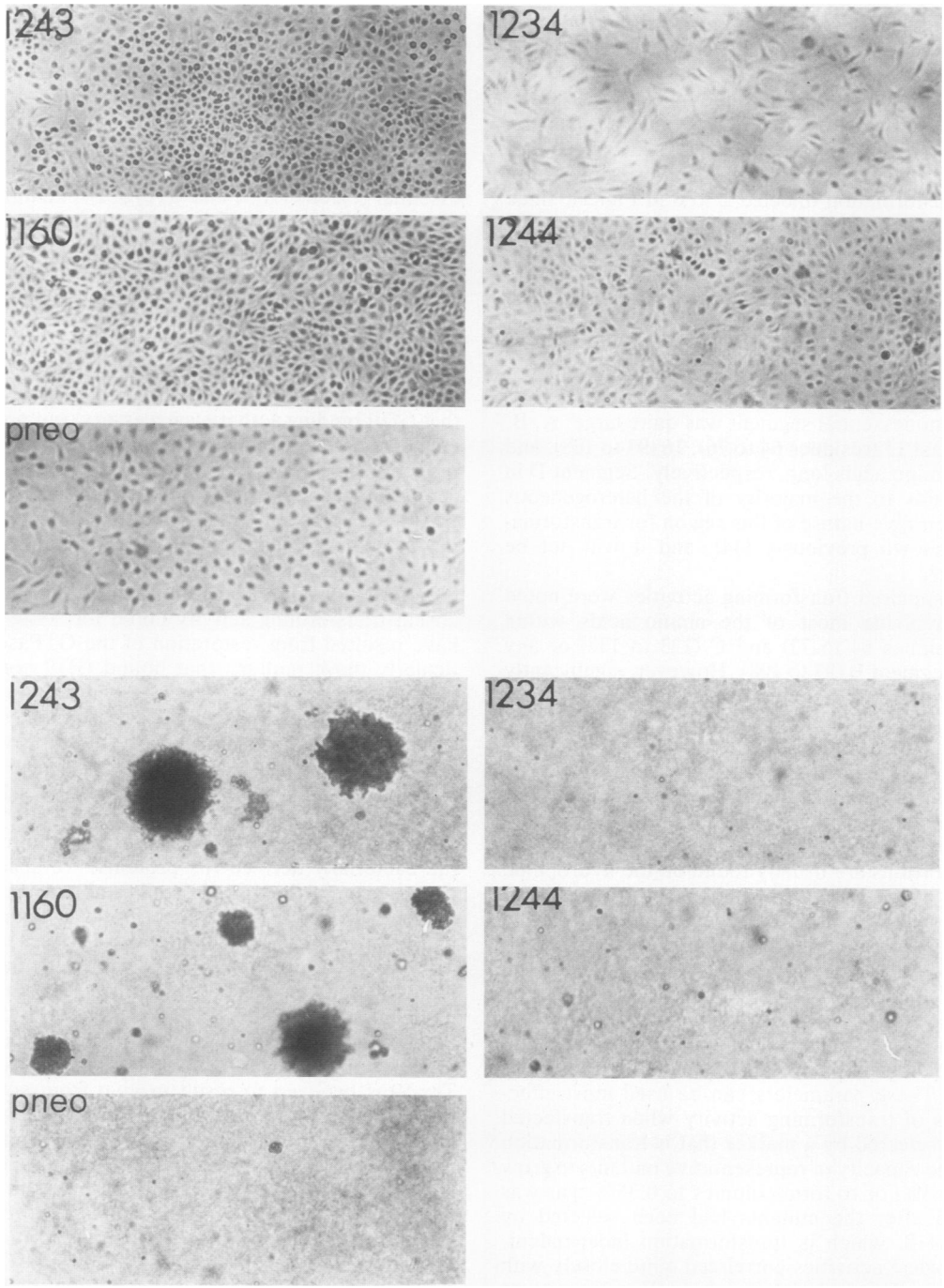

FIG. 2. Growth of cells in low serum (0.5%) and soft agar. Each mutant number corresponds to its designation in Table 1, which also lists its structure. pneo are cells transfected by the Neo^R-containing vector without a *ras* insert; 1160 is wild-type v-*ras*^{H} in the vector; 1196 and 1243 are highly transforming mutants; 1201, 1220, and 1244 transform less efficiently; 1197 and 1234 are td. After mass cultures of cells containing each mutant were selected by growth in the presence of the antibiotic G418 (0.5 mg/ml), 10^4 cells containing the designated mutant were seeded in 60-mm petri dishes. Cells were seeded and grown either as monolayer cultures in 0.5% fetal bovine serum or in 0.35% agar for anchorage-independent growth. The upper set of photomicrographs shows representative fields of the mutants 15 days after growth in 0.5% fetal bovine serum; the lower set shows representative fields after 25 days in agar; the graph on the adjacent page indicates the number of cells per dish after 18 days in 0.5% serum (determined by counting cells from duplicate dishes after trypsinization) and the proportion of colonies larger than 0.5 mm after 23 days growth in agar (determined with a micrometer attached to the inverted microscope).

in the NIH 3T3 cells are unstable in these mammalian cells. Results consistent with this interpretation were obtained with the two poorly transforming mutants (pBW1220 and pBW1244) that did not bind detectable levels of GDP; low levels of protein were detected in cells morphologically transformed by pBW1220 (Fig. 3A) and pBW1244 (Fig. 3B), but not in G418-selected cells transfected with either of these mutants (negative data not shown). The presence of a

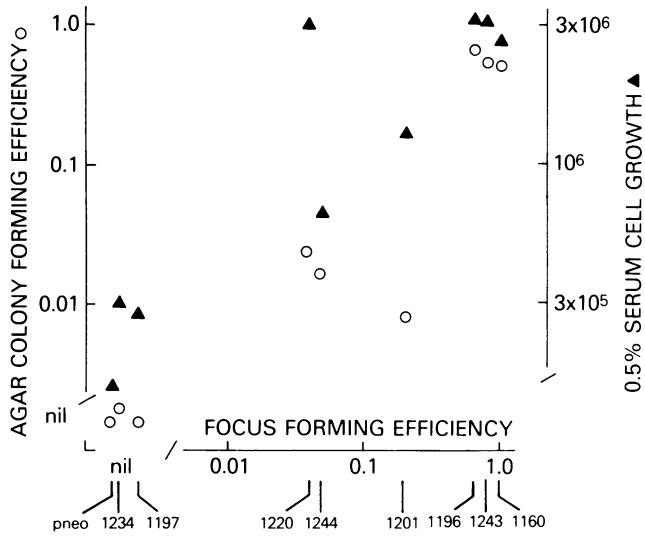

Fig. 2—continued

phosphorylated form of the protein from transformed cells from pBW1220 and pBW1244 shows that although nucleotide binding is undetectable in vitro, in vivo there is sufficient binding for autophosphorylation to take place.

When the transformation-competent mutants were studied, several of them were noted to react with only one of the two antibodies (Table 1; Fig. 3). Each mutant with a lesion in nonessential segment A (amino acids 64 to 76; mutant 968 in Fig. 3F) reacted with Y13-238 but not with Y13-259. The bacterially derived proteins from segment A mutants also failed to react with Y13-259 in an electroblot assay (data not shown). As we have shown elsewhere (31), this segment may encode the epitope recognized by this antibody since several segment A mutants from which residues 72 to 76 had been deleted did not bind Y13-259; however, a td mutant from which residues 72 to 82 had been deleted (pBW1202) did bind the antibody (further deletion [72 to 84] was once again negative). In contrast to the segment A mutants, each deletion mutant with a lesion in nonessential segment C (amino acids 120 to 138) was precipitated by Y13-259 but not by Y13-238 (mutant 1220 in Fig. 3A and 1239 in Fig. 3E), including one mutant (pBW674) from which only amino acid 129 had been deleted. However, this phenotype was not limited to mutations in this segment: a segment C mutant from which amino acids 93 to 103 had been deleted (1000, Fig. 3E) and mutants with duplication of amino acids 87 to 89 (pBW1265) and 154 to 155 (1241, Fig. 3A) also failed to be immunoprecipitated by this antibody.

These alterations in immunoreactivity suggest that the protein can tolerate significant conformational changes without loss of biological activity. Further support for this conclusion is found from the observation that two of the highly transforming segment B mutants (pBW1224 and pBW1248) encoded proteins that, despite their ability to bind GDP, were not detectably phosphorylated in the NIH 3T3 cells (data not shown).

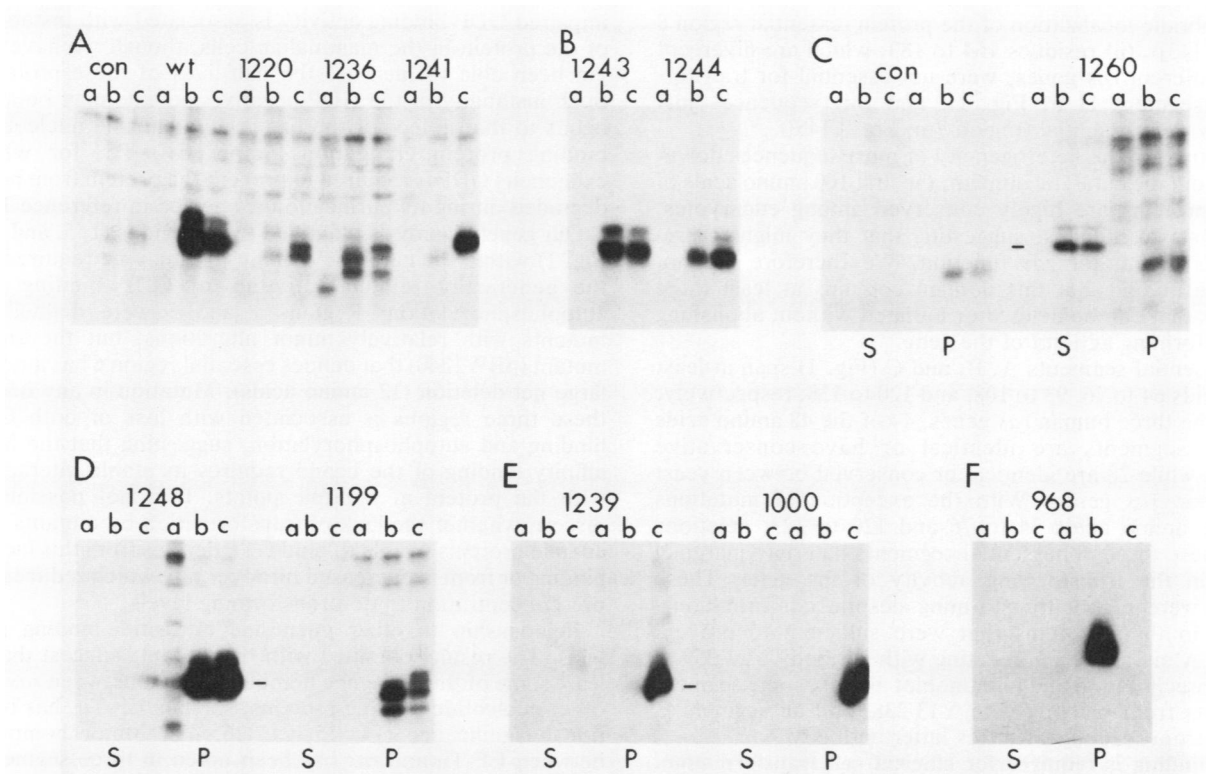

FIG. 3. Immunoprecipitation of mutant *ras* proteins from NIH 3T3 cells. Each mutant number corresponds to its designation in Table 1, which also lists its structure. con, Control NIH 3T3 cells; wt, wild-type v-*ras*^{H}; a, no antibody; b, *ras* antibody Y13-238; c, *ras* antibody Y13-259. Mutants were grown as morphologically transformed mass cultures, except for 1199 and 1260, which are td and were selected and grown in G418. After metabolic labeling with [^{35}S]methionine, extracts were made either without cell fractionation (A and B) or with fractionation (C through F) into a cytosolic supernatant (designated S below the panel) and a membrane pellet (designated P) and precipitated with the indicated antibody. The approximate location of the mutant *ras* protein is given by the horizontal line in panels C through E.

Subcellular localization of the mutant proteins. We also determined that lesions in the catalytic domain did not affect the subcellular location of the mature protein. Earlier studies had found that mutants with lesions in the C-terminus-encoded proteins that remained in the cytoplasm were td (pBW1260 in Fig. 3C), while the mature protein encoded by the wild-type gene or by transformation-competent mutants was associated with the membrane fraction (43). Using the same fractionation procedure, we found the protein encoded by representative transformation-competent mutants localized to the membrane fraction, as expected (Fig. 3C to F). A similar localization was found with td mutants from essential region 2 (mutant 1199, Fig. 3D), which was the only essential region in the catalytic domain in which mutants encoded detectable levels of protein in the NIH 3T3 cells. These results are consistent with earlier data suggesting that sequences of the C terminus of the protein represent the principal determinant of membrane association.

DISCUSSION

The v-*ras*^{H}-encoded protein contains many regions that are dispensable for transformation. This report represents the first systematic mutational analysis of a *ras* catalytic domain, which we consider to encompass the first 160 amino acids of the mammalian protein. Published studies of the catalytic domain have been limited to mutations in amino acids 11 to 13 and 59 to 63 (reviewed in references 13 and 35). Analysis of sequences downstream from the catalytic domain had indicated that (i) the last four amino acids of the protein (residues 186 to 189) were required for cell transformation and membrane localization of the protein (essential region 8 in Fig. 1 [43]); (ii) residues 164 to 183, which are divergent among different *ras* genes, were not essential for transformation (segment D in Fig. 1); and (iii) sequences just upstream were essential (region 7 in Fig. 1 [45]).

In contrast to the heterogeneity of most sequences downstream from the catalytic domain, the first 160 amino acids of the *ras* proteins are highly conserved among eucaryotes, from yeasts to humans, suggesting that they might be required as a unit for *ras* function. We therefore did not anticipate finding that this domain contains at least three segments that can be deleted or mutated without abolishing the transforming activity of the gene.

Nonessential segments A, B, and C (Fig. 1) span at least amino acids 64 to 76, 93 to 108, and 120 to 138, respectively. Among the three human *ras* genes, 44 of the 48 amino acids in these segments are identical or have conservative changes, while 28 are identical or conserved between yeast and human *ras* genes. With the exception of mutations involving amino acids 74 to 76 and 120 to 123, deletions within these three nonessential segments had only marginal effects on the transforming activity of the gene. These mutants were highly transforming despite conformational changes in their proteins that were sufficient to prevent segment A mutants from reacting with antibody Y13-259 in immunoprecipitation and immunoblot assays, some segment B mutants from reacting with Y13-238, and all segment C mutants from reacting with this latter antibody.

GDP binding is required for efficient cell transformation. Certain sequences outside these nonessential segments appear to be required for the in vitro GDP-binding activity of the protein, and this activity appears to be essential for efficient NIH 3T3 transformation. The importance of guanosine nucleotide binding for the transforming activity of *ras* has also been suggested by the observation that microinjection of *ras*-transformed cells with a *ras* antibody that prevents GDP binding in vitro can induce morphologic reversion (5).

With the exception of mutants pBW1220 and pBW1244, which had significantly impaired transforming activity, all mutants whose proteins did not possess detectable GDP binding in vitro were td. We believe that the failure to detect guanosine nucleotide binding in vitro with mutants pBW1220 and pBW1244 reflects the relative insensitivity of this assay (compared with NIH 3T3 cells focus formation), wich registers as negative when binding is less than 1% that of wild-type protein. Alternatively, the GDP-binding activity of some mutant proteins may be more sensitive than the wild-type protein to our extraction and purification procedure. In other systems, mutants are known that retain an essential activity in vitro when an in vitro assay suggests that this function would be absent (18). Since the GTP concentration in cells (about 10^{-3} M [6]) is so much higher than necessary for the p21 to be loaded with GTP (binding constant about 10^{-8} M [30, 34]), GTP binding might well take place in vivo on mutant proteins with binding constants that are changed 100-fold or more. It is presumably this high intracellular guanosine nucleotide concentration that enables mutants which in vitro show a greater than 90% reduction in binding to transform the NIH 3T3 cells with an efficiency approaching that of the wild-type gene.

When expressed in bacteria, the mutant proteins that failed to bind GDP in vitro had a stability that was similar to that of the wild-type protein. However, NIH 3T3 cells transfected with these td mutant genes did not contain detectable levels of these proteins. It therefore appears that impaired GDP-binding activity is associated with instability of the protein in the mammalian cells, though we have not yet been able to measure the half-lives of these proteins. Such instability of the GDP-negative mutants may be analogous to that noted for the bacterial guanosine nucleotide-binding protein elongation factor (EF) Tu, for which exogenous GDP is required to prevent the protein from being degraded during its purification (reviewed in reference 17).

Our genetic analysis identified three regions (1, 5, and 6 in Fig. 1) within the catalytic domain that may be required for the genetically related activities of GDP binding and autophosphorylation. Regions 1 and 5 were defined by mutants with relatively minor alterations, but the single mutant (pBW1240) that defines essential region 6 has a rather large net deletion (12 amino acids). Mutation in any one of these three regions is associated with loss of both GDP binding and autophosphorylation, suggesting that the high-affinity binding of the ligand requires its stable interaction with the protein at multiple points. It is not possible to discern whether the lack of transformation by mutants that encode proteins unable to bind GTP derives from this lack of binding or from an increased turnover rate which reduces the p21 concentration to nontransforming levels.

Relationship to other guanosine nucleotide-binding proteins. The results obtained with the mutants suggest that at least some of the sequence homology noted between *ras* and other nucleotide-binding proteins, such as EF-Tu, has functional significance (14, 15, 21, 26, 42). Major homology between EF-Tu and *ras* has been noted in three segments: amino acids 7 to 21, 57 to 61, and 109 to 121. In EF-Tu, which has not been subject to a mutational analysis but whose GDP-binding domain has been resolved crystallographically, the first two segments are implicated in phosphoryl binding, the latter in guanosyl binding. The activating mutations in *ras* have been localized previously to

these first two segments of homology. We did not test any mutants with lesions in the second of these segments, but essential regions 1 and 5 in Fig. 1, whose mutants are GDP negative, correspond to the other two regions of major homology. The *ras* mutants with lesions in essential region 5 establish the importance for GDP binding of sequences at both the 5′ and 3′ ends of the amino acid 109 to 121 segment of homology. The third essential region that was GDP negative (6 in Fig. 1) also shares some homology with EF-Tu. However, sequence homology by itself does not necessarily indicate that those *ras* sequences are essential either for guanosine nucleotide binding or cellular transformation, since EF-Tu and *ras* have some sequence homology in nonessential segment B (21).

It has been suggested that *ras* may function similarly to G proteins, which are membrane-associated guanosine nucleotide-binding proteins with GTPase activities that control enzymatic activities by regulating the reversible association of macromolecules (22, 28, 40). Results obtained with *ras* mutants may therefore have some relevance to the genetics of the G proteins. Tanabe et al. (40) have noted six segments of sequence homology between transducin-alpha and *ras* (amino acids 5 to 27, 58 to 73, 98 to 106, 111 to 116, 148 to 155, and 186 to 188). Three of these segments include the segments of major homology between EF-Tu and *ras* noted above. It therefore is likely that one function of sequences within these segments in transducin is to participate in guanosine nucleotide binding. The homology at the C terminus (amino acids 186 to 188) suggests that this segment in transducin also participates in membrane binding. The implications for transducin of our finding that amino acids 98 to 106 are not required for *ras* function, despite their homology with transducin, are not clear. This segment in transducin is believed to be required for its activation by ADP-ribosylation, which is a function that has not been described for *ras* proteins. We have not yet adequately studied *ras* mutants with lesions in amino acids 148 to 155. The only relevant mutant is pBW1241, which may be considered have a five-amino acid insertion between residues 155 and 156; it has a low transforming efficiency and forms foci of unusual morphology (45).

Implications for *ras* structure and function. Some aspects of normal *ras* protein structure and function can also be gleaned from analysis of the mutants. The segment B mutants, which did not bind antibody Y13-259, were useful to probe a normal *ras* function. In other studies (31), we have found that cells transformed by segment B mutants can overcome the block to cellular DNA synthesis induced by microinjection of Y13-259 into NIH 3T3 cells (29), while cells transformed by wild-type v-ras^H cannot overcome this block. This result implies that these transformation-competent mutant *ras* proteins can substitute functionally for at least this normal mammalian *ras* function and that segment B sequences are not required for this function. These lesions may also provide a way to test whether mutations in other regions we have found to be nonessential for the pathological activity of cell transformation may be essential for this physiological function.

In addition to these experimental studies, the hydropathic index of *ras* shown in Fig. 1 suggests several topological and functional features of *ras* protein when considered in conjunction with the transforming and biochemical activities of the mutants and the *ras* model derived from EF-Tu (15, 26). Most of the required regions appear to be hydrophobic, implying that disruption of interior portions alters the protein sufficiently to inactivate it biologically. The nonessential regions are apparently located at the surface of the molecule, corresponding to external alpha-helices in the EF-Tu model.

Essential region 2, which is highly conserved among *ras* proteins, is unusual in that it apparently represents a required exterior portion of the protein. Although there is no sequence homology between *ras* and EF-Tu in this region, acylated tRNAs are believed to bind to the analogous region of EF-Tu-GTP (14, 15, 17). The *ras* mutants with lesions in essential region 2 are td despite possessing all the known important biochemical features of *ras* proteins in that they synthesize a protein that is stable in NIH 3T3 cells, localizes to the membrane, and has GDP-binding, GTPase, and autophosphorylating activities that are similar to those of the wild-type v-ras^H protein. These mutants argue for the existence of an essential *ras* function that has not yet been defined. We speculate that the region 2 mutant proteins are td because they fail to interact with the elusive, putative target of the normal *ras* protein. Additional studies are required before this speculation can be substantiated or refuted.

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