

Genetic Analysis of the K-*rev*-1 Transformation-Suppressor Gene

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Flat revertants with reduced malignancy *in vivo* can be isolated from Kirsten sarcoma virus-transformed NIH 3T3 cells (DT line) following transfection with a normal human fibroblast cDNA expression library. We have recovered from one such revertant a 1.8-kb cDNA clone, K-*rev*-1, that exhibits an activity of inducing flat revertants at certain frequencies (2-5% of total transfectants) when transfected into DT cells. The K-*rev*-1 cDNA has the capacity to encode a protein with a calculated molecular weight of 21,000, having strong structural similarity to *ras* proteins (~ 50% homology), especially in their guanosine triphosphate/guanosine diphosphate-binding, effector-binding, and membrane-attachment domains. 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. Substitutions of the amino acid residues in the putative guanine nucleotide-binding regions (Asp¹⁷ and Asn¹¹⁶), in the putative effector-binding domain (residue 38), at the putative acylation site (Cys¹⁸¹), and at the unique Thr⁶¹ all decreased the transformation-suppressor activity. On the other hand, substitutions including Gly¹² to Val¹², Ala⁵⁹ to Thr⁵⁹, and Gln⁶³ to Glu⁶³ were found to significantly increase the transformation-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 guanine triphosphate/guanosine diphosphate-exchange mechanism probably in response to certain negative growth-regulatory signals.

Isolation of Morphologically Flat Revertants after Transfection

Our strategy for the isolation of flat revertants is outlined in Figure 1. DT is a transformed derivative of HGPRT-NIH 3T3 cells containing two copies of Kirsten murine sarcoma virus (Ki-MSV) genome (1). The occurrence of spontaneous revertants resulting from inactivation of viral oncogene, *v-Ki-ras*, is extremely low in this cell line. We transfected DT cells with a cDNA expression library (pcD2-human foreskin fibroblast library) (2) and then selected for G418-resistant colonies. The surviving colonies were pooled and treated by one of the several different procedures, each of which was designed to enrich the cell population that failed to express one or more of the properties usually associated with *v-Ki-ras*-induced transformation (3). The majority of the colonies surviving these treatments appeared to be morphologically transformed, and therefore, a final screening for flat revertants in the population of transformed cells was effected by microscopic observation of

individual colonies. Among the clones exhibiting relatively stable morphology and reduced tumorigenicity *in vivo*, seven clones were found to be totally or partially resistant to retransformation by superinfection with Ki-MSV (3).

Properties of the Revertants

By definition, each of the revertants exhibit a contact-inhibited growth pattern. Approximate numbers of transfected plasmids stably incorporated per cell, estimated by Southern blot analysis using a vector-specific probe, ranged from 1 to 10 copies. Also, differences in hybridization patterns observed in such experiments confirm the independent origins of these revertants. Doubling times of the revertants are generally longer than that of the parental DT line, while some revertants grow even more slowly than does the nontransformed NIH 3T3 line. Colony-forming efficiency (CFE) in medium with 1% fetal calf serum and in agar suspension culture is more or less reduced in these revertants. These *in vitro* properties correlate reasonably well with reduced tumorigenicity *in vivo* noted with these seven revertants (Table 1). Southern blot analysis indicated that two copies of the *v-Ki-ras* gene without any gross rearrangements are retained in six out of the seven revertants. High levels of expression of Ki-MSV RNA as well as p21^{ras} protein, comparable to those observed

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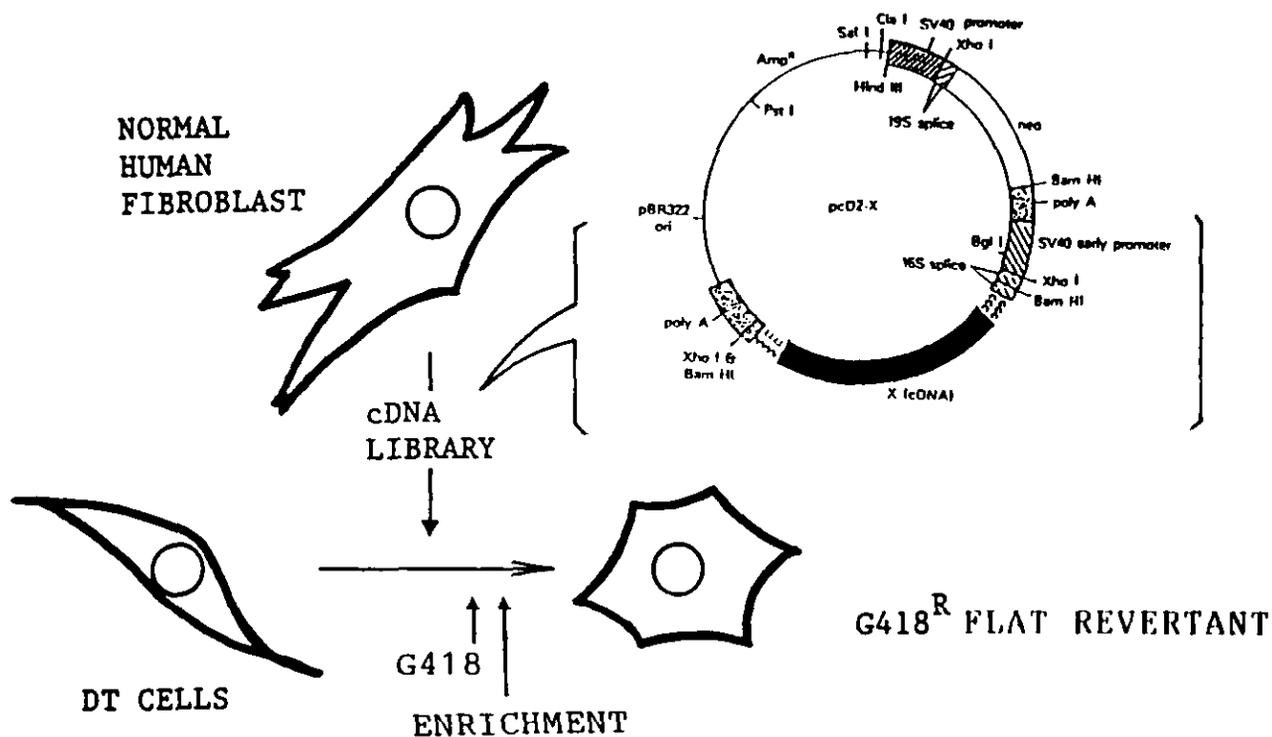


FIGURE 1. Detection of revertant-inducing cDNAs.

Table 1. Properties of NIH 3T3, DT, and the flat revertants isolated following cDNA library transfection.

Cell line	NIH 3T3	DT	R12	R14	R16	R29	R31	R37	R40
Doubling time, hr ^a	18	10	21	21	23	18	19	16	14
Saturation density, × 10 ⁴ cm ²	11	— ^a	14	10	4.4	8.7	11	29	19
In 1% fetal calf serum	< 0.1	11	1.2	< 0.1	1.4	1.1	< 0.1	< 0.1	4
Colony-forming efficiency, %, in soft agar ^b	< 0.01	68(L)	0.5(S)	1.2(S)	0.9(S)	< 0.2	1.7(S)	< 0.01	3.4 (S)
Tumorigenicity ^c	—	+++	—	—	—	±	+	—	±
Fibronectin expression ^d	+++	+	+++	+	+++	++	+++	+	++
Chromosome number, mean ± SD	50 ± 7	50 ± 11	55 ± 13	60 ± 24	57 ± 13	48 ± 12	43 ± 16	70 ± 12	50 ± 18
v-K-ras copy number	0	2	2	2	2	2	2	1	2
p21 Expression	+	++	+	+++	+	++	++	+	+++
Rescuable murine sarcoma virus ^e	—	+	+	+	+	+	+	+	+
Plasmid copy number	0	0	1	7-8	10-12	1-2	1-2	1	2-3

^aTransformed cells have no limit to their growth.^bRatio (%) of soft agar colonies to viable cells seeded as measured by colony-formation assay in liquid medium. Approximate colony sizes are indicated in parentheses as follows: L, > 500 cells; S, < 100 cells per colony at day 14.^cCells (5 × 10⁶) were inoculated subcutaneously into 4- to 6-week-old nude mice (CD-1; Charles River Breeding Laboratories), and the mice were periodically examined for evidence of tumors. (—) No evidence of tumor; (±) tumor of < 1 cm diameter; (+) tumor of 2 to 3 cm diameter; (++) tumor of > 3 cm diameter, in more than two of three inoculated animals. Experiments were terminated 28 days after inoculation. (+++) All animals died within 3 weeks, with large necrotic tumors.^dEstimated by immunoblot analysis.^eAssay performed as described (14).

in DT cells, were detected in all revertants. MSV rescue experiments demonstrated that all seven revertants tested here contain biologically active MSV genomes (Table 1). These observations indicate that reversions are probably not the result of inactivation of Ki-MSV genomes. When each revertant was fused to either NIH 3T3 cells or Ki-MSV-transformed TK-NIH cells, the majority of the cell hybrids expressed a nontransformed phenotype (3), indicating the occurrence of genetic alteration(s) in these flat revertants, which results in a

dominant suppression of the transformed phenotype associated with the v-Ki-ras gene.

Recovery of K-rev-1 cDNA from R16 Revertant

Southern blot analysis using the vector DNA as a probe showed that about 10 clones of human cDNA are present in one of the revertants, R16. The pcD2 vector has a unique Sal I site between the two drug resistance

markers, *amp* and *neo*. Although the *neo* gene is placed under the control of a eukaryotic promoter, it confers weak kanamycin resistance on *E. coli* as well. To recover transfected cDNAs, total DNA extracted from R16 cells was digested with *Sal*I, circularized by ligation at low DNA concentration, and transformed into highly competent *E. coli* (Fig. 2). Out of ten ampicillin-resistant bacterial clones, eight were kanamycin-resistant. Since plasmids retaining both selection markers after the above treatments are likely to be intact, we tested biological activities of these eight plasmid clones by transfection assay in DT cells. One plasmid clone, pK-*rev-1*, with an insert of 1.8 kb, was found to have an activity to induce flat revertants at frequencies of 2 to 5% of total G418-resistant colonies (4).

Structure of the *K-rev-1* cDNA

We have sequenced the cDNA insert of pK-*rev-1* plasmid by generating progressive deletions and by dideoxy sequencing procedures (4). The sense orientation of *K-rev-1* cDNA encodes only one long open reading frame (184 amino acid residues) that has the capacity to encode a protein with a calculated molecular weight of 21,000. A homology search of the Protein Identification Resource (NBRF, release 31.0) revealed that this reading frame shares strong structural similarity with *ras* proteins (Fig. 3). Similarities are especially strong in the regions known, in the Ha-*ras* protein, as the β phosphoryl-group-binding domain (residues 5–22), guanine-binding domains (residues 28, 116–120, 145–147) and so-

called effector-binding domain (residues 32–44). Also, *K-rev-1* and *ras* genes share the consensus sequence CAAX (A: nonpolar residue, X: any residue) at the carboxy-terminal regions, which is known to be essential for the membrane-attachment and the transforming activities of *ras* proteins. These findings prompted us to examine the effects of point mutations in those possible functional domains of *K-rev-1* protein on its biological activity.

Mutagenesis of *K-rev-1*

Mutations resulting in single amino acid substitutions were introduced to 10 sites in the coding region of *K-rev-1* cDNA (Fig. 4) by oligonucleotide-directed *in vitro* mutagenesis. The mutations can be divided into four categories: a) from normal *ras* type to activated *ras* type (Val¹², Thr⁵⁹); b) from normal *ras* type to inactivated *ras* type (Asp¹⁷, Ala³⁸, Asn³⁸, His¹¹⁶, Gly¹⁶⁷, Ser¹⁸¹); c) from *K-rev-1*-specific type to normal *ras* type (Gln⁶¹, Glu⁶³, Thr¹⁶⁰); and d) from *K-rev-1* specific type to activated *ras* type (Lys⁶¹). The mutant cDNAs were inserted into an eukaryotic expression vector pcEXV-1 and co-transfected with a marker plasmid (pL2*neo*) (2) into DT cells. Transfectant colonies were selected in medium containing G418, and the proportion of flat colonies to the total G418-resistant colonies were scored (Fig. 4).

The revertant-inducing activity of *K-rev-1* was significantly increased by one of the category 1 mutations Thr⁵⁹ (5.0-fold) and by another mutation Val¹² 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 Ala³⁸ and Asn³⁸ mutants, together with the fact that *K-rev-1* protein and *ras* proteins share an identical amino acid sequence in 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, Glu⁶³ and Thr¹⁶⁰, increased the frequency of reversion. It is interesting that wild-type *K-rev-1* has threonine at amino acid 61, because the Thr⁶¹ mutant of H-*ras* is known to be weakly transforming (5). In this experiment, two mutations at amino acid 61, the normal *ras* type (Gln) and the strongly activated *ras* type (Lys), both decreased the frequency of reversion, indicating the importance of the unique Thr⁶¹ residue for the transformation-suppressor activity of *K-rev-1*.

Discussion

In earlier studies, we observed that wild-type *K-rev-1* induced reversion only in a small fraction of DT cells. In the present study we have found that certain point mutants of *K-rev-1*, including *K-rev-1*(Val¹²) and *K-rev-1*(Glu⁶³), induce reversions at higher frequencies. These

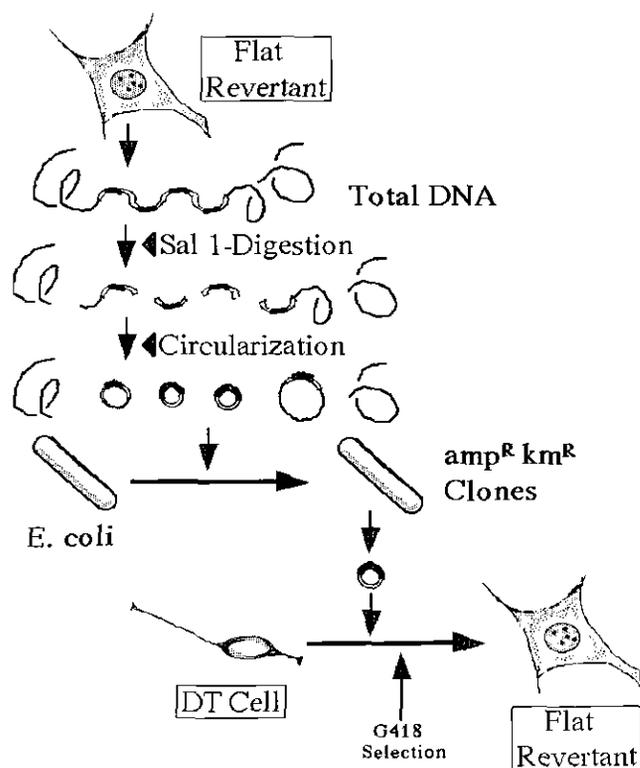


FIGURE 2. Recovery of the cDNAs.

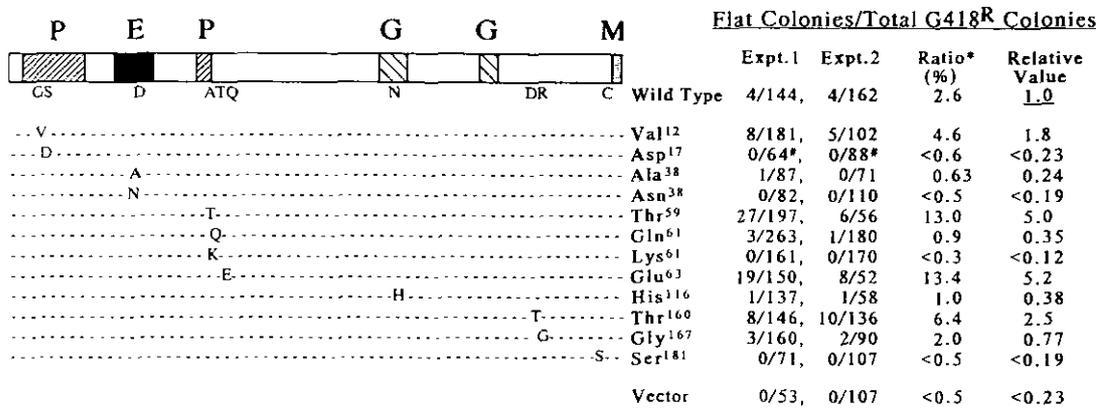


FIGURE 3. Structure of the *K-rev-1* cDNA. Comparison of the amino acid sequences of the predicted *K-rev-1* protein and *ras* proteins. Residue identical to *c-Ha-ras-1* is designated by a dash.

<i>c-Ha-ras1</i>	1	MTEYKLVVVG AGGVGKSALT IQLIQNHFDV EYDPTIEDSY RKQVVIDGET
<i>c-Ki-ras2</i>	1	-----
<i>N-ras</i>	1	-----
<i>R-ras</i>	27	SQTH-----C-----V-F--SY--S D-----T-ICSV--YP
<i>rho</i>		-----PE V-V--VFEN- VADIEVD-KQ
<i>Krev-1</i>	1	-R-----L- S-----V-FV-GI--E K-----EV-CQQ
<i>c-Ha-ras1</i>	51	CLLDILDTAG QEEYSAMRDQ YMRTEGEGFLC VFAINNTKSF EDIHQYREOI
<i>c-Ki-ras2</i>	51	-----H-----
<i>N-ras</i>	51	-----A--ML-----
<i>R-ras</i>	77	AR-----T-QFG--E--A-H--L-----DRQ--NEVGKLFPT--
<i>rho</i>		VE-ALV----D-DRL-PL SYPDTLVI-M C-SVDSPL-L -N-FEKWVPE
<i>Krev-1</i>	51	-N-E-----T-QFT----L--KN-Q--AL -YS-TAQST- N-LQDL----
<i>c-Ha-ras1</i>	101	KRVKDSDDVP MVLVGNKCDL AA RTVESRQ AQLDARSY G IPYIETSAKT
<i>c-Ki-ras2</i>	101	-----E-----PS --DTK-----F-----
<i>N-ras</i>	101	-----E-----YR PK--SS-DG TQ --GLP CVVM
<i>R-ras</i>	127	L---R--F- V---A--ESQ-Q-PRSE -SAFGA-H H VAYF-A---L
<i>rho</i>		VK HFCPN--I--A--K--RSDEHV (26 residues) YDYL-C----
<i>Krev-1</i>	101	L---TE---I-----EDE-V-GKE- C-N---QWNC CAPI-S---S
<i>c-Ha-ras1</i>	149	RGVVEDAFYT LVREI RQHK LRRKINPPDES SCPGCMSCCK CVLIS
<i>c-Ki-ras2</i>	149	-Q--D-----K--EEMSKDGGKK KKKSKTK CVIM
<i>N-ras</i>	149	-Q-----YR PK--SS-DG TQ --GLP CVVM
<i>R-ras</i>	176	-LN--DE--EQ ---AV -KYQ EQE-P-SPP- APRKGGGGCP CVLL
<i>rho</i>		KE--KEV-E- ATRAALQKRY CSQNGCINC CVLL
<i>Krev-1</i>	151	KIN--NEI--D ---Q-N-KTP VEKKKPKKKS CILL

FIGURE 4. Effects of various point mutations on the revertant-inducing activity of *K-rev-1* pL2neo DNA (0.5 μ g) and pcEXV(*K-rev-1* cDNA) (5 μ g) were co-transfected 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. Predicted domain structure of *K-rev-1* protein is presented at the top of the figure: P, phosphate binding; E, effector binding; G, guanine binding; M, membrane binding.

results are reminiscent of earlier observations that normal *ras* can transform NIH 3T3 cells only when over-expressed (6,7), whereas *ras* carrying certain point mutations such as Val¹² or Leu⁶¹ exhibit potent transforming activities.

It has been proposed that *ras* proteins are regulated by the guanosine triphosphate/guanosine diphosphate (GTP/GDP) exchange mechanism analogous to that for other well-characterized G-proteins [reviewed in Bar-

acid (8) and McCormick (9)]; 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 Val¹², 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* (10,11), 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.

McCormick (9) 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. Kituchi et al. (12) 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. Molloy et al. have reported the evidence that platelet-derived growth factor-receptor kinase phosphorylates *ras*GAP and alters its subcellular localization from the cytosol to the plasma membrane

(13), providing a potentially important insight into the nature of 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 signal.

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