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A Domain Responsible for the Transformation Suppressor Activity in K*rev*-1 Protein

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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 NIH3T3 cell line, DT. To explore the functional organization of K*rev*-1 protein, we constructed a series of chimeric genes consisting of fragments of H-*ras* and K*rev*-1 cDNAs, and tested their biological activities in DT cells. The results indicated that the determinant for the transformation suppressor activity resides in the N-terminal one-third of the K*rev*-1 encoded polypeptide within which a highly conserved, putative effector-binding domain is present.

Key words: Chimeric cDNA — H-*ras* gene — Flat revertant

The 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 NIH3T3 cell derivative, DT, following transfection with a human fibroblast cDNA expression library.[1,2] K*rev*-1 (also known as *rap* 1A[3] and *smg* p21[4]) 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* proto-oncogenes, namely, H-*ras*, K-*ras* and N-*ras*. 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. 1B for location), that have been determined by *in vitro* mutagenesis[5-10] and later by X-ray crystallography.[11,12] In the present study we took advantage of this strong structural similarity between K*rev*-1 protein and H-*ras* protein to map the region within K*rev*-1 protein responsible for its transformation suppressor activity.

A series of chimeric genes consisting of fragments of K*rev*-1 cDNA and H-*ras* cDNA were constructed (Fig. 1), and their ability to induce flat revertants when introduced into transformed DT cells was tested (Table I). We chose to use Val^{12} mutant of human K*rev*-1 as a parental gene in an attempt to obtain clear results, since we recently found that this mutant exhibits 1.5- to 2-fold higher revertant-inducing activity as compared to the wild-type gene having Gly^{12} residue.[23] For the same reason, the highly transforming Val^{12} mutant of human c-Ha-*ras*[13,14] was used as the other parent. The coding regions of these parental genes were divided into three portions: codons 1–59 (portion I), 60–111 (portion II), and 112–C-terminus (portion III). Portion I contains putative phosphate- and ribose-binding regions as well as the effector-binding region, portion II the unique pentapeptide (see below), and portion III the guanine-binding regions as well as the acylation site. We first created appropriate restriction sites (a *Pst*I site and a *Bst*XI site) at the junctions of these portions in both cDNAs to make it possible to reciprocally recombine fragments from the different cDNAs (for experimental details, see the legend to Fig. 1). To minimize the anticipated variation in the efficiency of mRNA and protein expressions among these chimeric DNAs, we cut down the length of the 5′-noncoding sequences derived from the parental cDNAs to as short as 15 bases.

When DT cells were transfected with the plasmid expressing wild-type K*rev*-1 cDNA and incubated in the medium containing 1 mg/ml G418 (Sigma) as a selective agent, 2.2 and 2.8% of the total G418-resistant colonies were morphologically flat in two separate experiments (Table I). Increased proportions of flat colonies were observed when we transfected the plasmid expressing K*rev*-1 (Val^{12}), one of the parental cDNAs used for constructing the chimeric genes. On the other hand, the other parent, H-*ras* (Val^{12}), was not able to induce any revertants. Chimeric gene C4, whose K*rev*-1 (Val^{12})-specific amino acids were limited to portion I, was almost as active as the K*rev*-1 (Val^{12}) parent, while the reciprocal construct C1 was inactive. The results localize the K*rev*-1-specific structure responsible for its revertant-inducing activity to the portion I. This conclusion was further supported by the results with the following two constructs.

Abbreviations: K*rev*-1, Kirsten-*ras*-revertant-1; GTP, guanosine triphosphate.

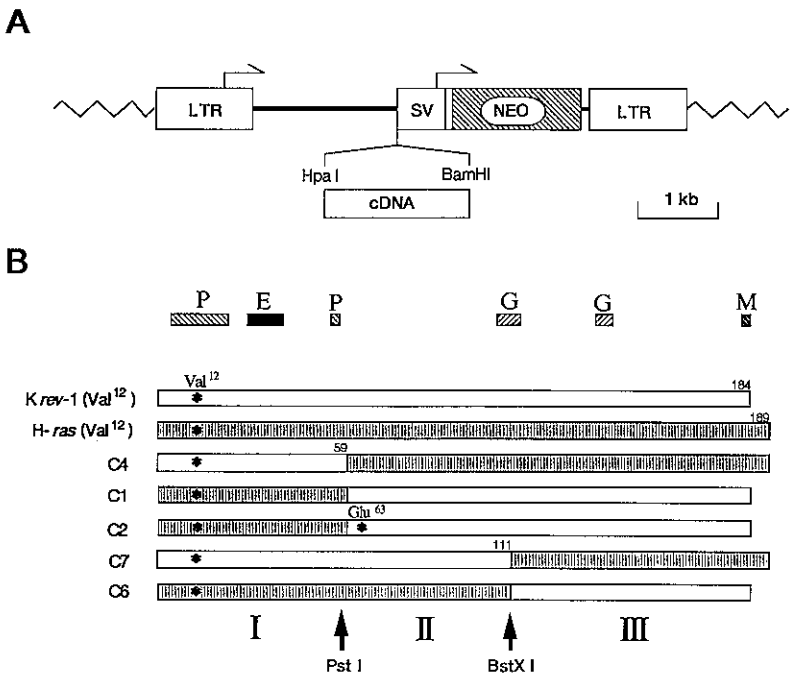

Fig. 1. DNA constructs used in this study. A. Structure of pLXSN vector.[21] In this retrovirus-based vector, the expression of inserts is driven by the mouse leukemia virus long terminal repeat (LTR), and that of the G418-resistant marker (NEO) is driven by the internal SV40 early promoter (SV). Zigzag lines indicate sequences derived from a bacterial plasmid pBR-322. B. Structures of chimeric K*rev*-1/H-*ras* genes. Domain structure of K*rev*-1 protein, as predicted by analogy with H-*ras* protein,[14] is shown at the top: P, phosphate-binding domain; E, effector-binding domain; G, guanine-binding domain; M, membrane-attachment (acylation) site. The structures of parental and chimeric gene products are shown in the lower part. Chimeric cDNAs were constructed as follows. The 2 kb *Bam*HI fragment of pK*rev*-1[2] was subcloned into pBluescript (SK+; Stratagene), in the sense orientation with respect to the T7 promotor, and the resulting plasmid was named pBK1. Two activated point mutants, pBK1(Val^{12}) and pBK1(Glu^{63}), were derived from pBK1 by using the oligonucleotide-directed *in vitro* mutagenesis system (Amersham).[23] To eliminate the *Pst*I site present in the upsteam multi-cloning sites in the vector, pBK1, pBK1(Val^{12}), and pBK1(Glu^{63}), were cut with *Hin*dIII, which also excises a small non-coding segment of K*rev*-1 cDNA, and religated to yield pBK1ΔH, pBK1(Val^{12})ΔH, and pBK1(Glu^{63})ΔH, respectively. A *Bst*XI site was created in the Pro^{110}-Val^{114} region within the K*rev*-1 coding sequences in these plasmids by mutagenesis using an oligonucleotide ACGGAAGATGTTCCCATGATTTTGGTT to yield pBK1ΔH-B, pBK1(Val^{12})ΔH-B, and pBK1(Glu^{63})ΔH-B, respectively. On the other hand, the normal H-*ras* and the activated H-*ras*(Val^{12}) cDNAs (provided by Dr. Allan Hall, Institute of Cancer Research, London) were subcloned into the *Eco*RI site of pBluescript (SK+) in the same orientation as pBK1 to yield plasmids named pBHa and pBHa(Val^{12}), respectively. A *Pst*I site was created in the Thr^{58}-Ala^{59} region within the H-*ras* coding sequence in pBHa by mutagenesis using an oligonucleotide CCTGGATACTGCAGGCCAGGAG (Ha-Pst), and the resulting plasmid was named pBHa-P. Since the *Bst*XI-recognition site has sequence ambiguity, the *Bst*XI site in the Pro^{110}-Val^{114} region of the H-*ras* coding sequence had to be modified to match this site with the corresponding site in the K*rev*-1 plasmid, pBK1ΔH-B, etc. This was done by mutagenizing pBHa(Val^{12}) using an oligonucleotide GACGACGTTCCCATGATTTTGGTTGGCAACAAGT. This modification changed the Val^{112} to Ile^{112} and the resulting plasmid was named pBHa(Val^{12})-B. The region bearing the amino acid change in this plasmid was not included in any of the final chimeric genes. A *Pst*I site was created in the Thr^{58}-Ala^{59} region in this plasmid by using oligonucleotide Ha-Pst (see above) to yield pBHa(Val^{12})-BP. The large *Pst*I-*Not*I fragment (vector plus portion I) of pBHa(Val^{12})-BP and the small *Pst*I-*Not*I fragment (portions II and III) of pBK1ΔH-B were ligated to yield a chimeric cDNA clone pBC1. Likewise, pBC2 was constructed by ligating the large *Pst*I-*Not*I fragment of pBHa-(Val^{12})-B and the small *Pst*I-*Not*I fragment of pBK(Glu^{63})ΔH-B. The small *Pst*I fragment (portions II and III) of pBHa-P was inserted into the *Pst*I site of pBK1(Val^{12})ΔH-B to yield pBC4. pBC6 was made by ligating the large *Bst*XI fragment (vector plus portions I and II) of pBHa(Val^{12})-B and the small *Bst*XI fragment (portion III) of pBHa(Val^{12})ΔH-B. pBC7 was made by ligating three fragments: the small *Bst*XI fragment (portion III) of pBHa(Val^{12})-B, the large *Bst*XI fragment (vector and non-coding region) of pBK1(Val^{12})ΔH-B, and the small *Bst*XI fragment (portions I and II) of pBK1(Val^{12})ΔH-B. The following coding fragment of each chimeric plasmid was inserted into the *Hpa*I-*Bam*HI sites of the retrovirus vector LXSN[21]: the *Eco*RV-*Bgl*II fragment of pBC1, pBC2, and pBC6, the *Dra*I-*Bgl*II fragment of pBC4, and the *Dra*I-*Bam*HI fragment of pBC7. The resulting plasmids, C1, C2, C6, C4, and C7, were purified and used for transfection assay on DT cells. The standard DNA manipulations were carried out as described by Maniatis *et al.*[22]

Table I. Revertant-inducing Activities of the Chimeric K*rev*-1/H-*ras* Genes

DNA	Flat colonies/Total G418^R colonies (%)[a]	
	Expt. 1	Expt. 2
Vector	0/304 (<0.4)	0/388 (<0.3)
K*rev*-1	10/459 (2.2)	11/391 (2.8)
K*rev*-1 (Val^{12})	13/352 (3.7)	12/285 (4.2)
H-*ras* (Val^{12})	0/244 (<0.4)	0/337 (<0.3)
C4	6/204 (2.9)	14/348 (4.0)
C1	0/253 (<0.4)	0/211 (<0.5)
C2	0/206 (<0.5)	0/168 (<0.6)
C7	13/372 (3.5)	14/359 (3.9)
C6	2[b]/248 (0.8)	4[b]/176 (2.3)

a) Biological activity was measured by transfecting plasmid DNA (5 μg) into DT cells as described elsewhere[1,2] and counting the numbers of total (on day 4–5) and flat colonies (on day 7–10) after selection in the medium containing 1 mg/ml G418. The data from two separate experiments are presented.
b) Morphologically less flat than others.

We have recently found that Gln^{63} to Glu^{63} mutation causes a five-fold increase in the revertant-inducing activity of K*rev*-1.[23] This mutation failed to confer revertant-inducing activity on a chimeric gene whose portion I was derived from H-*ras* cDNA (compare C1 and C2). This result suggests that amino acid 63 is not within the determinant for the revertant-inducing activity. In addition, chimeric gene C7, whose K*rev*-1-specific sequence was extended to portions I and II, was active, in accordance with the above conclusion. The reciprocal construct C6 exhibited a reduced but significant revertant-inducing activity. We noticed that the revertants induced by C6 DNA were morphologically not as flat as those induced by other constructs. The reason for this residual activity reproducibly detected with C6 gene is currently under investigation.

In the present experiments, products of the chimeric genes could not be characterized due to the lack of specific antibodies against K*rev*-1 protein. Therefore, we cannot rule out the possibility at this point that some of the negative results may be due to the lack of protein expression. However, in view of the positive results with the C4 construct we can draw the most important conclusion that portion I is required, and possibly sufficient, for the transformation suppressor activity of K*rev*-1.

K*rev*-1 and closely related genes, D*ras* 3, *rap* 1B, and *rap* 2, have a glutamine residue at the codon 63 position.[3,15] Moreover, the pentapeptide sequence surrounding this site (Thr^{61}-Glu-Gln-Phe-Thr^{65}) is characteristic of these members of the *ras*-super gene family, as compared to the same regions in classical *ras* proteins (Gln^{61}-Glu-Glu-Tyr-Ser^{65}). This fact prompted us to hypothesize the importance of this region for the unique biological activity of K*rev*-1.[2] However, the second portion of K*rev*-1, even in its activated form (Glu^{63}), failed to exhibit the suppressor activity when linked with the first portion of H-*ras* (Fig. 1B, Table I, C2). Although caution is necessary in interpreting this negative result (see above), this finding may indicate that the characteristic pentapeptide region is important for the regulation rather than the specificity of the functions of K*rev*-1 and other closely related proteins.

Another striking feature in the primary structure of K*rev*-1 protein is that it shares an identical amino acid sequence with classical *ras* proteins in the region known as the effector-binding domain (residues 32–40). Some mutations in this region of *ras* have been found to result in the loss of transforming activity[8,9] as well as in the lack of association with, or responsiveness to, the *ras*-GTPase-activating protein (GAP) that catalytically accelerates GTP hydrolysis by normal *ras* proteins but not that by transforming mutant, *ras* proteins.[16–19] These observations led to the proposal that GAP may be a component of the effector complex for *ras* proteins.[20] In terms 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 effector complex for *ras* and inhibit the transduction of the downstream growth signal. This model is consistent with our result here that portion I containing the conserved putative effector-binding domain and surrounding diverse regions[2] determines the transformation suppressibility of K*rev*-1.

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