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Genetic analysis of the Kirsten-*ras*-revertant 1 gene: Potentiation of its tumor suppressor activity by specific point mutations

(transformation suppressor gene/site-directed mutagenesis/HT1080 cells/*ras* genes)

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ABSTRACT **Kirsten-*ras*-revertant 1 (K*rev*-1) cDNA encodes a *ras*-related protein and exhibits an activity of inducing flat revertants at certain frequencies (2–5% of total transfectants) when introduced into a v-K-*ras*-transformed mouse NIH 3T3 cell line, DT. Toward understanding the mechanism of action of K*rev*-1 protein, we constructed a series of point mutants of K*rev*-1 cDNA and tested their biological activities in DT cells and HT1080 human fibrosarcoma cells harboring the activated N-*ras* gene. Substitutions of the amino acid residues in the putative guanine nucleotide-binding regions (Asp^{17} and Asn^{116}), in the putative effector-binding domain (residue 38), at the putative acylation site (Cys^{181}), and at the unique Thr^{61} all decreased the transformation suppressor activity. On the other hand, substitutions such as Gly^{12} to Val^{12} and Gln^{63} to Glu^{63} were found to significantly increase the transformation suppressor/tumor suppressor activity of K*rev*-1. These findings are consistent with the idea that K*rev*-1 protein is regulated like many other G proteins by the guanine triphosphate/guanine diphosphate-exchange mechanism probably in response to certain negative growth-regulatory signals.**

Kirsten-*ras*-revertant 1 (K*rev*-1) cDNA was recovered from one of the flat revertants isolated from populations of a v-K-*ras*-transformed NIH 3T3 cell derivative, DT, following transfection with a human fibroblast cDNA expression library (1). When transfected into DT cells, the plasmid expressing K*rev*-1 cDNA induces flat revertants at certain frequencies (2–5% of total transfectants), and relatively high levels of expression seem to be required to induce morphological reversion (2). K*rev*-1 (also known as *rap* 1A and *smg* p21) encodes a guanine-nucleotide binding protein with a molecular weight of 21,000 whose amino acid sequence shares strong similarity (around 50% amino acid identity) with the products of the classical *ras* protooncogenes—namely, H-*ras*, K-*ras*, and N-*ras* (2–4). Similarities are especially high in the essential regions known in H-*ras* protein as guanine nucleotide-binding regions, the effector-binding region, and the C-terminal acylation site (see Fig. 1 for locations), which have been determined by *in vitro* mutagenesis (5–10) and later by x-ray crystallography (11, 12).

The classical *ras* oncoproteins are also known to be activated by specific point mutations, such as amino acid substitutions at residues 12, 59, 61, and 63, to become highly transforming (reviewed in refs. 13 and 14). These mutations are believed to somehow disturb the guanine triphosphate (GTP)-hydrolyzing (GTPase) activity associated with *ras* proteins, thereby arresting these proteins in their active, GTP-bound form. In K*rev*-1 protein, some of the critical target amino acids for mutational activation of *ras* are identical (Gly^{12} and Thr^{59}), and others are distinct from the corresponding amino acids in normal *ras* proteins (Thr^{61}/Gln^{63} in K*rev*-1 vs. Gln^{61}/Glu^{63} in *ras*) (2).

In this study we took advantage of the strong structural similarity between K*rev*-1 protein and H-*ras* protein to investigate the similarity and the difference between the functional organizations of these biologically counteracting proteins. We have introduced a series of point mutations in K*rev*-1 cDNA; some correspond to those that inactivate *ras*, and others correspond to those that activate *ras*. The mutant cDNAs were expressed in DT cells and HT1080 human fibrosarcoma cells known to harbor the activated N-*ras* gene (15, 16), and the growth properties of the resulting cells were examined. Through this approach, we have identified five amino acid residues in K*rev*-1 protein that are essential for its transformation suppressor activities: Ser^{17}, the putative Mg^{2+}-binding site (12, 17); Asp^{38} within the putative effector-binding region; the unique Thr^{61} residue; Asn^{116} in one of the putative guanine-binding regions; and Cys^{181}, the putative acylation site. We have also found that mutations at residues 12 (Gly to Val) and 63 (Gln to Glu, Lys, etc.) significantly potentiate the suppressor activity of K*rev*-1. Our observations suggest that although K*rev*-1 and *ras* proteins have mutually counteracting biological activities, they seem to be regulated by similar biochemical mechanisms that probably involve the GTP/guanine diphosphate (GDP)-exchange reaction.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The 2-kilobase (kb) *Bam*HI fragment of pKrev-1 (2) was subcloned into pBluescript (SK+; Stratagene), in the sense orientation with respect to the T7 promoter, and the resulting plasmid, pBK1, was used for mutagenesis utilizing the oligonucleotide-directed *in vitro* mutagenesis system (Amersham). The oligonucleotides used for mutagenesis were as follows [mismatching nucleotide(s) is indicated by underline]: TTGGTTCAGTAGGCGTTGG (Val^{12}); GAGGCGTTGGGAAGGATGCTCTGACAGTTC (Asp^{17}); AACGATAGAAAATTCCTACAG (Asn^{38}); ACGATAGAAGCTTCCTACAGA (Ala^{38}); CTGGATACTACAGGGACAG (Thr^{59}); TGGATACTGCAGGGCAAGAGCAATTTACAG (Gln^{61}); ACTGCAGGGAAAGAGCAATTT (Lys^{61}); AGGGACAGAGGAATTTACAGC (Glu^{63}); TTTGGTTGGCCATAAATGTGA (His^{116}); ATGAGATATTTTATACCCTGGTCAGACAGA (Thr^{160}); ACAGATAAATGGGAAAACACC (Gly^{167}); AAAGAAATCAAGTCTGCTGCT (Ser^{181}); AGGGACAGAGNNNTTTACAGC (NNN: AAA for Lys^{63}, CGA for Arg^{63}); AGGGACAGAGNNNTTTACAGCAATG (NNN: GAC for Asp^{63}, GGA for Gly^{63}, GTA for Val^{63}, ACA for Thr^{63}, TAT for Tyr^{63}); GGGACAGAGCAATATACAGCAATGAGG (Tyr^{64}); ACTGCAGGGACAGAGTTTACAGCAATGAGG (63 deleted).

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Abbreviations: K*rev*-1, Kirsten-*ras*-revertant 1 gene; GTPase, guanosine triphosphatase; GAP, GTPase-activating protein.

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Mutant plasmids were screened by the dideoxy-sequencing method using Sequenase (United States Biochemical). Mutant cDNAs were excised from the plasmids with *Bam*HI, blunt-ended, and inserted into the blunt-ended *Eco*RI site of pcEXV-1 vector containing simian virus 40 early promoter (a gift from Allan Hall, Institute of Cancer Research, London). These DNA manipulations were performed as described by Maniatis *et al.* (18).

Biological Assays. The origin and the method for maintenance of DT cells have been described elsewhere (19). HT1080 cell line was a gift from Seong-Jin Kim (Laboratory of Chemoprevention, National Cancer Institute). The protocol for transfection assay optimized for DT cells has been described (1, 2) and was also applied for HT1080 cells in this study. Transfectants were selected in medium containing G418 (1 mg/ml) (Sigma); total G418-resistant colonies were counted on day 4 or day 5 after replating, and flat colonies were counted on day 14 or day 15 without knowing which DNA had been placed on the cells. Frequency of reversion in DT cells is defined as the ratio (%) of morphologically flat colonies to total G418-resistant colonies. Doubling times and saturation densities of the cells were determined by the method described by Crow *et al.* (20). Soft agar assay and tumorigenicity assay were performed as described (1).

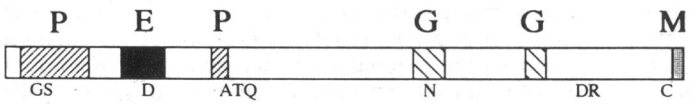
RESULTS

***In Vitro* Mutagenesis.** Mutations resulting in single amino acid substitutions were introduced in 10 codons of K*rev*-1 cDNA (Fig. 1) using oligonucleotide-directed *in vitro* mutagenesis, and their effects on the revertant-inducing activity were examined. The mutations can be divided into four categories: (*i*) from normal *ras* type to activated *ras* type (Val^{12}, Thr^{59}), (*ii*) from normal *ras* type to inactivated *ras* type (Asp^{17}, Ala^{38}, Asn^{38}, His^{116}, Gly^{167}, Ser^{181}), (*iii*) from K*rev*-1-specific type to normal *ras* type (Gln^{61}, Glu^{63}, Thr^{160}), and (*iv*) from K*rev*-1-specific type to activated *ras* type (Lys^{61}). The mutant cDNAs were inserted into a eukaryotic expression vector, pcEXV-1, and cotransfected with a marker plasmid (pL2neo) (21) into DT cells. Transfectant colonies were selected in medium containing G418, and the proportion of flat colonies to the total G418-resistant colonies was scored (Fig. 1).

The revertant-inducing activity of K*rev*-1 was significantly increased by one of the category 1 mutations, Thr^{59} (5.0-fold), and by another mutation, Val^{12}, to a smaller extent (1.8-fold). On the other hand, the activity is more or less diminished by the category 2 mutations, which indicates that these conserved amino acid residues probably play similar, if not identical, roles in the regulation of K*rev*-1 protein and of *ras* proteins. Also, the results with Ala^{38} and Asn^{38} mutants, together with the fact that K*rev*-1 protein and *ras* proteins share an identical amino acid sequence in the so-called effector-binding domain, suggest that these proteins might interact with a common, or structurally related, effector molecule(s) with this domain (residues 32–40). On the other hand, two category 3 mutations, Glu^{63} and Thr^{160}, increased the frequency of reversion. It is interesting that wild-type K*rev*-1 has threonine at amino acid 61, because the Thr^{61} mutant of H-*ras* is known to be weakly transforming (22). In this experiment, two mutations at amino acid 61, the normal *ras* type (Gln) and the strongly activated *ras* type (Lys), decreased the frequency of reversion, indicating the importance of the unique Thr^{61} residue for the transformation suppressor activity of K*rev*-1.

Phenotypes of the Transfectants. To further explore the biological activities of the activated K*rev*-1 mutants, we isolated individual revertant clones from the transfected DT populations and examined the growth properties of each clone *in vitro* and *in vivo* (Table 1). Independence of each clone was confirmed by the uniqueness in the pattern of DNA blot that was hybridized to the K*rev*-1-specific probe (Fig. 2*A*). We also estimated the levels of expression of the exogenously introduced K*rev*-1 and v-K-*ras* by RNA blotting technique (indicated pKrev-1 and v-K-*ras*, respectively, in Fig. 2*B*).

Two clones of flat revertants (WT-1 and -2) were isolated after transfection with wild-type K*rev*-1 (Table 1). WT-1 grew slowly and formed small colonies at a low frequency in soft agar but did not form tumors in nude mice under the conditions used. On the other hand, WT-2 was initially flat after cloning but threw off morphologically transformed cells with some frequency, and by the time we obtained enough cells for analysis the majority had become apparently transformed and formed large colonies in soft agar. Although the WT-2 cells do contain transfected K*rev*-1 DNA, the level of K*rev*-1 mRNA was very low, and this fact may explain the observed phenotypic instability of this clone. Three independent and reasonably stable revertants (12V-1 to -3) were isolated from DT cells transfected with K*rev*-1(Val^{12}). Although these revertants grew faster than WT-1 cells and formed more colonies with larger sizes in soft agar, their tumorigenic activity was greatly suppressed as compared to DT cells. Four stable revertants (59T-1 to -4) and one unstable (59T-5)



	Flat Colonies/Total G418^R Colonies			
	Expt.1	Expt.2	Ratio* (%)	Relative Value
Wild Type	4/144,	4/162	2.6	1.0
Val^{12}	8/181,	5/102	4.6	1.8
Asp^{17}	0/64#,	0/88#	<0.6	<0.23
Ala^{38}	1/87,	0/71	0.63	0.24
Asn^{38}	0/82,	0/110	<0.5	<0.19
Thr^{59}	27/197,	6/56	13.0	5.0
Gln^{61}	3/263,	1/180	0.9	0.35
Lys^{61}	0/161,	0/170	<0.3	<0.12
Glu^{63}	19/150,	8/52	13.4	5.2
His^{116}	1/137,	1/58	1.0	0.38
Thr^{160}	8/146,	10/136	6.4	2.5
Gly^{167}	3/160,	2/90	2.0	0.77
Ser^{181}	0/71,	0/107	<0.5	<0.19
Vector	0/53,	0/107	<0.5	<0.23

FIG. 1. Effects of various point mutations on the revertant-inducing activity of K*rev*-1. pL2neo DNA (0.5 μg) and pcEXV (K*rev*-1 cDNA) (5 μg) were cotransfected into about 10^6 DT cells, the transfectant colonies were selected in medium containing G418, and the numbers of total and flat colonies were scored. The predicted domain structure of K*rev*-1 protein is presented on the top: P, phosphate binding; E, effector binding; G, guanine binding; M, membrane binding. G418^R, G418 resistant.

Table 1. Properties of the DT cell clones harboring K*rev*-1 mutants

	mRNA		Doubling	Soft agar colony*		Tumorigenicity†		
	pKrev-1	v-K-*ras*	time, hr	%	Size	Day 10	Day 20	Day 30
NIH 3T3	−	−	17	<0.1		0/3	0/3	0/3
DT	−	+++	10	105	L	2/3 (SS)	3/3 (LLM)	1/1 (L)‡
WT-1	++	++	22	17	S	0/3	0/3	0/3
WT-2	+	+++	17	97	L	ND		
12V-1	+++	+	19	88	M	0/3	2/3 (SS)	2/3 (SS)
12V-2	++	+++	14	58	M	0/3	1/3 (S)	0/2§
12V-3	++	+++	18	16	M	0/3	0/3	1/3 (M)
59T-1	++++	++	30	17	S	0/3	1/3 (S)	1/3 (S)
59T-2	++	++++	29	16	S	0/3	0/3	0/3
59T-3	++	+	26	5.2	S	0/3	0/3	0/3
59T-4	+++	+++	33	<0.1		0/3	1/3 (M)	0/3
59T-5	+	++	19	8.6	S	0/3	2/3 (SS)	3/3 (SSM)
63E-1	+++	+++	18	12	M	0/3	1/3 (S)	1/3 (L)
63E-2	++	+++	23	70	S	0/3	0/3	0/3
63E-3	++	+++	22	95	M	0/3	0/3	1/3 (S)

ND, not done.

*Ratio (%) of soft agar colonies to viable cells, measured by colony-formation assay in liquid medium. Average sizes of the colonies are indicated as follows: large (L), >500 cells; medium (M), 100–500 cells; small (S), <100 cells per colony on day 14.

†The approximate size of each tumor (v) was estimated by multiplying the width (w), length (l), and height (h) of the tumor ($v = w \times l \times h$, in mm) and is indicated as follows: S, $v < 300$; M, $300 < v < 1000$; L, $1000 < v$.

‡Two animals were dead with large necrotic tumors.

§One animal was dead with a small tumor.

revertant were isolated with K*rev*-1(Thr^{59}). All of the stable clones expressed moderate to high levels of K*rev*-1 mRNA and grew very slowly. Growth in soft agar and tumorigenicity in nude mice were strongly suppressed in these revertants. The instability of 59T-5 cells may be explained by its low level expression of K*rev*-1 mRNA. Three stable revertants (63E-1 to -3) obtained with K*rev*-1(Glu^{63}) exhibited phenotypes similar to those with K*rev*-1(Val^{12}). In summary, these revertants are not only flat in morphology but also exhibit reduced malignancy *in vitro* and *in vivo* as compared to parental DT cells. Also, a balance between the expression of K*rev*-1 and of v-K-*ras* seems to correlate with the phenotypic stabilities of these clones.

Biological Activities of K*rev*-1 and Its Mutants in HT1080 Cells. Since activation of endogenous *ras* genes is known to be involved in many human malignancies, it was of particular interest to see whether K*rev*-1 has any effects on the growth or the tumorigenicity of naturally occurring tumors. As an initial attempt to answer this question, we transfected the K*rev*-1 cDNA or some of its mutants along with the pL2neo plasmid into a human fibrosarcoma cell line, HT1080, and examined the growth properties of the transfectants (Table 2). Because HT1080 cells exhibit flatter morphology than DT cells, and there is a considerable degree of morphological heterogeneity among the individual colonies, it was impossible to determine the frequency of morphological reversion after the transfection in this case. Instead, we determined the growth rate, the colony-forming ability in soft agar, and the tumorigenicity in nude mice of the pooled transfectants that had survived G418 selection. Wild-type K*rev*-1 seems to slow the growth of HT1080 cells, although the cells remain tumorigenic. The same is true with K*rev*-1(Thr^{59}). Consistent with its lack of activity in DT cells, K*rev*-1(Asn^{38}) did not slow the growth of HT1080 cells. K*rev*-1(Ser^{181}) reproducibly exhibited some residual activity in the assay *in vitro* and *in vivo*, and presently the reason for this phenomenon is unknown. On the other hand, K*rev*-1(Val^{12}) and K*rev*-1(Glu^{63}) had prominent effects on the growth of HT1080 cells *in vitro* as well as *in vivo*. The cells harboring K*rev*-1(Val^{12}) grew very poorly in soft agar, and they only formed a nodule in one of three nude mice inoculated. This nodule was small and regressed afterward. The growth rate of the cells harboring K*rev*-1(Glu^{63}) was slower than those harboring wild-type K*rev*-1, and they formed fewer colonies in soft agar. These cells initially formed small nodules in nude mice but failed to establish tumors. Thus, the Val^{12} and the Glu^{63} mutants of K*rev*-1 exhibited strong tumor suppressor activities in this human sarcoma cell line harboring the activated, endogenous N-*ras* gene.

Effects of Mutations in Codon 63. Fasano *et al.* (24) have discovered a unique activating mutation of H-*ras*, Glu^{63} → Lys^{63}, induced by random mutagenesis. Effects of substitutions at this site to other amino acids in *ras* proteins are unknown. K*rev*-1 and closely related genes, D*ras* 3, *rap* 1B, and *rap* 2, have a glutamine residue at this site (3, 25), and the pentapeptide sequence surrounding this site (Thr^{61}-Glu-Gln-Phe-Thr^{65}) is characteristic of these members of the *ras* supergene family. This fact prompted us to speculate on the importance of this region for the unique biological activity of K*rev*-1 and to further study the effects of other mutations in codon 63. We have constructed and tested nine K*rev*-1 mutants carrying single amino acid changes at codon 63 and one mutant at codon 64 (Table 3). Deletion of the amino acid 63 abolished the revertant-inducing activity of K*rev*-1. Among the eight substitutions tested, three (Glu^{63}, Asp^{63}, and Arg^{63}) were found to significantly potentiate the revertant-inducing activities, whereas the Val^{63} mutation abolished the activity. Little effect was observed with the other substitutions. Thus, substitutions of codon 63 to certain charged amino acids seem to be effective in potentiating the K*rev*-1 activity.

DISCUSSION

We have observed that K*rev*-1(Val^{12}) and K*rev*-1(Glu^{63}) exhibited substantial tumor suppressor activities in HT1080 cells, whereas wild-type K*rev*-1 exhibited only a small effect on the growth properties of these cells. Similarly, wild-type K*rev*-1 induced reversion only in a small fraction of DT cells, whereas K*rev*-1(Val^{12}) and K*rev*-1(Glu^{63}) induced reversions at higher frequencies. These results are reminiscent of earlier observations that normal *ras* can transform NIH 3T3 cells only when overexpressed (26, 27), whereas *ras* carrying

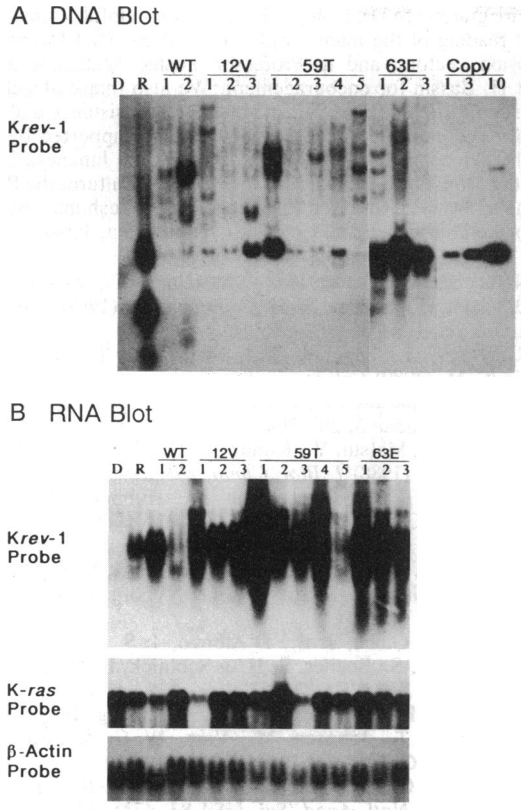


FIG. 2. Occurrence and expression of the transfected K*rev*-1 gene in the flat revertant clones. (*A*) Total DNA extracted from DT (D), R16 (R), or flat revertants induced with either wild-type K*rev*-1 (WT, 1–2), K*rev*-1(Val^{12}) (12V, 1–3), K*rev*-1(Thr^{59}) (59T, 1–5), or K*rev*-1(Glu^{63}) (63E, 1–3) was digested with *Bam*HI and *Bgl* II and analyzed by the Southern DNA blot hybridization technique using the 2.0-kb *Bam*HI fragment of pKrev-1 plasmid (2) as a probe. (*B*) Total RNA (15 μg) extracted from the same set of cell lines was analyzed by the Northern blotting technique. The same blot was probed sequentially with the following three probes: 2.0-kb *Bam*HI fragment of pKrev-1 (K*rev*-1 probe), 0.39-kb *Sac* II/*Xba* I fragment of Kirsten murine sarcoma virus provirus (K-*ras* probe), and 1.7-kb *Pst* I fragment of pA1 (23) (β-actin probe). Blotting and hybridization were performed as described (2).

certain point mutations such as Val^{12} or Leu^{61} exhibit potent transforming activities.

In this study, we are assuming that the point mutations described here do not affect the levels of protein expression. This assumption has not been rigorously proven mainly because of the lack of specific antibodies available at this moment. However, we anticipate some difficulty in interpreting data even if we were able to measure the levels of mutant proteins in the transfectants, because there might be a selective pressure against the cells overexpressing these potentially growth-inhibitory proteins. For these reasons, it seems reasonable to interpret our biological data on the above assumption, at least, for the moment.

Table 3. Revertant-inducing activities of K*rev*-1 cDNAs carrying various mutations in codons 63 and 64

K*rev*-1 mutant	Nature of the amino acid	Frequency of reversion,* %
Vector	—	<1.2
Gln^{63} (WT)†	Polar	2.9 ± 0.3
63 deleted	—	<1.4
Glu^{63}	Acidic	13.5 ± 3.4
Asp^{63}	Acidic	4.0 ± 1.3
Lys^{63}	Basic	10.1 ± 1.8
Arg^{63}	Basic	7.7 ± 0.4
Gly^{63}	Polar	4.3 ± 1.0
Thr^{63}	Polar	3.7 ± 0.5
Tyr^{63}	Polar	4.0 ± 2.9
Val^{63}	Nonpolar	<0.5
Tyr^{64}	(H-*ras* type)	4.8 ± 0.2

The amino acid sequences around this site are
K*rev*-1: Gly^{60}-Thr-Glu-Gln^{63}-Phe-Thr-Ala^{66};
H-*ras*: Gly^{60}-Gln-Glu-Glu^{63}-Tyr-Ser-Ala^{66}.
Values are expressed as mean ± SD.
*Combined data from three separate experiments.
†Wild type.

We observed that only a fraction of DT cells could be reverted, even with the activated form of K*rev*-1 genes, whereas strong effects were observed on the malignancy of HT1080 cells in the bulk transfection assay with these genes. In fact, when we inoculated a pooled DT cell population that had been transfected with the activated K*rev*-1 cDNA followed by G418 selection, we failed to see any differences in the rate of tumor formation as compared to the control DT cells harboring vector DNA (data not shown). Although we do not know the reason for this apparent discrepancy between the results with these two systems, the following facts should be taken into account in interpreting the results: (*i*) DT cells have much higher tumorigenicity in nude mice (10^3 cells per site may form a tumor) than HT1080 cells (at least 10^6 cells per site are needed to obtain a tumor); (*ii*) HT1080 cells have a fairly stable karyotype, whereas DT cells, as parental NIH 3T3 cells, have unstable and hyperploid karyotypes; (*iii*) the species differences—namely, we are looking at the effects of human K*rev*-1 cDNA on mouse DT cells and human HT1080 cells; and (*iv*) the transcription of v-K-*ras* is driven in DT cells by mouse leukemia virus long terminal repeat, a strong promoter in rodent cells, whereas that of N-*ras* is driven by its native promoter in HT1080 cells. The transcription of K*rev*-1 cDNAs used in this study is driven by the simian virus 40 early promoter whose efficiency is known to

Table 2. Growth properties of the HT1080 cells transfected with wild-type or mutant K*rev*-1

DNA	G418^R colonies*	Soft agar colonies, %	Doubling time, hr	Tumorigenicity†		
				Day 15	Day 32	Day 45
Vector	79	69	23	3/3 (SSS)	3/3 (LMS)	3/3 (LLM)
Wild type	39	76	34	2/3 (MS)	3/3 (LLM)	3/3 (LLL)
Val^{12}	51	10	32	0/3	1/3 (S)	0/3
Asn^{38}	95	75	23	3/3 (MSS)	3/3 (LLL)	3/3 (LLL)
Thr^{59}	108	41	36	3/3 (SSS)	3/3 (LLM)	3/3 (LLL)
Glu^{63}	81	28	42	2/3 (SS)	1/3 (S)	0/3
Ser^{181}	105	62	35	1/3 (M)	2/3 (MS)	3/3 (LMS)

*One microgram of pL2neo DNA and 5 μg of pcEXV-K*rev*-1 DNA were cotransfected into about 10^6 HT1080 cells, G418-resistant (G418^R) cells were selected for 16 days, and total G418-resistant colonies were pooled and expanded. Three biological assays (soft agar assay, growth curve determination, and tumorigenicity assay) were performed using the same batches of cells.
†The size of each tumor is designated as described in footnote † to Table 1.

be higher in human cells than in rodent cells. Therefore, the ratio of the levels of expression of *ras* and of K*rev*-1 may be quite different between these two systems.

It has been proposed that *ras* proteins are regulated by the GTP/GDP-exchange mechanism analogous to that for other well-characterized G-proteins (reviewed in refs. 14 and 28)—namely, a hypothetical upstream signal stimulates the protein to release GDP and to bind with GTP. Only this active, GTP-bound form of the protein is able to interact with its effector molecule whose nature in mammalian cells is currently unknown (see below). The system is switched off by a specific GTPase-activating protein (GAP), which converts the GTP-bound *ras* protein to the inactive, GDP-bound form, by potentiating the intrinsic GTPase activity of *ras* proteins. The activating mutations in *ras* are thought to inactivate the intrinsic GTPase activity and/or to decrease the affinity to GAP. In light of this model, one could speculate that K*rev*-1/GTP-complex, which is expected to be stabilized by the activating mutations such as Val^{12}, may bind to the *ras* effector and inhibit the transduction of the downstream growth signal. We have recently found by using chimeric K*rev*-1/H-*ras* genes that a region (residues 1–59) including the conserved putative effector-binding domain (residues 32–40) is responsible for the transformation suppressibility of K*rev*-1 (unpublished data), which is consistent with this model. However, an alternative model that K*rev*-1 protein is involved in a negative signal transduction pathway that is separate from the positive pathway for *ras* protein seems equally probable at this moment.

In the three-dimensional structure of H-*ras* protein reported by Pai *et al.* (12), residues 61–64 form a highly mobile loop sticking out from the globular surface of this protein, and the preceding amino acid (Gly^{60}) has a direct interaction with the γ-phosphate of GTP (12). Der *et al.* (22) have shown earlier that substitutions of codon 61 of H-*ras* to various other amino acids led to the reduction of its GTPase activity and the potentiation of its transforming activity. From these observations, one could speculate that the highly mobile loop (i.e., residues 61–63) in *ras* proteins may play an important role in the regulation of its GTPase activity and that changes in the amino acid sequence in this loop may not necessarily destroy or alter the overall structures and functions of these proteins. This hypothesis is consistent with our findings that various types of codon 63 mutants of K*rev*-1 exhibited increased transformation suppressor activity (Table 3).

McCormick (28) has proposed that GAP may be the effector itself for *ras* proteins, since the GAP-binding domain in *ras* coincides with the genetically identified effector-binding domain, and the enzymatic activity of GAP is consistent with the model if one assumes that the effector receives and terminates the signal. Kikuchi *et al.* (29) have recently reported that they could detect in the bovine brain two distinct species of GAP specific to *smg* p21, the bovine homologue of K*rev*-1, and that the *smg* p21 GAPs failed to activate the GTPase activity of H-*ras* protein, whereas the original *ras* GAP failed to activate *smg* p21 GTPase. These findings indicate that, at least, the switching off mechanisms for *ras* and for K*rev*-1 are separate. Recently, Molloy *et al.* (30) have reported evidence that platelet-derived growth factor receptor kinase phosphorylates *ras* GAP and alters its subcellular localization from the cytosol to the plasma membrane, providing a potentially important insight into the nature of the upstream signal for *ras* protein(s). The main conclusion of the present study that the mode of regulation for K*rev*-1 protein is probably very similar to that for *ras* proteins raises the possibility that K*rev*-1 protein may also be regulated by certain upstream, negative growth-regulatory signals.

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