Genetic Definition of *ras* Effector Elements

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The products of *ras* genes may function as GTP-binding signal transducers, but the nature of their targets is largely unknown. To define genetically the cellular effector(s) of *ras* in rat fibroblast transformation, somatic variants that suppress the nontransforming phenotype of v-H-*ras* effector domain mutations were sought. Variant cell lines perturbed in the *ras* effector pathway were recovered, and the properties of one suggest that the primary target of *ras* action may be altered. In this cell variant, no single residue in the *ras* protein effector domain must be wild type to bring about transformation. In parental rat cells, conservative substitutions are tolerated in six of nine residues. Functional interaction with the target may not require a high degree of structural specificity in the *ras* protein effector domain.

The products of *ras* genes appear to act as signal transducers, cycling between GDP-bound “off” and GTP-bound “on” forms (8, 9). In the GTP-bound state, the effector domain of the *ras* protein assumes a configuration that is thought to combine with the target of *ras* action and in some way alter its activity. On the basis of strict conservation in evolution and genetic analysis, residues 32 to 40 in the product of the Harvey sarcoma *ras* gene were proposed to constitute this effector domain (32, 43). Curiously, while the sequence of the effector domain is absolutely conserved in evolution, the biological functions of *ras* have diversified radically. In *Saccharomyces cerevisiae*, there is abundant evidence that *ras* regulates adenylate cyclase activity (10, 16, 28, 36, 41). Adenylate cyclase activities are not *ras* regulated in other species, however, and the noncatalytic domains in *S. cerevisiae* that endow this enzyme with *ras* responsiveness are not conserved in the adenylate cyclase of *Schizosaccharomyces pombe* or mammals (5, 18, 25, 35). The diverse biological functions of *ras* include facilitating conjugation in *S. pombe* (18), specification of vulval fate in *Caenorhabditis elegans* (6, 21), maturation of *Xenopus* oocytes (7), stimulation of proliferation in many vertebrate cells (3), and inhibition of proliferation and stimulation of neurite outgrowth in certain mammalian neural cell types (4, 30). In the cases examined, an intact effector domain is required for these functions. An identical effector domain may serve yet other functions in other G proteins, however. The same sequence in the product of the Krev/rap1A gene, for example, can serve to antagonize *ras* transforming function (23).

Substantial progress has been made toward identifying the cellular elements that regulate the balance between the on and off forms of vertebrate *ras* proteins, collectively referred to as p21. Enzymes that influence rates of p21 nucleotide exchange have been identified (13, 40, 44), and two enzymes that increase the rate of GTP hydrolysis, i.e., GAP (38) and the product of the neurofibromatosis type 1 gene (2, 29, 45), have been discovered. It has been argued that these latter two GTPase-activating proteins may be effectors of p21, and, in fact, both proteins do utilize the p21 effector domain to interact with p21 (1, 11, 15, 24, 29, 31, 33, 39, 45). This issue has not been settled, although the balance of evidence supports the idea that these GTPase-activating enzymes are

regulators, not effectors, of *ras* function (8). For example, an effector domain mutation that is temperature sensitive for transformation encodes a protein that is not demonstrably temperature sensitive for enhanced hydrolysis by the two GTPase-activating proteins mentioned above, arguing that the p21 effector domain requirements for transformation and for GTPase stimulation differ (12).

In order to identify elements of the mammalian *ras* effector pathway, somatic mutations in rat fibroblasts that suppress nontransforming substitutions in the effector domain of v-H-*ras* were sought. In principle, the method selects for cellular mutants that have an altered target such that a productive interaction with the structurally abnormal p21 effector domain is established.

MATERIALS AND METHODS

Mutations and vectors. Effector domain mutations in v-H-*ras* were constructed by the cassette method as described previously (34), and each mutation was transferred to a GV16-based retroviral vector (22) to facilitate expression in fibroblasts. In most cases, the structures of these mutations were confirmed by the dideoxy sequencing method with GV16 plasmid templates. The Glu-33, Leu-36, Cys-39, and Leu-41 mutations were isolated as proviral clones in experiments aimed at isolating temperature-sensitive mutations from a pool of virus representing a collection of v-H-*ras* effector domain mutations; the sequence was determined by analysis of three or more different M13 subclones carrying sequences recovered by polymerase chain reaction from genomic DNA of single provirus clones. The Glu-33 mutation is only slightly temperature sensitive. The other three temperature-sensitive mutations have been described elsewhere (12). Virus stocks were prepared by rescuing these temperature-sensitive alleles with Moloney murine leukemia virus. The pBW1406 linker substitution mutation (34) encodes a p21 species with a substantially remodelled effector domain (see Table 2) and was used as a negative control virus. The pBR1631 (c-H-*ras*) retroviral vector has been described elsewhere (34). The pBW1225 vector expresses the Cys-186-to-Ser mutation, encoding the same nonisoprenylated, non-membrane-localized form of p21 as pBW858 (42). To transduce the *tk* gene with v-H-*ras*, the *Bcl*I-to-*Cl*aI fragment of GV16 containing the *neo* sequence was replaced with the *Bgl*II-to-*Pvu*II fragment containing the *tk* coding region from herpes simplex virus type 1 (14). Proviral clones

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used in focus selection experiments were derived by infection with the *tk* version of the virus, while the "reinfection" tests used the *neo*-expressing version.

Cells and viruses. Cells were cultured at 37°C in a 5% CO₂ environment. Nonselective medium was Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. To select for cells that expressed *tk*, HAT medium (nonselective medium plus 1.0×10^{-5} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine) was used. When cells were transferred from HAT medium into some other medium, they were first cultured in medium containing hypoxanthine and thymidine only. To select for cells that had lost *tk*, nonselective medium was supplemented with 50 µg of 5-bromodeoxyuridine per ml. To select for cells that expressed *neo*, nonselective medium was supplemented with 400 µg of geneticin per ml.

Purified plasmids were transfected into psi-2 cells (27) by the calcium phosphate method (20) to generate helper-free retroviral stocks. Rat2 cells (37) were plated at a density of $2 \times 10^5/25\text{-cm}^2$ flask the day before infection. Virus stocks were passed through 0.45-µm-pore-size filters to remove cells and then adsorbed to target cells in the presence of 8 µg of Polybrene per ml for 1 h. After a further 2 days of incubation in nonselective medium, cells were subcultured into selective medium and the medium was renewed every 3 to 4 days until colonies could be enumerated, scored for transformation phenotypes, isolated with cloning cylinders, or treated with trypsin to generate pooled populations of proviral colonies.

To isolate cell variants altered in the *ras* effector pathway, subconfluent flasks containing single proviral clones expressing particular v-H-*ras* effector domain mutations were treated overnight with 5 mM ethane methylsulfonate (EMS) in HAT medium. Cultures were incubated in HAT medium for a further 1 to 2 days and then subcultured at a 1:4 dilution. Monolayers were maintained for 3 to 4 weeks with weekly feedings of HAT medium. Transformed foci were picked with Pasteur pipettes, and fully transformed cell lines were then derived by cloning by limiting dilution.

For virus rescue tests, viral sequences were rescued from proviral clones by infecting the clones with Moloney murine leukemia virus. Rat2 cells were infected with rescued virus, and Tk⁺ clones were then selected in HAT medium to determine whether the rescued virus had transforming activity. For soft-agar growth assays, 10^5 cells were plated in nonselective medium containing 0.3% agar supported by a 0.6% agar layer. For cell fusion experiments, suspensions containing 10^6 transformed variant (TK⁺ Neo⁻) and 10^6 genetically marked rat2 (TK⁻ Neo⁺) cells were exposed to 50% polyethylene glycol. After incubation for 1 to 2 days in nonselective medium, hybrids were selected in HAT medium supplemented with G418.

p21 analysis. To assess the levels of p21 expression, cells were plated at 2×10^6 in a 60-mm dish. After they had attached, the cells were rinsed in methionine-deficient Eagle medium containing 2% dialyzed fetal bovine serum and then incubated overnight in this medium containing 100 µCi of [³⁵S]methionine (800 Ci/mol; New England Nuclear; translation grade) per ml. Cells were then rinsed in phosphate-buffered saline and lysed by scraping them in buffer containing 2 mM phenylmethylsulfonyl fluoride, 1.0% (vol/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 0.1 M NaCl, 0.005 M MgCl₂, and 0.02 M Tris (pH 7.4). After nuclei were removed by centrifugation, an aliquot representing 1.0×10^7 to 3.0×10^7 cpm of trichloroacetic acid-precipitable material was incubated on ice for 1 h with protein A-linked

Sepharose to remove material that bound nonspecifically. The unbound material was then adsorbed to 1 to 2 µg of anti-p21 monoclonal antibody Y13-238 (19) for 1 h at 0°C, and the immunoprecipitates were collected with protein A-linked Sepharose coated with rabbit anti-rat immunoglobulin G. Precipitates were washed with a low-salt buffer (0.1% [wt/vol] sodium dodecyl sulfate, 1% [vol/vol] Triton X-100, 0.5% sodium deoxycholate, 0.001 M EDTA, 0.1 M NaCl, 0.01 M sodium phosphate, pH 7.6), twice with a high-salt buffer (the same but containing 1.0 M NaCl), and once again with low-salt buffer before being boiled in sodium dodecyl sulfate-containing sample buffer. Samples were resolved on 15% polyacrylamide gels containing sodium dodecyl sulfate, treated with En³Hance (New England Nuclear), and exposed to film at -80°C in the presence of an intensifying screen. The identity of p21 was confirmed by its mobility relative to mobilities of prestained low-molecular-weight markers (Bethesda Research Laboratories). This method was used to demonstrate that all mutant versions of *ras* constructed in this study encode stable p21 species in rat2.

RESULTS

General strategy. The strategy for isolating somatic mutations that might define the *ras* effector is outlined in Fig. 1A. A helper-free retroviral vector system (Fig. 1B) was used to introduce v-H-*ras* with various effector domain substitutions in *cis* with *tk* (Fig. 1C) into rat2, a TK⁻ nontransformed cell line (37). Nontransformed proviral clones were established after selection in HAT medium. For each *ras* allele, about half of the TK⁺ clones were shown to express readily detectable levels of viral p21. For each of the alleles Phe-32, Ser-35, Met-36, Ala-36, and Ile-40, representative p21^{v-H-*ras*}-expressing proviral clones were treated with the chemical mutagen EMS, and fully transformed derivative clones were then selected by using a focus assay. Three general types of genetic events can result in cell transformation in this system. Class I variants arise from mutations within the proviral sequence that restore transforming activity to v-H-*ras*. Class II variants represent the activation of cellular transforming sequences that function independently of the retrovirally transduced sequence. Class III variants result from the alteration of cellular sequences such that *trans*-acting suppressors of the *ras* effector domain mutation are created. Variants in this last category may define elements of the *ras* effector pathway.

Transformed cell lines derived from picked foci (prefaced rv for *ras* variant) were cloned by limiting dilution and then subjected to several tests to ascertain the nature of the events responsible for the cell transformation. One population of each variant was infected with Moloney murine leukemia virus. Copies of the *ras* and *tk* sequences were thereby rescued and transferred into parental rat2 cells to determine whether a class I event was involved. For non-class I variants, a second population was subjected to selection in 5-bromodeoxyuridine to generate TK⁻ derivatives. Loss of tk⁺ is often accompanied by loss of v-H-*ras* expression. By examining the morphology and growth properties of individual clones that had been "cured" of v-H-*ras* expression, class II and III variants could be readily distinguished. Finally, cured versions of the class III variants (denoted with the suffix BUR for bromodeoxyuridine resistant) were subjected to a reinfection test; the nontransformed, cured derivatives were infected with the nontransforming virus bearing the *ras* mutation that provided the context for selecting the parental transformed variant. In

FIG. 1. (A) Scheme for the isolation of cell variants with alteration in the *ras* effector pathway. Triangles and circles represent nontransformed and transformed rat2 cells, respectively. Solid arrows depict changes in cell genotype and phenotype, while gene transfer is indicated with dotted lines. $ras^{-}tk^{+}$, retroviral vector transducing an effector domain mutation in v-H-*ras* and a wild-type thymidine kinase gene; MLV, murine leukemia virus; 5BU, 5-bromodeoxyuridine. (B) Structure of the retroviral vector in proviral form. Dotted boxes, long terminal repeats; solid box, *ras*; open box, selectible marker, either *tk* or *neo*, driven by the simian virus 40 promoter and enhancer; E, *EcoRI* restriction sites within the provirus; wavy lines, host sequence flanking the provirus. Transcription is from left to right. (C) Mutations used in this study. The top line represents the normal p21^{v-H-*ras*} effector domain as well as residue 41. The mutations below in boldface type were those used to select cell variants. Double mutations are arranged in underlined pairs in the bottom three rows.

essence, the system yields a nontransforming cell variant that allows transformation by an otherwise nontransforming *ras* allele, a sort of binary transforming gene, with the cellular element potentially defining the target of *ras* action.

Properties of the system. Several conclusions and inferences can be drawn from the results of focus selection data (Table 1). First, the parental rat2 population transforms very

TABLE 1. Selection of transformed variants

Cell line	<i>ras</i> allele ^a	EMS	No. of:		Variant class ^b			Variant
			75-cm ² cultures ^c	Foci ^d	I	II	III	
rat2		+	36	1	*	1	*	
R/4	c-H- <i>ras</i>	+	32	9	5	1	0	
R2a	Ala-36	+	39	(3)	0	3	0	
R3a	Met-36	+	7	(4)	1	1	2	rv2
8tk3	Ile-40	+	64	0	0	0	0	
11tk1	Phe-32	+	32	(6)	0	3	0	
11tk3	Phe-32	+	32	18	*	*	*	
11tk3	Phe-32	—	32	1	*	*	*	
12tk5	Ser-35	+	32	47	13	3	1	rv68

^a Retrovirally transduced.

^b Variant classes: I, viral revertants; II, cellular, v-H-*ras* independent; III, cellular, v-H-*ras* dependent. *, virus rescue and curing test were not performed.

^c Saturation densities were in the range 1×10^7 to 2×10^7 per flask.

^d Numbers in parentheses are approximate and may be underestimated by a factor of 2.

infrequently, even after treatment with EMS. Only one focus was observed, corresponding to a frequency of about 3.1×10^{-9} based on a saturation density of about 10^7 cells per flask. The rarity of this type of event is also reflected in the low yield of class II variants from the proviral clones.

Class I variants were observed in some but not all situations. For example, the Ser-35 mutations can revert to Thr by a G-to-C transversion (Table 2). Five apparent Ser-35 revertants and the single Met-36 revertant were analyzed by DNA sequence analysis of polymerase chain reaction-derived templates and were shown to be true revertants (data not shown). As expected, class I variants were not observed in cases where the mutation differed by two nucleotides from wild type. As a control, foci were selected from a nontransformed proviral clone expressing a retrovirally transduced c-H-*ras* proto-oncogene. Class I variants, representing activation of the transduced c-H-*ras*, predominated (Table 1). While it is clear that gene mutations can be selected in this system, it is not obvious whether the mutagen is responsible. EMS has been shown in cultured mammalian cells to induce G-to-A transitions almost exclusively (26).

Class III variants were selected separately in the context of both Met-36 and Ser-35. Although class III variants were not selected in the context of Phe-32, it may be noteworthy that foci were recovered more frequently from lines expressing this v-H-*ras* sequence than in parental rat2 cells and that these foci were dependent to a large extent on mutagen treatment. One of the class III variants selected in the context of Met-36 (rv2) and the one selected in the context of Ser-35 (rv68) and their cured derivatives were studied further to gain some insight into the *ras* effector pathway.

Properties of class III variants. Cell variants rv2 and rv68, selected in the context of Met-36 and Ser-35, respectively, are remarkable in that they do not harbor a transforming v-H-*ras* sequence nor do the cured derivatives, rv2BUR and rv68BUR, exhibit obvious transformation phenotypes. Typical morphological observations are shown in Fig. 2. The proviral clone expressing Ser-35 is morphologically indistinguishable from rat2. The transformed variant, rv68, is essentially like rat2 transformed with wild-type v-H-*ras*, while the cured derivative, rv68BUR, is essentially nontransformed, although it forms a slightly disorganized monolayer at satu-

TABLE 2. Allele specificity of class III v-H-*ras* effector variants

<i>ras</i> allele ^a	From	To	Activity in ^b :		
			rat2	rv2BUR	rv68BUR
v-H- <i>ras</i> ⁺			+++	+++	+++
c-H- <i>ras</i> ⁺			—	—	—
pBW1406			—	—	—
pBW1225			—	—	—
Phe-32	TAT	TTT	—	—	—
His-32	TAT	CAC	+	+++	+++
Trp-32	TAT	TGG	++	+++	+++
Glu-33	GAT	GAG	+++	+++	+++
Gln-33	GAT	CAA	++	+++	+++
Asn-33	GAT	AAC	++	+++	+++
Ser-34	CCC	AGT	—	—	+++
Gly-34	CCC	GGG	—	—	++
Ser-35	ACT	AGT	—	—	+++
Met-35	ACT	ATG	—	—	+++
Met-36	ATA	ATG	—	+++	+++
Ala-36	ATA	GCG	—	—	—
Leu-36	ATA	CTA	++	+++	+++
Val-36	ATA	GTA	++	+++	+++
Ala-37	GAG	GCT	—	—+++	+++
Asp-37	GAG	GAT	+++	++	+++
Ala-38	GAC	GCG	—	—	—
Glu-38	GAC	GAG	—	++	+++
Cys-39	TCC	TGC	++	+++	+++
Ile-40	TAC	ATT	—	—	—
Phe-40	TAC	TTT	+	+++	+++
Leu-41	CGG	CTG	++	+++	+++
Glu-33 ^c and Cys-39	GAT	GAA	+++	+++	+++
Lys-33 ^c and Cys-39	TCC	TGC	+	+++	+++
Gly-34 ^c and Glu-38	CCC	GGG	—	—	+++
	GAC	GAG			

^a The c-H-*ras* and v-H-*ras* vectors have been described previously (34). pBW1406 is a linker substitution mutation that specifies the p21 sequence 1-31-Leu-Ile-Arg-38-198, where the numbers indicate the unaffected residues (34). The pBW1225 v-H-*ras* allele specifies a Cys-186-to-Ser substitution, and the encoded protein is nonisoprenylated and non-membrane localized. Other mutations were created on the v-H-*ras* background.

^b On the basis of morphological transformation of Neo⁺ colonies, the activities of v-H-*ras* alleles were classified as follows: +++, strongly transforming; ++, leaky; +, weak; —, null. No colonies were observed in mock-infected controls.

^c These *ras* alleles are double mutations.

ration density. Similar observations were made with rv2 and its cured derivative (not shown).

As an independent test of transformation, these cell lines were assessed for their capacity for anchorage-independent growth. While neither rat2 nor the nontransformed proviral clones grow in soft agar, rv2 and rv68 grow as robustly as do rat2 cells transformed by wild-type v-H-*ras*. In soft-agar assays, rv68BUR sometimes forms minute colonies after several weeks of incubation, while rv2BUR does not grow at all. Neither of these cured derivatives spontaneously reverts to a fully transformed state in focus assays, in soft-agar assays, or in colony selection experiments (see below).

Fusion of either rv2 or rv68 with genetically marked rat2 (Neo⁺) cells resulted in hybrid clones that exhibited morphologically transformed phenotypes, suggesting that these variants represent dominant cell mutants that complement the v-H-*ras* effector defects.

Verification of *ras* expression and proviral structure in cell effector variants. To verify that the clones under study had the desired genetic constitution, p21 levels were compared. Rat2 express a relatively small amount of cellular p21 (Fig.

FIG. 2. Representative cell morphologies. (a) 12tk5, a proviral clone derived by infecting rat2 cells with the v-H-*ras* Ser-35 *tk* vector followed by selection in HAT medium. Rat2 cells have an indistinguishable morphology (not shown). (b) rv68, a fully transformed clone derived from 12tk5 by treatment with EMS followed by focus selection and cell cloning. (c) rv68BUR, a cured clonal derivative of rv68 obtained after selection in 5-bromodeoxyuridine.

3). The proviral clone 12tk5 (Ser-35) expresses more total p21, and it expresses the slow-migrating, phosphorylated form that is diagnostic of the v-H-*ras* protein. The fully transformed variant rv68 expresses somewhat more viral protein than its progenitor, 12tk5, but this increased expression cannot in itself account for the properties of this cell line. Importantly, the cured derivative rv68BUR expresses

FIG. 3. p21 analysis of cell lines. Cell lines were labeled with [35 S]methionine, and p21 was immunoprecipitated (3.0×10^7 cpm of protein per lane), resolved by electrophoresis, and detected by fluorography. Exposure was for 20 days at -80°C . Lanes: 1, rat2; 2, 12tk5 (Ser-35 expressing); 3, rv68; 4, rv68BUR; +, Y13-238 antibody; -, no primary antibody. Arrows indicate the positions of the p21 species.

only the cellular form of p21. Similar results were obtained with rv2 and rv2BUR (data not shown).

DNA blot analysis with a restriction enzyme that cuts within the provirus (*EcoRI*) was used to establish that the proviral clones harbored a single provirus and that this sequence was in fact removed during the curing process. Since each proviral clone harbors a single v-H-*ras* sequence, a unique proviral restriction fragment containing v-H-*ras* is generated upon digestion with *EcoRI* (Fig. 1B). This blot hybridization analysis, furthermore, eliminated several trivial explanations for the origins of the cell variants. Amplification of proviral sequences, reciprocal homologous recombination between v-H-*ras* and c-*ras* sequences, and virus or cell contamination were ruled out as possible explanations for the origins of these variants (data not shown).

Allele specificities of rv2 and rv68. The availability of cured derivatives of rv2 and rv68 made possible a detailed examination of the interaction between the cell variants and *ras* sequences that served as the context for their selection. Furthermore, the response of the cell to alternative *ras* alleles could be examined in the absence of the context allele. Accordingly, rat2, rv2BUR, and rv68BUR cells were infected with retroviruses designed to transmit various alleles of *ras* in *cis* with *neo*. As expected, after infection of rv2BUR and rv68BUR with Met-36 and Ser-35 virus, respectively, most of the proviral clones selected on the basis of *neo* expression exhibited a transformed morphology, while these alleles were nontransforming in rat2 cells. Retransformation of these two variants by their respective context alleles seems to occur slowly; often the clones appear to be sectorized with both flat and morphologically transformed regions developing from a single infected cell. In mass culture, however, isolated Neo^+ clones are morphologically indistinguishable from the original transformed variants. The responsiveness of the cured derivatives to retransformation is a stably heritable property of these cell lines, since the phenotype is not influenced by passage history.

In contrast to the results of *neo* selection experiments, neither variant generated typical transformed foci after reintroduction of their context *ras* allele followed by incubation in nonselective medium. Even more surprisingly, neither cured cell line generated foci when infected with wild-type v-H-*ras* virus. By comparison, rat2 cells infected with wild-

type virus generated foci in numbers comparable to the numbers of drug-resistant colonies obtained in parallel cultures. Furthermore, overtly transformed drug-resistant colonies were readily obtained after the cured variants were infected with wild-type virus. In some focus selection experiments, it was observed that rv2BUR infected with wild-type virus developed microfoci consisting of one or a few relatively large cells. Diffuse patches of refractile cells sometimes accumulated on monolayers of rv68BUR after infection with wild-type virus. Because of these unexpected results of focus assays, the morphology of Neo^+ colonies as well as the properties of cell populations derived from such colonies were used to examine the response of the cell variants to other *ras* sequences.

The response of rv2BUR and rv68BUR to a variety of *ras* mutations is summarized in Table 2 along with data obtained from parallel infections done with rat2. Although it was not possible to assign quantitative values for transforming activities, it was straightforward to categorize *ras* sequences into several types on the basis of Neo^+ cell morphologies and growth patterns in mass culture. Mutations that did not affect rat2 Neo^+ colony morphology were considered null, while mutations that had very slight effects on morphology were termed weak. Mutations that gave rat2 colonies composed of disorganized refractile cell were termed strong, while leaky alleles gave compact but obviously transformed colonies.

Several conclusions could be drawn. First, many alleles classified as weak or leaky in rat2 cells were strongly transforming in rv2BUR and rv68BUR cells. Furthermore, several alleles classified as null in rat2 cells had substantial transforming activity in rv68BUR cells. The transformation responsiveness does appear to be specific for the activated viral form of v-H-*ras*, as no clear signs of transformation were observed with the c-H-*ras*-transmitting vector, although these two forms of *ras* have identical effector domain sequences. Also, no transformation was seen with a mutation that encodes a *ras* protein that does not localize to the plasma membrane. Furthermore, several effector domain mutations are null in all three cell types. Thus, the enhanced transforming propensity of the two cell variants requires a *ras* transforming sequence that is functional in most respects, but a remarkable degree of flexibility in the structure of the effector domain is evident. Indeed, no single effector domain residue must be wild type to effect transformation of 68BUR. To strengthen this last conclusion, rat2 and 68BUR cells expressing the null mutations were compared with uninfected cells in soft-agar growth assays. In this assay, rv68BUR cells demonstrates unequivocal responsiveness to v-H-*ras* effector domain null mutations (Fig. 4).

DISCUSSION

Selection of variants with altered *ras* effector function. A genetic selection strategy has been designed to isolate transformed cell variants in the context of nontransforming v-H-*ras* effector domain mutations, and the combined results of virus rescue and genetic curing tests have been used to classify the variants. The recovery of class I variants (viral revertants) demonstrates the potential for isolating mutant clones representing unique nucleotide substitutions. The relatively low yield of class II variants (v-H-*ras*-independent cellular transformation events) is somewhat surprising. These variants represent the combined contribution from activation of all endogenous oncogenic sequences including the c-*ras* proto-oncogenes. Possibly, the relatively high level

FIG. 4. Anchorage-independent growth of rat2 and rv68 expressing various *ras* species. Top row: rat2 and drug-resistant (Tk^+ or Neo^+) populations expressing the indicated *ras* alleles were plated in soft agar and photographed after 16 days of incubation. Bottom row: rv68BUR and proviral clones expressing the indicated *ras* alleles. —, uninfected controls.

of expression of the virally transduced sequence weights the system such that most transformation events are either class I or class III (v-H-*ras*-dependent cellular events).

Cell variants with altered *ras* effector function were selected in the context of both Met-36 and Ser-35. The products of effector domain mutations in v-H-*ras* are expected to be found in the GTP-bound state in vivo, since the oncogenic mutations (Arg-12, Thr-59) as well as the effector domain substitutions render the proteins nonresponsive to GTPase-activating proteins. Therefore, it seems most likely that these cell variants affect elements that function downstream of p21; either the target of *ras* itself or some crucial element of the *ras*-regulated pathway(s) is affected. Since the selection system can be used to isolate single nucleotide substitutions in v-H-*ras*, it seems plausible that rv2 and rv68 represent cellular mutants. This notion is supported by the apparent dominance of the variants' transformed phenotype in cell hybrids and by the stability of the transformation-sensitive phenotypes in the nontransformed, cured derivatives. It is also noteworthy that the frequency of class III variants selected in the context of Ser-35 is about 1 order of magnitude less than the frequency of mutations affecting the second nucleotide of codon 35 of v-H-*ras*. Certainly the system has the potential to select cell mutants affected in the *ras* effector pathway. However, the term variant seems more

appropriate until concrete evidence of DNA changes has been garnered.

Marshall and co-workers (28) reported a similar analysis for *S. cerevisiae*. When strains of yeast that could grow on a fermentable carbon source while expressing a *RAS2* Ser-42 mutation (equivalent to the Ser-35 mutation in v-H-*ras* used here) were selected for, one mutant with a substitution in *CYR1*, the adenylate cyclase structural gene, was recovered. Partially purified adenylate cyclase from this mutant strain was shown to be stimulated in vitro by the *RAS2* Ser-42 protein. The *CYR1* substitution affects a site within one of the two extracatalytic domains that endows this enzyme with *RAS2* responsiveness (35). These findings support the conclusion that *RAS* genes control cyclic AMP levels in *S. cerevisiae*, but they do not rule out the possibility that *RAS* works indirectly through an adenylate cyclase-associated protein (17). Even if *RAS2* protein does physically interact with the *CYR1* product, it is not clear that the *CYR1* substitution affects the site that combines with the *RAS2* product. The mutation might affect intramolecular signaling such that a weak interaction between *RAS2* protein and *CYR1* product is amplified to produce a physiologically significant output from the catalytic domain.

These three formal possibilities, i.e., mutational alteration in the p21 combining site of the immediate target of *ras*

FIG. 5. Genetics of *ras* effector function. (A) p21 bound to GTP (square) does not combine with the target (notched circle). (B) Upon binding of GTP, p21 changes conformation (triangle) and combines with the target, and output is generated (arrow). (C) p21 encoded by an effector domain mutation (notched triangle) does not interact with the wild-type target. (D) A cellular effector mutation encodes a target with a compensatory structural change (small solid triangle), and productive interaction with the structurally abnormal p21 is restored. (E) A cellular mutation affecting the immediate target of *ras* action affects intramolecular signaling such that a weak p21-target interaction is amplified into a substantial signal. (F) A cellular mutation affecting an element that functions downstream of the p21-target interaction (notched rectangle) results in amplification of a weak signal (dotted line) to significant levels.

action, alteration of an intramolecular signaling region of the direct target of *ras* action, and alteration of a distinct polypeptide that responds indirectly to *ras* signals, are schematically shown in Fig. 5. In principle, the specificity of the effector variant with respect to *ras* effector alleles could help distinguish between these possibilities. For example, a very subtle alteration in the p21 combining site might be exquisitely allele specific, such that only the context *ras* sequence could elicit a response (Fig. 5D). Alternatively, a variant affecting a downstream cellular element that responds indirectly to p21-target interaction might be capable of amplifying very weak signals generated by the combination of a wild-type target with many different p21 species of reduced effector potency (Fig. 5F). The latter type of variant is not expected to respond to *ras* products that cannot combine with the wild-type target, however. The increased responsiveness of rv2 and rv68 to weak and leaky mutations throughout the effector domain suggests that they may affect elements that function downstream of p21-target interaction. This interpretation seems quite likely in the case of rv2, since Met-36 has detectable transforming activity in NIH 3T3 cells (34). On the other hand, its dramatic responsiveness to several null v-H-*ras* mutations suggests that rv68 may represent a cell mutation that influences the way in which p21 combines with its target, in which case rv68 may genetically define the actual target of *ras* action. Obviously, the strength of this last inference rests on the assumption that *ras* mutations defined as null in rat2 cells are indeed effector null mutations at the biochemical level.

Two unexplained observations deserve comment. First, the foci were relatively frequent in Phe-32-expressing cell

lines (Table 1), and, while these transformed clones may have initially depended on Phe-32 expression for their transformed phenotype, the ones analyzed were clearly class II variants (v-H-*ras* independent). Second, the failure to observe foci after infection of the cured variants with viruses expressing either their respective context alleles or wild-type v-H-*ras* is perplexing. Possibly *ras* is toxic to these cells in the setting of a focus assay. Alternatively, transformation of these variants may require substantial proliferation after the initiation of v-H-*ras* expression, as suggested by the mosaic appearance of the Neo⁺ colonies obtained after infection of rv68BUR. Neither of these explanations is very satisfactory, however, since the variants were initially selected as foci. The phenomenon does highlight the fact that the *ras* effector pathway in these variants is disturbed, however.

In summary, a genetic selection scheme to identify somatic variants that are altered in the *ras* effector pathway has been devised, and one variant cell line with some of the properties expected of a p21 target mutant has been isolated. In this cell line, rv68, any single wild-type residue in the p21 effector domain can be altered without loss of transforming function. If the variant affects a cellular element that functions downstream of p21-target interaction, the conclusion discussed above applies to interactions between p21 and the wild-type target. One implication of this study is that the number of molecular structures that could function as agonists or antagonists of *ras* effector function may be very large.

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