Decreased GTPase activity of K-ras mutants deriving from human functional adrenocortical tumours

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Summary Our previous studies have shown that seven out of 15 patients with adrenocortical tumours contained K-ras gene mutation. In addition, the mutation type was a multiple-site mutation, and the hot spots were located at codons 15, 16, 18 and 31, which were different from those reported before (codons 12, 13 and 61). To understand whether the mutation hot spots in human adrenocortical tumours were associated with activation of K-Ras oncogene and the alterations of its biocharacteristics, mutant K-Ras genes were cloned from tumour tissues and then constructed with expression vector pBKCMV. Mutant K-Ras genes were expressed at high levels in Escherichia coli and the resultant K-Ras proteins were shown to be functional with respect to their well-known specific, high-affinity, GDP/GTP binding. The purified K-Ras protein from E. coli were then measured for their intrinsic GTPase activity and the GTPase activity in the presence of GDP/GTP exchange reaction that is facilitated by guanine nucleotide-releasing factors (Segal et al., 1993; Overbeck et al., 1995; Lin et al., 1996). On the other hand, the GTP-bound form is converted to the GDP-bound form by the intrinsic GTPase activity, which is accelerated by GAPs (Molloy et al., 1995; Miao et al., 1996; Winston and Hunter, 1995). Like other guanine nucleotide-binding proteins, ras cycles between an active GTP-bound form and an inactive GDP-bound form. The GDP-bound form is converted to the GTP-bound form through a GDP/GTP exchange reaction that is facilitated by guanine nucleotide-releasing factors. The wild-type K-Ras protein (p21BN) exhibits about ten times higher intrinsic GTPase activity than the activated protein (p21BM3), which mutated at codon 60. With regards to the codon 15, 16, 18 and 31 mutant K-Ras proteins (p21BM2), the GTPase activity in the presence of GAP is much lower than that of the normal K-Ras protein, whereas the intrinsic GTPase activity is nearly the same as that of the normal K-Ras protein. These results indicated that mutations at these hot spots of K-Ras gene were indeed activated K-Ras oncogene in adrenocortical tumours; however, their association with tumors needs further experiments to prove. © 2000 Cancer Research Campaign

Keywords: GTPase; K-ras mutants; functional adrenocortical tumours

Among the studies of oncogenes in human tumours, activation of ras oncogene has been found to be the most common event in various cancers including bladder cancers, breast cancers, colon cancers, kidney cancers, liver cancers, lung cancers, ovarian cancers, pancreatic cancers and gastric cancers (Yamashita et al., 1995; Muleahy et al., 1998; Caduff et al., 1999). In addition, activation of K-ras gene was found in more than 40% of colon cancer, pancreatic cancers and lung cancers (Anker et al., 1997; Gao et al., 1997). Our previous study has also showed that mutations of the K-ras gene were found in 55% of adrenocortical tumours and the mutation hot spots were focused on codons 15, 16, 18 and 31 (Lin et al., 1998), which were different from those (codons 12, 13 and 61) in other studies (Grendy et al., 1997). The K-ras gene encoded a protein of 21 kDa, p21, which was consisted of 188 amino acids (Santos and Nebreda, 1989). Ras protein as a critical relay switch that controls signalling pathways connecting the cell surface with the nucleus (Chen et al., 1996; Feliciello et al., 1996; Hughes et al., 1997). Ras protein is activated by growth factors, such as epidermal growth factor (EGF), insulin and insulin growth factor (IGF) (Leitner et al., 1997). It then triggers the activation of a cascade of serine/threonine kinase, which includes Raf-1 and MAPK (mitogen-activated protein kinase) (Porras and Santos, 1996). Activated MAPK then translocates to the nucleus where it phosphorylates and activates transcription factors which, in turn, cause changes in gene expression responsible for growth stimulation (Winston and Hunter, 1995). Like other guanine nucleotide-binding proteins, ras cycles between an active GTP-bound form and an inactive GDP-bound form. The GDP-bound form is converted to the GTP-bound form through a GDP/GTP exchange reaction that is facilitated by guanine nucleotide-releasing factors (Segal et al., 1993; Overbeck et al., 1995; Lin et al., 1996). On the other hand, the GTP-bound form is converted to the GDP-bound form by the intrinsic GTPase activity, which is accelerated by GAPs (Molloy et al., 1995; Miao et al., 1996; Wittinghofer et al., 1997). Since hydrolysis of GTP was an important event in regulation of p21 activity, it was suggested that mutation of ras gene might affect normal GTP hydrolysis. Numerous researchers have analysed the ras gene and reported that the mutation at codon 61 cause the mutant proteins to be unresponsive to the stimulation by the GTPase activating proteins, furthermore, increase the ras-transforming potential (Adari et al., 1988). However, other results showed that when a substitution occurred at codons 12, 13 or 59 of the ras gene, the Ras protein changed and furthermore lost its ability to bind with GTP (Barbacid, 1987). Consequently, mutant Ras persists in an active, GTP-bound state, leading to constitutive and deregulated stimulation of growth regulatory process that contributes to the aberrant growth properties of tumour cells (Downward, 1996). Our previous findings indicated that K-ras gene was mutated at codons 15, 16, 18 and 31. Because these mutations occurred frequently in the patients with adrenocortical tumours, we believe that they are associated with the development of adrenal tumours. Therefore, it is important to know whether these mutations affect the GTPase activity of Ras in the presence or absence of GAP to understand the mechanism of the carcinogenesis of adrenal tumours. In this way, we first examined the intrinsic GTPase activity of mutant Ras.
Table 1  K-ras gene expression constructs from human adrenocortical tumours and their gene products

| Plasmid   | Vector       | Codon base | Amino acid | Protein |
|-----------|--------------|------------|------------|---------|
| pKWTCMV  | pBKCMV       | Wild type  | p21BN      |         |
| pK1516MCMV | pBKCMV       | 15GGC→AGA | Gly→Thr   | p21BM1  |
| pK568MCMV | pBKCMV       | 16AAG→GAG | Lys→Glu   | p21BM2  |
| pK60MCMV | pBKCMV       | 18GCC→AGG | Gly→Thr   | p21BM3  |

K-Ras protein (p21K-Ras) and its GTPase activity in the GAP presence. We hope the results can provide a further understanding of the correlation between K-ras oncogene and adrenal tumours.

MATERIALS AND METHODS

Construction of mutant K-ras expression plasmids

mRNA was extracted from tumour tissue and reverse transcribed (RT) to cDNA with MMLV reverse transcriptase and oligo (dT). Then, cDNA was amplified by polymerase chain reaction (PCR) with primers specific for coding region and 5’ and 3’ non-coding region of K-ras including KpnI and SacII site as follows: 5’-TTGTACCCCTAAAAATGACTGAATATAAACTTGT-3’ and 5’-CGAGCTCGACCACTTGTACTAGTATGCC-3’. A 615 bp PCR product was amplified and inserted into the vectors pBKCMV (Stratagen, La Jolla, CA, USA) to prepare K-ras gene expression constructs for prokaryotic and eukaryotic cells. For prokaryotic cells, the vector pBKCMV was used. This vector contained a lac promoter which could regulate K-ras expression. In our experiments, wild-type and mutant K-ras genes, including mutations at codons 15, 16, 18 and 60, separately were cloned into the pBKCMV vector and named as pKWTCMV, pK1516MCMV, pK568MCMV, pK60MCMV (Table 1).

Purification of mutant K-ras protein synthesized by E. coli

The bacterially synthesized K-Ras protein was purified as described earlier (Gross et al., 1985). Ten grams of E. coli cells were sonicated in 50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) (5 ml g–1 of cells), and the sonicated cell extract was then centrifuged for 10 min at 4000 g to remove unbroken cells and cell debris. The particulate fraction was pelleted by ultracentrifugation for 2 h at 30000 rpm. The pellet was homogenized in an excess of buffer A (50 mM Tris–HCl, pH 7.4, 5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PMSF) and the suspension was centrifuged as above. The pellet was suspended in buffer A (2 ml g–1 of cells), and Ras protein was solubilized by the addition of an equal volume of 7 M guanidine-HCl. The clear supernatant containing Ras protein was obtained after centrifugation for 2 h at 30000 rpm and stored at –20°C before use.

Construction of GAP expression plasmid

Using the PCR we constructed the expression plasmid for minimal fragments of p120<sup>AP</sup>-GAP-273 (residues Met<sup>74</sup> – His<sup>96</sup>) from the bovine brain cDNA. The oligonucleotides used were as follows: 5’-CCGGAATTCGCCACCCGCCGACCCGATGGAAAATACTAGCGCC-3’ and 5’-GAGGCCGGCTTATTTAAGCCGCTGGCGG-3’. PCR products were isolated, digested with EcoRI/Not I and ligated into the GST fusion vector pGEX-4T-2 (Amersham, Pharmacia Biotech UK Ltd). The resulting plasmid, pGEX-GAP, was transformed into E. coli strain TG1. The correct orientation of the GAP cDNA was confirmed by sequencing.

Expression, purification and cleavage of GST-GAP fusion protein

The expression of fusion protein was performed basically as described by Guan and Dixon (1991). Twenty millilitres of an overnight culture was inoculated into 1 litre of 2XYT (10 g yeast extract, 16 g tryptone and 5 g sodium chloride (NaCl) in 1 litre) containing 50 µg ml–1 ampicillin. The culture was incubated vigorously at 37°C until an absorption of one at 600 nm was reached. IPTG was then added and cells were harvested by centrifugation and suspended in 10 ml PBST (2 mM EDTA, 0.1% β-mercaptoethanol, 0.2 mM PMSF and 5 mM benzamidine) after 3 h incubation. Cells were lysed and bacterial lysate was centrifuged at 4°C to remove the insoluble fraction. One millilitre of the bacterial supernatant containing soluble proteins was mixed with 2 ml 50% (v/v) glutathione agarose beads and incubated 30 min at 4°C with gentle shaking. The agarose beads were washed four times with 10 ml PBST. The fusion protein was eluted by competition with glutathione using 2 × 2 min washes with 1 ml of 50 mM Tris (pH 8.0) containing 10 mM glutathione. Free glutathione was removed by dialysis against 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% β-mercaptoethanol. Approximately 2-mg fusion protein was incubated with 4 µg of thrombin at room temperature for 20 min in 2 ml of thrombin cleavage buffer (50 mM Tris, (pH 8.0), 150 mM NaCl, 2.5 mM calcium chloride (CaCl2), 0.1% β-mercaptoethanol). Glutathione S-transferase and uncleaved fusion protein were removed by directly adding 2 ml of glutathione agarose (50% v/v) to the cleavage reaction. The sample was incubated for 30 min at 4°C and centrifuged for 10 min. The supernatant, which contained purified GAP-273 protein was recovered. GAP1-273 activating activity of GAP-273 was assayed by adding H-Ras (Santa Cruz Biotechnology, Inc) expressed in E. coli.

Immunoblot

K-Ras protein from E. coli transformed with K-ras expression plasmids was purified and electrophoresed through vertical slab gels composed of 10% polyacrylamide containing 0.1% sodium dodecyl sulphate (SDS) prepared in 3 mm Tris–HCl, pH 8.8. Following electrophoresis, the protein was electroblotted to a nitrocellulose membrane (Schleicher & Schusl GmbH, Dassel, Germany). The membrane fixed with protein was incubated with mouse monoclonal antibodies (mAbs) specific for the K-Ras p21 product (25 mg ml–1) (Santa Cruz Biotechnology, Inc.), and the excess unbounded antibodies were then washed off. The membrane binding with the mouse mAbs was incubated with a solution of secondary goat anti-mouse IgG conjugated with alkaline phosphatase (1:3000, Bio-Rad, Hercules, CA, USA). The
tagged Ras protein was detected by enhanced chemiluminescence (ECL) detection system (Amersham, Pharmacia Biotech UK Ltd). Blots were then exposed to Kodak XAR film (Eastman Kodak, Rochester, NY, USA).

**Measurement of GAP activity**

GAP assay was measured as described by Li et al (19). Two picomoles of Ha-Ras-[γ-32P]GTP (30 Ci mol⁻¹, ICN) was incubated at 30°C with the indicated amounts of GAP-273 in 20 μl of a solution containing 50 mM Tris–HCl (pH 8.0), 5 mM magnesium chloride (MgCl₂), 0.25 mM GTP, 5 mg ml⁻¹ bovine serum albumin and 5% glycerol. The reactions were quenched by keeping the sample on ice. After the addition 0.5 ml of ice-cold washing buffer (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl₂), the sample was passed through a nitrocellulose filter (0.45 μm, Schleicher & Schuell GmbH, Dassel, Germany). The filter was washed three times with 1 ml of ice-cold washing buffer, and the radioactivity trapped on the filter was determined using a liquid scintillation counter. The amount of GTP hydrolysed during the reaction was determined as a decrease in the reactivity trapped on the filter. The data were calculated from at least three independent experiments.

**GTPase assay**

Purification K-Ras protein (0.8 μg) was incubated with 60 μg of K-Ras p21 mAb (Santa Cruz Biotechnology, Inc.) in 100 μl of GTP buffer containing 0.1% Triton X-100, 50 μM PMSF and 0.3 mg ml⁻¹ BSA at 0°C for 60 min. Then, 2 μl of protein A-Sepharose beads (Amersham, Pharmacia Biotech UK Ltd), coated with rabbit anti-rat IgG and equilibrated in GTP buffer/0.1% Triton, was added and the sample was mixed for 25 min at 4°C. The beads were washed extremely with GTP buffer/0.1% Triton–HCl, 100 mM NaCl, 5 mM MgCl₂, and 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl₂, the sample was separated on a 10% polyacrylamide gel stained with Coomassie blue (Figure 1A). Then, 3.5 M LiCl and 1M formic acid solution. GTP and GDP spots were then cut off from the TLC plate separately and the radioactivity was determined. The rate of GTP hydrolysis was obtained from semilogarithmic plotting of the amount of bound GTP against time.

**RESULTS**

The translational products of mutant K-ras gene constructs from human adrenocortical tumours made by E. coli, were showed in the 12.5% SDS polyacrylamide gel in Figure 1A. Then, 3.5 M guanidine–HCl was used to extract the K-Ras protein from the washed particulate fraction and a few other proteins might also be extracted. However, the intensity of the bands in Figure 1 showed that 85% of the proteins were K-Ras proteins. Further immunoblot analysis proved that those proteins could interact with mAbs specific for K-Ras p21 proteins, confirming that they were p21K-Ras proteins (Figure 1B). Comparison with the M.W. marker revealed that these K-Ras protein were 23 000 dalton. The 273-residue fragment from p120GAP from Met714 to His986 could be expressed in E. coli as a glutathione S-transferase (GST) fusion protein. Figure 2 shows that the protein was isolated as GST fusion protein and cleaved from GST by thrombin. It remained soluble after purification. The specific activity of GAP-273 for

![Figure 1](image-url)
The GAP-273 (30 kDa) in lane 3 and glutathione S-transferase (26 kDa) in lane 4 are major components of the reaction mixture catalysed hydrolysis of Ha-Ras GTP was shown in Figure 3. In Figure 3, GAP-273 purified from GST fusion protein is actually able to catalyse the hydrolysis of Ras GTP. Therefore, we used this purified GAP-273 protein to regulate the GTPase activity of mutant K-Ras proteins in our experiments.

The GTPase activities of these K-Ras proteins were measured both in the absence and presence of GAP-273. When the four different K-Ras proteins were analysed for their intrinsic GTPase activity, we found that the difference of K-Ras proteins of similar purity and quantity were shown in GTPase reaction, however, they have very high reproductivity. p21BN K-Ras protein, which is the wild-type K-Ras protein, reacted very similarly to p21BM1 and p21BM2 in intrinsic GTPase activity in which about 20% of the substrate GTP is hydrolysed by p21BN protein after 1.5 h (Figure 4). However, GTPase reactions of p21BM3 were 10 times lower than that of p21BN (Figure 5).

K-Ras mutant protein, p21BM3 and wild-type p21BN were purified with the same procedure and analysed by immunoprecipitation of intrinsic GTPase activity, suggesting that they should have similar purity and the same experimental errors. However, the GTPase activity of the two proteins were 8–15 times different. Therefore, we thought that these GTPase reaction were actually caused by K-Ras protein. In addition, other recombinant protein of similar size made in E. coli under the control of the same expression plasmid and solubilized from the particulate fraction of bacterial cell extracts in an identical manner to that used for K-Ras protein had minor contaminants similar to those in the K-Ras protein preparations yet exhibited no trace of GTPase activity. The above results indicated that the difference in GTPase activity was actually caused by purified K-Ras protein, only the K-Ras protein mutated at codon 60 from human adrenocortical tumour influenced the intrinsic GTPase activity.

In the GTPase activity assay with the GAP stimulation, the mutant p21BM1 K-Ras protein mutated at codon 15 and 16, in both the absence and presence of purified GAP-273 protein, it had the same GTPase activity as wild-type p21BN K-Ras protein. Although the intrinsic GTPase activity of p21BM3 K-Ras protein, which mutated at codon 60, decreased the intrinsic GTPase activity, the GAP-stimulated GAPase activity of this mutant protein was similar to that of the wild-type K-Ras protein. By contract, the mutant K-Ras protein, which mutated at codons 15, 16, 18 and 31, had the same intrinsic GTPase activity to this of wild-type K-Ras protein, but their GTPase activity was significantly lower than wild-type K-Ras protein after stimulation with GAP. Thus, we concluded that of the mutant K-Ras proteins from human adrenocortical tumours, only the mutant K-Ras protein which mutated at codons 15, 16, 18 and 31, influence GAP-sensitivity.

DISCUSSION

The K-ras gene has two alternative 3’ exons designated 4A and 4B (Capon et al, 1983). p21K-rasA and p21K-rasB are similar but different in the 24/25 C terminal residues. Numerous reports have shown that K-rasB appears to be ubiquitously expressed (Slamon and Cline, 1984; Leon et al, 1987); in contrast, the expression of K-rasA is induced during differentiation of pluripotent embryonal stem cells in vitro (Pells et al, 1997). In addition, our previous study of human adrenocortical tumours showed that mutant and expressed K-ras genes are mainly K-rasB gene. Therefore, we cloned K-rasB cDNA from tumour cells into a vector and then transformed the K-ras gene constructs into E. coli. The K-Ras proteins expressed in E. coli transformed with K-ras gene constructs were observed through SDS-gel electrophoresis. The results showed that these K-Ras proteins migrate with an apparent molecular weight slightly larger than that of natural p21Kras protein. This is probably due to the inability of E. coli to carry out the post-translational modification with p21Kras undergoes in mammalian cells (Lantenerberger et al, 1983).

In the cell the conformational state of guanine nucleotide binding proteins is regulated by two kinds of interacting molecules, guanine nucleotide exchange factors and GTPase activating proteins (called GAPs). Three specific GAPs for p21Kras were p120GAP (the prototype of this class of proteins and the first one to be isolated) (Vogel et al, 1988; Lim et al, 1992), neurofibromin (NF1) (Hattori et al, 1992) and a mammalian homologue (GAP1m)
of the Drosophilic GAP1. p120GAP and NF1 protein can be distinguished with respect to their catalytic properties (Martin et al., 1990; Xu et al., 1990; Nur-E-Kamal and Maruta, 1992). p120GAP increases the GTPase reaction of p21Ha-Ras more than tenfold catalytic activity for Ras GTP, whereas NF1 protein has been reported to have a lower activity but a higher affinity. In this study, we demonstrated that GAP-273 (codons 174–986) could stimulate the GTP hydrolysis of Ha-Ras protein. This is consistent with the result made by Ahmadian et al (1996). In 1996, Ahmadian et al confirmed that the specific activity of GAP-273 for catalysed hydrolysis of Ras bound GTP was almost the same as that measured with full-length p120GAP. Therefore, we used GAP-273 as the GAP of mutant K-Ras in this study.

Regarding the analysis of GTPase activity and GAP sensitivity, the results showed that only mutation at codon 60 influenced intrinsic GTPase activity. This may be due to the fact that codon 60 (Gly) is the only amino acid without a branch among the amino acids on phosphate binding region (codons 57–63) (Sung et al., 1995). In the mutant K-Ras protein, the cysteine substitution caused this amino acid (Gly) to add a sulphur, which affected its conformation and furthermore affected its binding with GAP, then enhancing the activity of GTPase (Boriack-Sjodin et al., 1998). The same phenomenon was also observed in the K-Ras protein with mutation at codon 28 (Powers et al., 1989). On the other hand, the mutant K-Ras protein with mutation at codons 15, 16, 18 and 31 had lower GAP sensitivity than normal. These results can be explained by the reports of Sigal et al. (1986), Powers et al. (1989) and Shirouzu et al. (1992). In 1986, Sigal et al found p21 protein Lys-16 was the decision site of the GTP/GDP-binding site. If Lys-16 is replaced by Asn, the affinity between GTP and GDP will decrease 100 times, without affecting GTP/GDP-binding specificity. In 1989, Powers et al designed a mutant p21 protein with Ala instead of Gly-15 for studying the characteristics of the p21 protein. The results showed that this alteration could also affect the normal functions of the p21 protein. In addition, Shirouzu et al. (1992) found that Ras protein with Glu-31→Lys mutation was found to have drastically decreased GAP sensitivity. Thus they

Figure 4  Time course of the GAP-273-stimulated GTP hydrolysis of wild-type K-Ras protein, p21BN. Shown is the GTP hydrolysis reaction catalysed by recombinant K-Ras protein, p21BN in the absence and presence of GAP-273. The reaction was conducted at 30°C and samples were taken at the indicated times. The reaction products were analysed by TLC, followed by autoradiography (A) and quantification of the radioactive GTP and GDP spots using a PhosphoImager (Molecular Dynamics) (B)

Figure 5  Time course of the GAP-273-stimulated GTP hydrolysis of mutant K-Ras proteins. The GTPase activity was determined by 25 μl of the solution containing (8, 5'-3H) α-32P-GTP bound Ras protein was incubated with (filled symbol) or without (open symbol) GAP-273 at 30°C. At different time, 5 μl of the mixture was analysed by TLC. GTP and GDP spots were then cut off from the TLC plate separately and the radioactivity was determined. The rate of GTP hydrolysis was obtained from semilogarithmic plotting of the amount of bound GTP against time
concluded that p21 Ras protein Glu-31 is an important residue for the simulation of GTPase activity by GAP<sup>in vitro</sup>. However, the K-Ras protein with mutation at codons 15 and 16 from another tumour did not reveal a decrease in GAP sensitivity. This may be caused by different amino acid substitution between the two mutant K-Ras protein.

In this study, we observed that mutant K-ras gene from human adrenocortical tumour has indeed decreased the GTPase activity of K-Ras protein. Our advanced study showed that when the normal adrenocortical cell transfected with mutant K-ras expression plasmids derived from human adrenocortical cells by electroporation, the levels of cortisol secreted by transfected cells were 15–25 times higher than normal cells (Lin et al., 1999). However, the mechanisms by which K-Ras protein affects the adrenocortical tumour development needs more study.

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