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A *ras* Effector Domain Mutant Which Is Temperature Sensitive for Cellular Transformation: Interactions with GTPase-Activating Protein and NF-1

JEFFREY E. DECLUE,[1] JAMES C. STONE,[2] RACHEL A. BLANCHARD,[2] ALEX G. PAPAGEORGE,[1] PATRICK MARTIN,[1] KE ZHANG,[1] AND DOUGLAS R. LOWY[1]*

Laboratory of Cellular Oncology, Building 37, Room 1B-26, National Cancer Institute, Bethesda, Maryland 20892,[1] and Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada[2]

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A series of v-*ras*^{H} effector domain mutants were analyzed for their ability to transform rat 2 cells at either low or high temperatures. Three mutants were found to be significantly temperature sensitive: Ile-36 changed to Leu, Ser-39 changed to Cys (S39C), and Arg-41 changed to Leu. Of these, the codon 39 mutant (S39C) showed the greatest degree of temperature sensitivity. When the same mutation was analyzed in the proto-oncogene form of *ras* (c-*ras*^{H}), this gene was also found to be temperature sensitive for transformation. Biochemical analysis of the proteins encoded by v-*ras*^{H}(S39C) and c-*ras*^{H}(S39C) demonstrated that the encoded p21^{ras} proteins were stable and bound guanine nucleotides in vivo at permissive and nonpermissive temperatures. On the basis of these findings, it is likely that the temperature-sensitive phenotype results from an inability of the mutant (S39C) p21^{ras} to interact properly with the *ras* target effector molecule(s) at the nonpermissive temperature. We therefore analyzed the interaction between the c-*ras*^{H}(S39C) protein and the potential target molecules GTPase-activating protein (GAP) and the GAP-related domain of NF-1, on the basis of stimulation of the mutant p21^{ras} GTPase activity by these molecules in vitro. Assays conducted across a range of temperatures revealed no temperature sensitivity for stimulation of the mutant protein, compared with that of authentic c-*ras*^{H} protein. We conclude that for this mutant, there is a dissociation between the stimulation of p21^{ras} GTPase activity by GAP and the GAP-related domain NF-1 and their potential target function. Our results are also consistent with the existence of a distinct, as-yet-unidentified effector for mammalian *ras* proteins.

The mammalian *ras* genes (c-*ras*^{H}, c-*ras*^{K}, and c-*ras*^{N}) are closely related members of a multigene superfamily whose encoded proteins bind guanine nucleotides (5, 11). The *ras* genes were first discovered as the v-*ras*^{H} and v-*ras*^{K} transforming genes of the Harvey and Kirsten murine sarcoma viruses, respectively. Although overexpression of c-*ras* can transform established cells in vitro and be oncogenic in vivo, v-*ras*^{H} and v-*ras*^{K} contain point mutations which greatly increase these biological activities (3). The c-*ras* genes in a wide variety of human and animal tumors have been found to contain analogous mutations that augment their transforming activity (4).

ras genes encode membrane-associated 21-kDa proteins (p21^{ras}) which bind GTP and GDP with high affinity and can hydrolyze GTP to GDP. GTP-bound p21^{ras} is biologically active, while the GDP-bound form is inactive (7, 21, 29). Hydrolysis of GTP therefore negatively regulates p21^{ras}. Under steady-state conditions, most normal p21^{ras} is bound to GDP, while GTP is bound to a much higher proportion of mutationally activated p21^{ras}, which correlates with its increased transforming potential (10, 20, 29).

Mutational analysis of p21^{ras} has identified a region (amino acid residues 32 to 44 and surrounding residues) which appears to be required for proper signaling to the downstream effector of p21^{ras}. p21^{ras} mutants carrying lesions in this "effector domain" may be defective in their ability to transform cells, yet they retain normal GTP binding, are fully processed, and localize properly to the cell plasma membrane (25, 27, 32, 36). Despite these findings, the molecule(s) which functions as the target effector for mammalian p21^{ras} remains to be identified.

Two proteins which apparently interact physiologically with p21^{ras} have been identified. The GTPase-activating protein (GAP) was shown to greatly stimulate the intrinsic GTPase activity of normal p21^{ras} (29). In contrast, activated versions of p21^{ras} were resistant to GAP stimulation, allowing them to remain in the GTP-bound state, although they still interacted with GAP (31). Several defective effector domain mutants of p21^{ras} are impaired in their interaction with GAP (1, 6, 22). On the basis of these results, the interaction between p21^{ras} and GAP mimics the interaction between p21^{ras} and its target. Recently, the gene altered in the human genetic disorder neurofibromatosis (the *NF-1* gene) has been identified. The *NF-1* protein shares a region of homology with GAP, and like GAP this region (called the NF-1 GAP-related domain [GRD]) has been shown to stimulate the GTPase activity of normal p21^{ras} (2, 17, 33). The possible effector function of GAP and NF-1 is now under active investigation.

The use of conditional transformation mutants represents one approach to probing the interaction between p21^{ras} and its putative target. To date, only one temperature-sensitive (*ts*) *ras* mutant has been identified; the product of this gene was shown to be unstable at the nonpermissive temperature (24). In the present study, we describe the isolation and characterization of effector domain mutants which are *ts* for cell transformation. A detailed analysis of one of these mutants (Ser-39 to Cys, referred to as S39C below) has revealed that it is *ts* when present in either the normal c-*ras*^{H} form or the mutationally activated v-*ras*^{H} context. Unlike the previously described *ts* mutant of *ras* (24), the p21

* Corresponding author.

encoded by this mutant is not thermolabile. The mutant therefore appears to be defective for target interaction at the nonpermissive temperature. We have used this mutant to examine, in vivo and in vitro, the interaction of its encoded protein with the potential target molecules GAP and NF-1 GRD. The results indicate that the interaction of the mutant (S39C) $p21^{ras}$ with GAP and with NF-1 GRD, measured by assaying their ability to stimulate the intrinsic GTPase activity of the mutant protein, does not mimic the $p21^{ras}$-target interaction.

MATERIALS AND METHODS

Construction of *ts* v-ras^H mutants and a c-ras^H(S39C) mutant. To construct v-ras^H that would be *ts* for transformation, a pool of mutants was constructed by a "doped" synthetic oligonucleotide method (13). A previously described v-ras^H plasmid vector, which has a deletion in the *ras* effector region that can be repaired to encode the wild-type v-ras^H by insertion of a double-stranded 34-mer encoding the wild-type sequence (27), was used to construct the mutants. The mutant pool was generated by intentionally "contaminating" each phosphoramadite with 2% (vol/vol) of the other three phosphoramadite reagents immediately before constructing the pair of oligonucleotides. This level of contamination was designed to maximize the frequency of single- and double-nucleotide substitutions within the 34-mer. A pool of v-ras^H plasmids harboring the effector mutations was recovered, and these were placed under the control of the Moloney murine sarcoma virus long terminal repeat in the expression vector pGV16 (14), which contains the neo^R selectable marker and can be rescued as a retrovirus.

The S39C mutation in c-ras^H was constructed with oligonucleotide-directed mutagenesis and placed in a prokaryotic expression vector, pJCL-30 (15), and in pGV16 as described for many *ras* mutants (32). To allow for more efficient virus rescue than in the pGV16 vector (which contains two different promoters and whose RNA is more than 10 kb), the wild-type c-ras^H and c-ras^H(S39C) were removed from pGV16 by digestion with *Sst*II and *Xho*I and inserted in place of v-ras^H in a wild-type Harvey murine sarcoma virus vector (30).

Rescue and titration of transforming *ras* viruses. To isolate v-ras^H *ts* mutants, multiple samples of the pGV16-based plasmids were transfected into psi-2 cells (16) by the calcium precipitation technique, and several independent pools of retrovirus were recovered. Rat 2 cells (28) were infected (multiplicity of infection, less than 0.01) with a virus preparation, and G418-resistant colonies were selected at 37°C. Colonies that exhibited a slight degree of transformation were isolated, expanded in G418, and subcultured at 33°C and 39 to 39.5°C. Colonies that were distinctly less transformed at one of the two temperatures were infected with Moloney murine leukemia virus (MuLV) to rescue the presumptive *ts* mutant v-ras^H, and the temperature sensitivity of the rescued virus was tested in a focus assay in rat 2 cells. In the focus assay, triplicate dishes, seeded the previous day with 2×10^5 cells, were infected for 1 h with 0.2 ml of the virus preparation or of 10-fold serial dilutions of the virus preparation in the presence of polybrene (8 μg/ml) and incubated overnight at 37°C, and then one dish each was placed at 33°C, 37°C, and 39 to 39.5°C and incubated for 9 to 11 days. Dishes were then stained with crystal violet (0.7%), and foci were counted in a blinded manner.

The effector regions of the v-ras^H alleles that were confirmed by this procedure to be *ts* were then sequenced by recovering copies of the effector region by the polymerase chain reaction. Approximately 2 μg of genomic DNA extracted from the originally isolated colony served as the template in the polymerase chain reaction. After subcloning the appropriate polymerase chain reaction product into M13mp18 (18), the sequence of the variant v-ras^H allele was determined by the dideoxy chain termination method. In each case, at least three independent M13 subclones with identical v-ras^H sequences were analyzed before a variant sequence was assigned.

To generate the c-ras^H and c-ras^H(S39C) viruses, the genes expressed in the Harvey vector were cotransfected with pSV_2Neo (26) in the ratio 30:1 into NIH 3T3 cells. G418 selection and virus harvests were carried out as described above for the v-ras^H mutants, except that amphotropic MuLV was used as the helper virus. Titers of the c-ras^H viruses were determined in the same manner as for v-ras^H viruses, except that NIH 3T3 cells were infected with 0.5 ml of virus (or 1:10 serial dilutions) and analyzed after 10 to 12 days at 34 and 39.5°C.

Radiolabeling and immunoprecipitation of $p21^{ras}$. A total of 2×10^6 cells were plated in T-25 flasks, grown for 24 h at either 34 or 39.5°C, and labeled for 20 h in 2 ml of Dulbecco modified Eagle medium lacking methionine (Flow Laboratories) plus 10% regular Dulbecco modified Eagle medium, 2% fetal bovine serum, and 200 μCi of [^{35}S]Trans-label (ICN) per ml. Cells were rinsed once with cold phosphate-buffered saline (PBS) (GIBCO) and lysed in 600 μl of lysis buffer, as described elsewhere (35). Portions of cell lysates containing equal numbers of trichloroacetic acid-precipitable counts per minute were compared by incubation at 4°C with the anti-*ras* monoclonal antibody Y13-238 (9) and for 90 min with protein A-Sepharose which had been coated with rabbit anti-rat immunoglobulin (Organon Teknika Corp.). Immunoprecipitates were washed and processed as described previously (19).

Analysis of guanine nucleotide binding in vivo. Cells were plated as described above and labeled for 18 h in 2 ml of Dulbecco modified Eagle medium lacking phosphate (Flow Laboratories) and containing 10% dialyzed fetal bovine serum and 1 mCi of $^{32}PO_4$ (Amersham). The cells were rinsed twice with cold PBS and lysed as described above. The lysates were passed through a 22-gauge needle, pelleted for 15 min in a Microfuge, and subjected to immunoprecipitation as described above. Immunoprecipitates were washed three times with 10 mM $NaPO_4$ (pH 7.6)–100 mM NaCl–20 mM $MgCl_2$–1 mM EDTA–1% Triton X-100–0.5% Na deoxycholate–0.1% sodium dodecyl sulfate (SDS) and once with 10 mM Tris-HCl (pH 7.5)–20 mM $MgCl_2$ and boiled in 10 μl of 10 mM EDTA–1% SDS. The supernatant was spotted on a thin-layer chromatography plate (Polygram 801053), chromatographed in 1.3 M LiCl, dried, and exposed for autoradiography.

Protein purification and in vitro assays with GAP and NF-1 GRD. $p21^{ras}$ proteins were purified from *Escherichia coli* as described previously (32). Soluble p21 was further purified by chromatography on an Ultrogel AcA54 column (IBF Biotechnics) and eluted in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5)–0.5 M NaCl–1 mM EDTA–0.01% *N*-octylglucoside–1 mM dithiothreitol–5 mM β-mercaptoethanol. p21-containing fractions were pooled and dialyzed against 0.02 M Tris-HCl (pH 8.0)–50% glycerol and stored at −20°C. GTP binding was performed as follows: 1 μg of p21 was suspended in 50 μl of 0.1 M $NaPO_4$ (pH 6.8)–4 mM $MgCl_2$–0.5 mM EDTA–0.0025% Na deoxy-

TABLE 1. Focus formation by *ras* viruses

Plasmid or virus	No. of focus-forming units/ml at:		
	Permissive temp	Nonpermissive temp	Ratio
pBW1406	$<2.5 \times 10^0$	$<2.5 \times 10^0$	
v-*ras* with mutation at:			
Leu-36	2.6×10^2	5×10^0	5.2×10^1
Cys-39	7.8×10^2	$<2.5 \times 10^0$	$>3 \times 10^2$
Leu-41	8.6×10^2	7.5×10^0	1.1×10^2
Wild-type v-*ras*	2.1×10^3	7.6×10^2	2.7
Amphotropic MuLV	$<2 \times 10^0$	$<2 \times 10^0$	
c-*ras*(S39C)	4×10^3	1.5×10^2	2.7×10^1
Wild-type c-*ras*	1×10^4	8×10^3	1.2

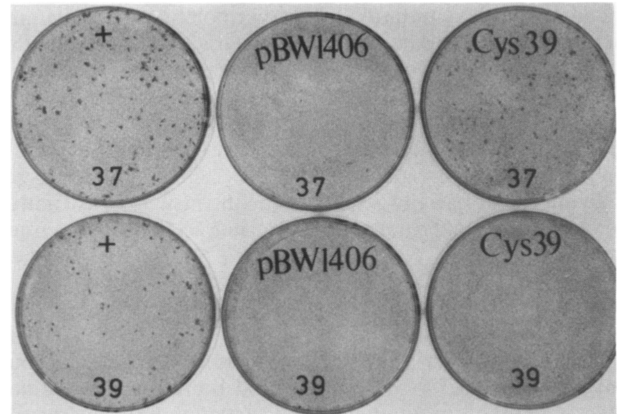

FIG. 1. *ts* focus formation activity by v-*ras*^{H}(S39C) virus. Rat 2 cells were infected with Moloney MuLV pseudotyped virus preparations expressing v-*ras*^{H}, pBW1406 (an effector null mutant), or v-*ras*^{H}(S39C). The cultures were fixed and photographed after 11 days of growth at the temperature indicated at the bottom of each plate.

cholate–0.5 M dithiothreitol–500 μg of bovine serum albumin (BSA) per ml. Following incubation at 37°C for 10 min, 2 μl of [γ-^{32}P]GTP (ICN) was added, and the mixture was incubated at 30°C for 15 min and then placed at 0°C.

Affinity-purified full-length human GAP and human NF-1 GRD, purified from baculovirus-infected insect cells, were provided by F. McCormick (Cetus Corp) (17). p21^{ras} (30 to 100 ng) was incubated with 120 pg of purified GAP in 20 mM HEPES (pH 7.5)–1 mM $MgCl_2$–1 mM dithiothreitol–500 μg of BSA per ml at various temperatures for 10 min. The reactions were stopped by the addition of 450 μl of ice-cold 25 mM Tris-HCl (pH 7.5)–0.25 mM $MgCl_2$, and the [^{32}P] GTP-bound p21^{ras} was captured on filters (BA85; Schleicher & Schuell) and counted in liquid scintillant. Assays with NF-1 GRD were carried out as for GAP except that 2 mM $MgCl_2$ was used and 3.8 ng of NF-1 GRD was incubated with the p21^{ras} for 20 to 30 min.

RESULTS

Identification of *ts ras* effector domain mutants. To analyze a large body of mutants with lesions in the effector domain of v-*ras*^{H}, a doped oligonucleotide mutagenesis procedure was employed. The mutant alleles were linked to the neomycin resistance (*neo*^{R}) gene in a retroviral vector and introduced into psi-2 cells, and supernatants from these cells were used to infect rat 2 cells. Cell clones derived from colonies of G418-resistant cells which showed evidence of *ts* transformation were infected with Moloney MuLV, and the derived virus stocks were used to infect rat 2 cells at various temperatures (Table 1). Three different mutations, at amino acid residues 36 (Ile to Leu), 39 (Ser to Cys), and 41 (Arg to Leu), were shown to confer a *ts* phenotype when expressed in the context of v-*ras*^{H} (Table 1). Of these three, the mutant showing the greatest degree of temperature sensitivity was the one bearing the mutation at codon 39; this mutant was therefore analyzed further.

v-*ras*(S39C) and proto-oncogene (S39C) are *ts* for transformation. Figure 1 displays focus formation by the v-*ras*^{H} (S39C) virus in infected rat 2 cells. The formation of foci at the nonpermissive temperature was decreased more than 300-fold compared with that at the permissive temperature (Fig. 1; Table 1). The control wild-type v-*ras*^{H} virus showed a less than threefold decrease in focus formation at the nonpermissive temperature (Table 1). Figure 2 depicts the morphology of neo^R cells expressing wild-type v-*ras*^{H}, a transformation-defective effector domain deletion mutant of v-*ras*^{H}, and the v-*ras*^{H}(S39C) gene. Cells transformed by the wild-type v-*ras*^{H} gene displayed a refractile, transformed morphology which was similar whether the cells were grown at the low or high temperature. In contrast, cells expressing v-*ras*^{H}(S39C) were transformed at the permissive temperatures, but when grown for several days at the nonpermissive temperature the cells became flat and resembled control (nontransformed) cells.

The wild-type v-*ras*^{H} and v-*ras*^{H}(S39C) genes were linked to *neo*^{R} and transfected into NIH 3T3 cells, and mass cultures of cells resistant to geneticin were analyzed for their ability to grow in soft agar (Table 2). NIH 3T3 cells expressing wild-type v-*ras*^{H} grew with nearly equal efficiency at either 34 or 39.5°C (Table 2). In contrast, the cells expressing v-*ras*^{H}(S39C) displayed a nearly ninefold reduction in colony-forming frequency at 39.5°C compared with that at 34°C. We conclude that transformation by the v-*ras*^{H}(S39C) mutant is *ts*.

The proto-oncogene (c-*ras*)-encoded form of the protein can in principle be studied at permissive and nonpermissive temperatures for its interaction with GAP and NF-1 in vitro by measuring GTPase acceleration and in vivo by determining the proportion of GTP-bound mutant protein. We therefore sought to determine whether the c-*ras* form of the S39C mutant would also be *ts* for transformation. To test this possibility, a c-*ras*^{H}(S39C) mutant gene was constructed and placed in a retroviral plasmid vector whose RNA, when expressed in mouse cells, could be pseudotyped by a helper retrovirus. NIH 3T3 cells were then transfected at 37°C with this plasmid and infected with amphotropic MuLV, and the virus preparation was studied for its ability to induce foci in NIH 3T3 cells at 34 and 39°C. As shown in Table 1, focus formation by the c-*ras*^{H}(S39C) virus was reduced at the high (nonpermissive) temperature, while the isogenic control wild-type c-*ras*^{H} virus showed no evidence of temperature sensitivity. In addition, cell lines transformed by the c-*ras*^{H}(S39C) virus reverted to a nontransformed morphology when grown at the nonpermissive temperature (data not shown).

Stability and guanine-nucleotide binding of v-*ras*^{H}(S39C)- and c-*ras*^{H}(S39C)-encoded proteins in vivo. To rule out the possibility that the *ts* phenotype of c-*ras*^{H}(S39C) and v-*ras*^{H}(S39C) resulted from thermolability of their encoded proteins, wild-type and mutant p21^{ras} proteins from cells

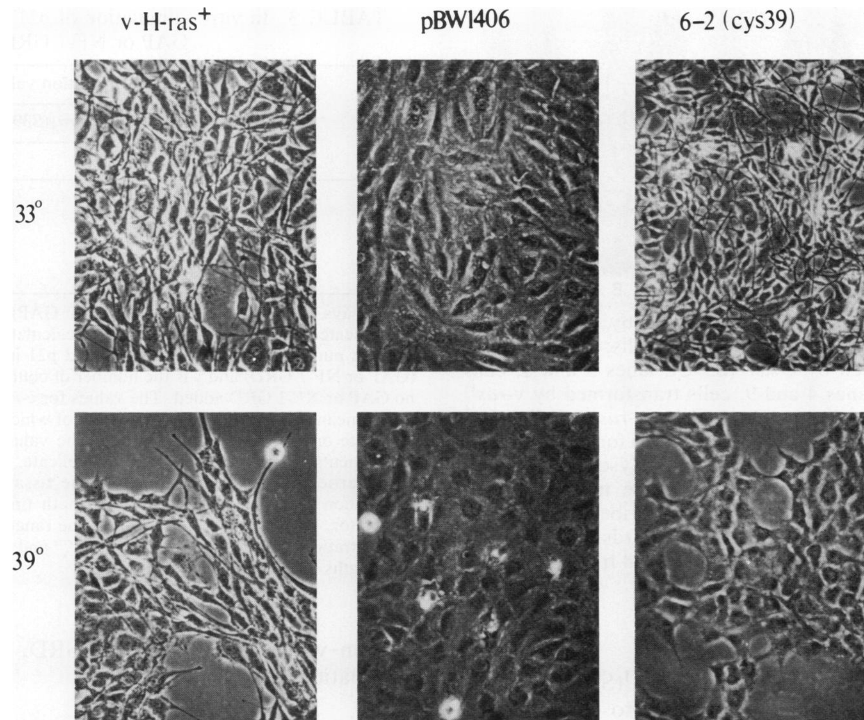

FIG. 2. *ts* morphology of rat 2 cells expressing v-*ras*^{H} and v-*ras*^{H}(S39C). Rat 2 cells expressing wild-type v-*ras*^{H}, pBW1406 (an effector null mutant), or v-*ras*^{H}(S39C) without helper virus were photographed after culture at the indicated temperature for 2 weeks.

grown at permissive and nonpermissive temperatures were analyzed (Fig. 3). NIH 3T3 cell lines transformed by c-*ras*^{H}, c-*ras*^{H}(S39C), v-*ras*^{H}, and v-*ras*^{H}(S39C) were grown at 34 or 39.5°C, metabolically labeled, and subjected to immunoprecipitation with an anti-ras antibody. As shown in Fig. 3, the parental NIH 3T3 line expressed a low level of p21^{ras} at both low and high temperatures (lanes 1 and 2), while cells transformed by wild-type c-*ras*^{H} (lanes 3 and 4) or c-*ras*^{H} (S39C) (lanes 5 and 6) expressed elevated levels of p21^{ras} protein at both temperatures. The c-*ras*^{H}(S39C)-transformed line had been chosen for its high level of p21 (well above that required for transformation) to facilitate the analysis of its in vivo guanine nucleotide binding (see below). As expected, cells transformed by v-*ras*^{H}(S39C) and wild-type v-*ras*^{H} expressed two ras-specific bands; the slower migrating band represents a phosphorylated form characteristic of v-*ras*^{H}-encoded proteins (23). A slight decrease in the level of viral protein was noted at the nonpermissive temperature, but this was the case for both the wild-type v-*ras*^{H} and v-*ras*^{H}(S39C) forms. The v-*ras*^{H}(S39C)-encoded protein was also found, in pulse-chase experiments on rat 2 cells, to be stable at the nonpermissive temperature (data not shown). We conclude that protein thermolability cannot account for the temperature sensitivity of the S39C mutant in c-*ras*^{H} or v-*ras*^{H} form.

Since transformation by *ras* is dependent on the ability of p21^{ras} to bind guanine nucleotide, we examined the level and form of nucleotide bound to wild-type and S39C p21^{ras} in intact cells by ^{32}P labeling and immunoprecipitation (Fig. 4). Endogenous c-*ras* protein (lanes 1 and 6), overexpressed

TABLE 2. Agar colony formation by v-*ras*-transformed NIH 3T3 cells

v-*ras* gene	Colony-forming frequency[a] at:		
	Permissive temp	Nonpermissive temp	Ratio
None	$<10^{-5}$	$<10^{-5}$	
v-*ras*(S39C)	3.1×10^{-4}	3.6×10^{-5}	8.6
Wild-type v-*ras*	5.7×10^{-4}	3.2×10^{-4}	1.7

[a] Per cell plated in agar (10^5 cells plated in each dish). The permissive temperature was 34°C; the nonpermissive temperature was 39.4°C.

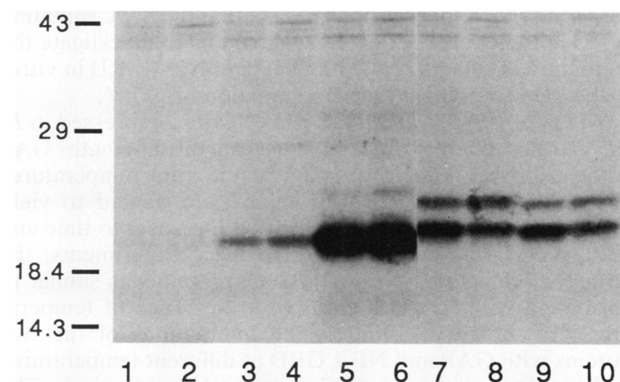

FIG. 3. Expression of wild-type and S39C mutant p21^{ras} proteins in transformed cells. Lanes 1 and 2, NIH 3T3 cells; lanes 3 and 4, cells transformed by c-*ras*^{H}; lanes 5 and 6, cells transformed by c-*ras*^{H}(S39C); lanes 7 and 8, cells transformed by v-*ras*^{H}(S39C); lanes 9 and 10, cells transformed by v-*ras*^{H}. Cells were plated at either 34°C (lanes 1, 3, 5, 7, and 9) or 39.5°C (lanes 2, 4, 6, 8, and 10), grown for 24 h, and then labeled for 22 h with ^{35}S-methionine. After lysis, p21^{ras} was immunoprecipitated and analyzed on a 15% polyacrylamide gel. Autoradiography was for 3 days at −70°C. Migration of molecular weight standards (in thousands) is shown at the left.

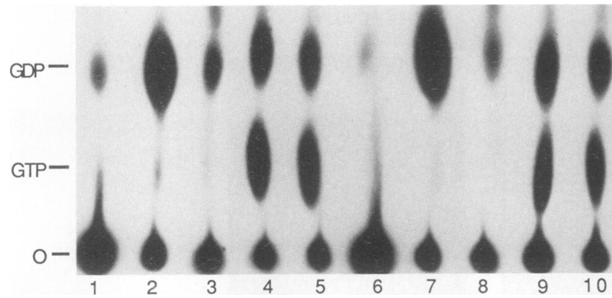

FIG. 4. In vivo guanine nucleotide binding by wild-type and S39C mutant p21^{ras}. Lanes 1 and 6, NIH 3T3 cells; lanes 2 and 7, NIH 3T3 cells transformed by c-*ras*^{H}(S39C); lanes 3 and 8, cells transformed by c-*ras*^{H}; lanes 4 and 9, cells transformed by v-*ras*^{H} (S39C); lanes 5 and 10, cells transformed by v-*ras*^{H}. Cells were plated at either 34°C (lanes 1 to 5) or 39.5°C, grown for 24 h, and then labeled for 18 h with $^{32}PO_4$. The cells were lysed, p21^{ras} was immunoprecipitated, and the bound ^{32}P-guanine nucleotide was analyzed by ascending chromatography as described in Materials and Methods. Migration of GDP and GTP standards in this system is indicated at the left. Autoradiography was for 8 h at −70°C.

TABLE 3. In vitro stimulation of p21^{ras} GTPase by purified GAP or NF-1 GRD

Temp (°C)	Stimulation value[a] for:					
	c-*ras*		c-*ras*(S39C)		v-*ras*	
	GAP	NF-1	GAP	NF-1	GAP	NF-1
34	61.5	68.4	55.1	63.1	5.2	14.0
37	64.1	62.0	56.7	58.8	5.4	−1.3
40	65.7	62.3	57.4	59.4	3.5	12.7
44	61.3	50.2	51.0	41.6	4.8	12.7

[a] Assays were performed for 10 min (for GAP) or 20 to 30 min (for NF-1) at the indicated temperatures. Values were calculated as $100 \cdot (1 - x/y)$, where x is the number of counts of GTP-bound p21 in the presence of stimulator (GAP or NF-1 GRD) and y is the number of counts in identical reactions with no GAP or NF-1 GRD added. The values for c-*ras* and c-*ras*(S39C) are given as the mean from two experiments, each of which represented the average of duplicate or triplicate determinations. The values for v-*ras* represent single experiments, also done in duplicate or triplicate. Preliminary experiments had been carried out to ensure that for the assays presented here, GTPase stimulation was linear with respect to both time and the concentration of stimulator. In each experiment, across the range of temperatures, the molar concentrations of wild-type or mutant p21^{ras} and of the GTPase stimulator and the lengths of the assays were the same.

c-*ras*^{H} protein (lanes 3 and 8), and c-*ras*^{H}(S39C) protein (lanes 2 and 7) were all bound exclusively to GDP at both permissive and nonpermissive temperatures. In contrast, the wild-type v-*ras*^{H} (lanes 5 and 10) and v-*ras*^{H}(S39C) (lanes 4 and 9) proteins bound to GTP and GDP in a ratio of approximately 60 to 40 at both low and high temperatures. These results demonstrate that (i) the *ts* phenotype of v-*ras*^{H}(S39C) and c-*ras*^{H}(S39C) is not due to an inability of the encoded p21 protein to bind guanine nucleotide at the nonpermissive temperature and (ii) the protein encoded by c-*ras*^{H}(S39C) interacts normally in vivo at permissive and nonpermissive temperatures with at least one GTPase-accelerating protein, such as GAP or NF-1.

Interaction of c-*ras*^{H} protein with GAP and NF-1 GRD in vitro. Based on the experiments described, the most likely explanation for the temperature sensitivity of the *ras* S39C mutant is an inability to stimulate the p21^{ras} target at the nonpermissive temperature. The fact that the S39C mutation was *ts* in the context of c-*ras*^{H} enabled us to investigate the interaction of this protein with GAP and NF-1 GRD in vitro, on the basis of their GTPase stimulation of p21^{ras}.

Wild-type p21$^{c\text{-}ras}$ and S39C p21$^{c\text{-}ras}$ were expressed in *E. coli*, purified, and incubated with purified (full-length) GAP or the catalytic domain of NF-1 at different temperatures (17). GAP and NF-1 GRD proteins were diluted to yield GTPase stimulation that was linear with respect to time and the concentration of stimulator. In these experiments, the intrinsic GTPase of the c-*ras*^{H}(S39C) protein was similar to that of the wild-type protein across the range of temperatures. As shown in Table 3, the interactions of the two proteins with GAP and NF-1 GRD at different temperatures in the range of 34 to 44°C were nearly identical. The c-*ras*^{H}(S39C) protein was slightly (3 to 10%) more resistant to GAP and NF-1 GRD than the wild-type c-*ras*^{H} protein throughout the range of temperatures tested; no specific decrease in stimulation of the mutant protein was seen when the reactions were carried out at or above 40°C. As has been reported previously, there was no significant stimulation of the GTPase activity of the wild-type v-*ras*^{H}-encoded protein by either GAP or NF-1 GRD (Table 3). These experiments demonstrate that the in vitro interaction of the c-*ras*^{H}(S39C) protein with GAP and NF-1 GRD, on the basis GTPase stimulation, is not thermolabile.

DISCUSSION

Here we have described effector domain mutants in *ras* which result in a *ts* phenotype for cell transformation. We have characterized in detail the S39C mutant, which in preliminary experiments displayed the greatest difference in biological activity at permissive and nonpermissive temperatures. The S39C mutation had little effect on the ability of v-*ras*^{H} to transform cells at the permissive temperature, yet at the nonpermissive temperature focus formation by the mutant gene was reduced by more than 2 orders of magnitude. According to the currently favored model of *ras* transformation, the integrity of the effector domain is required for proper interaction of p21^{ras} with its target. We therefore believe that the mutant protein is relatively defective for this interaction when expressed at the nonpermissive temperature. This interpretation was substantiated by the findings that (i) the v-*ras*^{H}(S39C)-encoded protein was stable at the nonpermissive temperature and (ii) the mutant (S39C) p21$^{v\text{-}ras}$ protein was bound to GTP in the same proportion as wild-type p21$^{v\text{-}ras}$ protein, regardless of the temperature.

The same mutation, when expressed in the context of the proto-oncogene form of *ras*, also resulted in a *ts* phenotype. This result is consistent with the mutation impairing the target interaction of the c-*ras*^{H}(S39C) protein at the nonpermissive temperature. As was true of the v-*ras*^{H}(S39C)-encoded form, the protein encoded by c-*ras*^{H}(S39C) was stable at the nonpermissive temperature. Furthermore, the in vivo analysis of guanine nucleotide binding revealed that the c-*ras*^{H}(S39C) protein was bound almost exclusively to GDP at both low and high temperatures. This finding implied that the mutant protein interacted properly with cellular negative regulators of ras-GTP (e.g., GAP and NF-1) at permissive and nonpermissive temperatures. We were able to verify this conclusion by analyzing the interaction of purified mutant protein with GAP and NF-1 GRD in vitro. We observed no temperature sensitivity in the GTPase stimulation of the c-*ras*^{H}(S39C) protein by GAP or the region of NF-1 with homology to GAP.

When some proteins encoded by *ras* mutants with inactivating lesions in the effector domain have been assayed for their interaction with GAP, the interaction was found to be impaired (1, 6, 22). Since the biological activity of these mutants correlated with their ability to interact with GAP, these results supported the hypothesis that GAP might also be the target effector of *ras*. Indeed, interaction between ras and GAP does produce an effector function in an in vitro system employing atrial cell membranes (34). However, studies on the overexpression of GAP in fibroblasts (35) have revealed that GAP is limiting as a negative regulator but probably does not function by itself as the target effector for *ras*.

The results described here show that the interaction of the c-ras^{H}(S39C) protein with GAP or with NF-1 GRD, as measured by GTPase stimulation, does not mimic the interaction with the *ras* target. Dissociation between GTPase stimulation by GAP and potential GAP target function has also been observed with *rap*1A mutants and certain *ras*/*rap*1A chimeras; *ras* residues located C terminal to the effector region (downstream from residue 60) make them subject to GTPase stimulation by GAP (8, 12, 34a), yet they are nontransforming because they encode *rap*1A in the region surrounding residues 32 to 44 (36). These exceptions to the general rule that GTPase stimulation by GAP usually correlates with *ras* effector function establish that GAP-mediated GTPase stimulation represents an indirect assay of potential *ras* effector function. Our results are consistent with the existence of a distinct, as-yet-unidentified molecule serving as the target effector for mammalian *ras*. However, they do not preclude the possibility that GAP does indeed represent the *ras* target but that the putative target function of GAP is at least partially nonoverlapping with its GTPase-stimulatory activity. The same arguments apply to the less well characterized NF-1 gene product. Further investigation will be required to clarify these points.

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