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

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Flatt 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 transfected) 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 Asn34), in the putative effector-binding domain (residue 38), at the putative acylation site (Cys81), and at the unique Thr49 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/guanine 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 p21ras protein, comparable to those observed

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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

![Figure 1](image-url)

**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<sup>a</sup> | 18 | 10 | 21 | 21 | 23 | 18 | 19 | 16 | 14 |
| Saturation density, × 10<sup>4</sup> cm<sup>-2</sup> | 11 | —<sup>b</sup> | 14 | 10 | 4.4 | 5.7 | 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<sup>a</sup> | < 0.01 | 68(L) | 0.5(S) | 1.2(S) | 0.9(S) | < 0.2 | 1.7(S) | < 0.01 | 3.4(S) |
| Tumorigenicity<sup>c</sup> | — | ++ | — | — | — | ± | + | — | ± |
| Fibronection expression<sup>d</sup> | ++ + | + | ++ + | + | ++ + | + | ++ + | + | ++ |
| 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 | 1 | 2 |
| p21 Expression | + | ++ | + | ++ + | + | ++ | + | ++ + |
| Rescueable murine sarcoma virus* | — | + | + | + | + | + | + | + |
| Plasmid copy number | 0 | 1 | 7-8 | 10-12 | 1-2 | 1-2 | 1 | 2-3 |

<sup>a</sup>Transformed cells have no limit to their growth.

<sup>b</sup>Ratio (%) 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.

<sup>c</sup>Cells (5 × 10<sup>5</sup>) 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.

<sup>d</sup>Estimated by immunoblot analysis.

<sup>e</sup>Assay performed as described (14).

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 Sall, 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 dyeoxy 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, Asn63, Gly67, Ser61); c) from K-rev-1-specific type to normal ras type (Glu61, Gln61, 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 (pL2neo) (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 Asn63 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, Glu61 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(Glu61), induce reversions at higher frequencies. These
results are reminiscent of earlier observations that normal ras can transform NIH 3T3 cells only when overexpressed (6,7), whereas ras carrying certain point 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-raf-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-raf-1 (10,11), which is consistent with this model. However, an alternative model that K-raf-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-raf-1, and that the smgp21GAPs failed to activate the GTPase activity of H-ras protein, whereas the original rasGAP failed to activate smg-p21 GTPase. These findings indicate that, at least, the switching-off mechanisms for ras and for K-raf-1 are separate. Molloy et al. have reported the evidence that platelet-derived growth factor receptor kinase phosphorylates rasGAP 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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