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Identification of amino acid residues of Ras protein that are essential for signal-transducing activity but not for enhancement of GTPase activity by GAP

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To determine the amino acid residues required for the signal-transducing activity of the human c-Ha-Ras protein, we introduced point mutations at residues 45–54 near the 'effector region' (residues 32–40). We transfected PC12 cells with these mutant genes and also micro-injected the mutant proteins, bound with an unhydrolyzable GTP analog, into PC12 cells. Both procedures showed that Val[45]→Glu and Gly[48]→Cys mutations impaired the ability of the Ras protein to induce morphological change of PC12 cells. These mutations did not affect the guanine nucleotide-binding activity or GTPase activity in the absence or presence of bovine GTPase-activating protein (GAP). Therefore, the Val[45] and Gly[48] residues should be included by definition in the effector region responsible for the signal transduction, while only a subset of the effector-region residues is required for enhancement of the GTPase activity by GAP.

c-Ha-*ras*; Krev-1; Effector region; GTPase activating protein; Mutagenesis

1. INTRODUCTION

The Ras protein is a guanine nucleotide-binding protein. 'Activated' Ras proteins, which have much lower GTPase activity than that of the normal Ras protein, promote transformation of NIH 3T3 cells [1], terminal differentiation of rat pheochromocytoma (PC12) cells [2,3] and maturation of *Xenopus* oocytes [4]. The GTP-bound form of the Ras protein has been shown to be the active form for these signal transducers [5]. The signal-transducing activities are impaired by mutations at amino acid residues 32–40 [6]. Mutations at residues 32–40 do not influence the guanine nucleotide-binding activity or the GTPase activity, so these amino acid residues are considered to constitute a region that is involved in interaction with the target effector of the Ras protein (the 'effector region' or 'effector-binding region') [6].

The K*rev*-1 gene [7], which is identical to the *smg*21 gene [8] and the *rap*1A gene [9], has been found to be a suppresser gene of transformation by an activated K-*ras* gene. The amino acid sequence of the Krev-1 protein is very similar to that of the Ras protein. In particular, residues 32–40 of the Krev-1 protein are identical with the corresponding effector-region residues 32–40 of the Ras protein. However, mutations corresponding to the 'activating' mutations of the *ras* genes did not result in transforming activity of the K*rev*-1 gene [10]. Therefore, amino acid residues other than those at positions 32–40 are required for the signal-transducing activity of the Ras protein. Zhang et al. showed that a chimeric protein consisting of residues 41–60 of the Krev-1 protein and the other residues from the Ras protein have no transforming activity [11]. Therefore, some of the residues at positions 41–60 of the Ras protein must be essential for the signal-transducing activity, and may belong to the effector region.

The GTPase activity of the Ras protein is enhanced by the GTPase-activating protein (GAP) [12,13]. The enhancement of the GTPase activity requires residues 32–40 [14,15]. However, it is not yet clear whether GAP is a target effector or a negative regulator of the Ras protein [16]. GAP for the Ras protein has higher affinity to the Krev-1 protein than to the Ras protein, but does not enhance the GTPase activity of the Krev-1 protein [17,18]. This indicates that some residues other than residues 32–40 are required for 'GAP sensitivity' (the ability to undergo enhancement of the GTPase activity of the Ras protein by GAP).

The amino acid residues at positions 45, 46, 48, 49, 50, 52 and 54, near the previously identified effector region, differ in the Ras and Krev-1 proteins [7]. Therefore, in the present study, we constructed a series of

Abbreviations: GMPPNP, guanilyl(β,γ-imide)diphosphonate.

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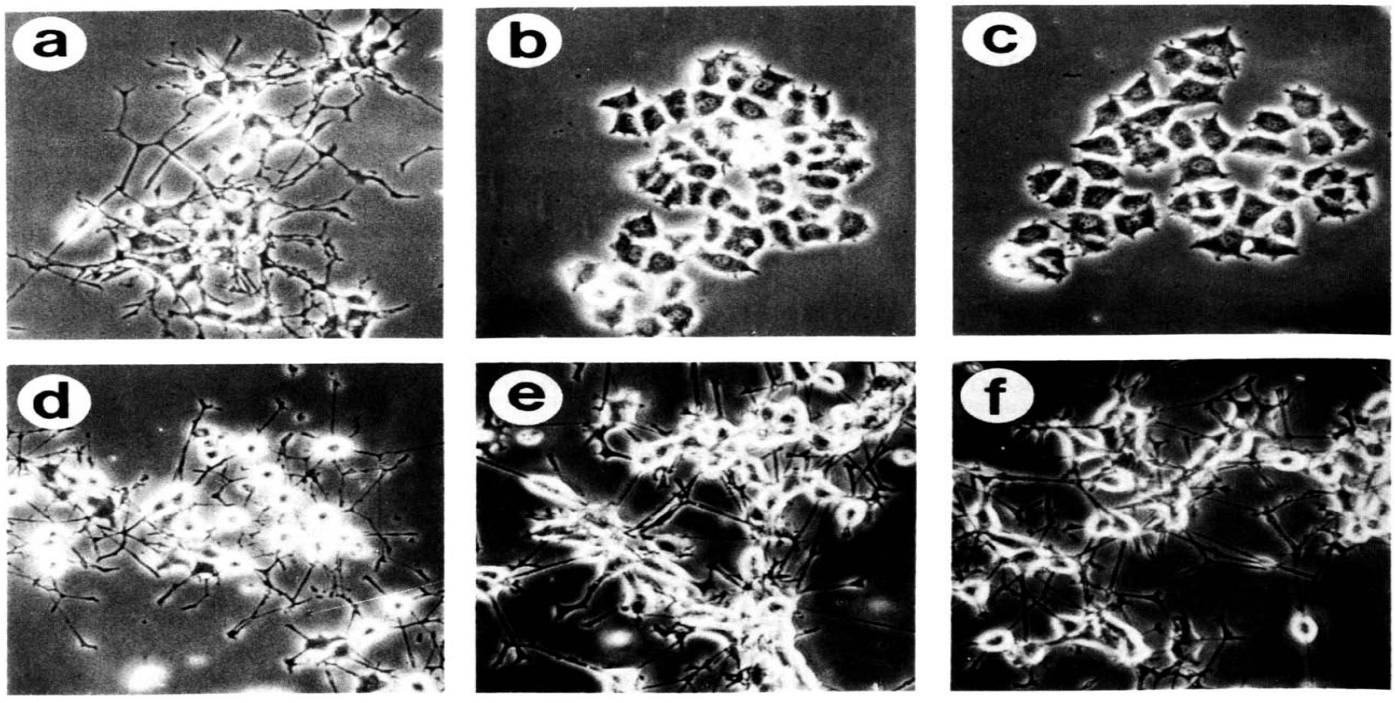

Fig. 1 Morphological change of PC12 cells induced by mutant *ras* genes (Val^{12}-type). The photographs were taken 24 h after the addition of dexamethasone. PC12 cells were transfected with the original gene (a), or the gene carrying the second mutation(s), Val^{45}→Glu (b), Gly^{48}→Cys (c), Glu^{49}→Gln (d), Thr^{50}→Gln (e) or Leu^{52}→Met / Asp^{54}→Glu (f).

mutant Ras proteins by site-directed mutagenesis at one or two of these positions (except position 46 occupied by Ile in Ras and by Val in Krev-1); the residue of the Ras protein was replaced by the corresponding residue of the Krev-1 protein. Then, we analyzed the signal-transducing activities of these mutant Ras proteins in PC12 cells and also their GAP sensitivity in vitro.

2. MATERIALS AND METHODS

2.1. *Preparations of normal and mutant Ras proteins*

Mutations were introduced into a synthetic human c-Ha-*ras* gene [18] by oligonucleotide-directed mutagenesis using a Muta-Gene kit (Bio-Rad). Mutant Ras proteins were overproduced in *E. coli* and purified as described [19] with the modifications that the buffer A contained 5 mM $MgCl_2$ and no *n*-octyl glucoside.

2.2. *Measurements of dissociation rates of guanine nucleotides and GTP-ase activities of Ras proteins in the absence and presence of GAP*

The dissociation rates of GDP and GTP from each mutant Ras protein (Gly^{12}-type) were measured as described previously [20]. The intrinsic GTPase activity and the GAP sensitivity of each mutant protein were analyzed as described previously [21].

2.3. *Transfection of PC12 cells with mutant* ras *genes*

An oncogenic *ras* gene (Gly^{12}→Val) additionally carrying one of the mutations described above was subcloned into the mammalian expression vector pMAM-*neo* (Clontech). PC12 cells were transfected with DNA (5 μg) by the calcium phosphate precipitation method and G418-resistant clones were obtained. Expression of the mutant *ras* gene was induced by dexamethasone, and neurite outgrowth of PC12 cells was measured.

2.4. *Micro-injection of the GMPPNP-bound Ras proteins into PC12 cells*

The purified Ras protein (Gly^{12}-type) in the GDP-bound form (1 mg) was incubated with GMPPNP, an unhydrolyzable analog of GTP, (0.2 μmol) and 1 mM EDTA for 5 min at 37°C. Then free nucleotides were removed by ultrafiltration in a Centricon-10 (Amicon). This cycle was repeated three times. HPLC analysis confirmed that 95% of the protein-bound nucleotides were converted from GDP to GMPPNP. PC12 cells were re-plated 24 h before micro-injection at cell densities of 1×10^5 cells/dish. Mutant Ras proteins in the GMPPNP-bound form (10 mg/ml) were injected into 100–200 cells with an injectoscope IMF-2 (Olympus).

3. RESULTS

3.1. *Signal-transducing activities of mutant* ras *genes*

We investigated the signal-transducing activities of the mutant *ras* genes to promote neurite outgrowth of PC12 cells (Fig. 1). All the mutant genes tested had a mutation in the region of residues 45–54 in addition to the 'activating' Gly^{12}→Val mutation ('Val^{12}-type' mutants). The presence of these mutant proteins in the transfected PC12 cells was confirmed by Western blotting analysis using an anti-Ras monoclonal antibody,

Table I

Signal-transducing activities of Ras proteins

Mutation between positions 45 and 54	Neurite outgrowth (%)[a]	
	Gene expression (Val^{12}-type)	Microinjection with GMPPNP (Gly^{12}-type)
None	97	67
Val^{45}→Glu	0	4
Gly^{48}→Cys	0	5
Glu^{49}→Gln	91	ND[b]
Thr^{50}→Gln	84	ND
Leu^{52}→Met / Asp^{54}→Glu	82	ND

[a]The numbers of PC12 cells that extended neurites were counted and are shown as percentages of the total number of the cells transfected with the mutant *ras* gene or micro-injected with the GMPPNP-bound mutant Ras protein.
[b]Not determined.

NCC-RAS-004 [22]. On expression of the Val^{12}-type *ras* gene with a second mutation of Glu^{49}→Gln, Thr^{50}→Gln or Leu^{52}→Met/Asp^{54}→Glu in PC12 cells, the neurite-outgrowth efficiency was as high as that on expression of the original Val^{12}-type *ras* gene (more than 80% of the cells extended neurites) (Table I and Fig. 1). In contrast, PC12 cells expressing the Val^{12}- type *ras* gene with a Val^{45}→Glu or Gly^{48}→Cys mutation extended no neurites (Table I and Fig. 1). Thus, of the five mutations tested, Val^{45}→Glu and Gly^{48}→Cys mutations were found to impair the signal-transducing activity.

3.2. *Signal-transducing activities of microinjected GMPPNP-bound Ras proteins in PC12 cells*

We then used Ras proteins with glycine at position 12 (Gly^{12}-type mutants). As with the wild-type, Val^{45}→Glu mutant and Gly^{48}→Cys mutant Ras proteins, we exchanged bound GDP for GMPPNP, and microinjected the GMPPNP-bound Ras proteins into PC12 cells. The Val^{45}→Glu and Gly^{48}→Cys mutant Ras proteins induced neurite outgrowth in only a small fraction (4–5%) of the micro-injected PC12 cells, whereas the wild-type Ras protein induced neurite outgrowth in 67% of the injected cells (Table I).

3.3. *Guanine nucleotide-binding activities and GTPase activities in the absence and presence of GAP*

Next, we examined the biochemical activities of these mutant Ras proteins (Gly^{12}-type). We measured the rates of dissociation of the bound GDP and GTP from the Ras proteins by counting the radioactivities of [8,5′-^{3}H]GTP or [8,5′-^{3}H]GDP remaining bound to the Ras proteins after incubation in the presence of Mg^{2+}, and estimating the dissociation rates of GDP and GTP from semilogarithmic plots (Table II). Results showed that the mutations had little effect on the dissociation rate. We also measured the GTPase activities of the mutant

Table II

Dissociation rates of GDP and GTP, and GTPase activities in the absence and presence of GAP of the Ras proteins

Mutation between positions 45 and 54	Dissociation rate[a] ($k_d \times 10^3$/min^{-1})		GTPase activity ($k_c \times 10^2$/min^{-1})	
	GDP	GTP	−GAP	+GAP
None	5.5	4.4	1.7	4.8
Val^{45}→Glu	7.6	5.8	1.4	4.8
Gly^{48}→Cys	4.6	4.1	1.9	5.1
Glu^{49}→Gln	5.3	4.8	1.8	5.3
Thr^{50}→Gln	5.8	4.6	2.1	4.8
Leu^{52}→Met / Asp^{54}→Glu	6.4	5.3	3.0	5.8

[a]Rates of dissociation of protein-bound [8,5′-^{3}H]GDP or [8,5′-^{3}H]GTP in the presence of 100 mM $MgCl_2$ and 20 mM GTP.

Ras proteins in the absence and presence of GAP. The intrinsic GTPase activities of all the mutant Ras proteins were similar to that of the wild-type Ras protein, except for that of the Leu^{52}→Met / Asp^{54}→Glu mutant protein, which has a 2-fold higher GTPase activity (Table II). The GTPase activities of these mutant Ras proteins in the presence of GAP were practically the same (Table II). The ratios of the GTP-hydrolysis rate in the presence of GAP to that in its absence were also similar (67–120%). Therefore, this region does not appear to be related to the interaction with GAP.

4. DISCUSSION

In the present study, we have identified two residues (Val^{45} and Gly^{48}) that were required for the signal transduction, and that are located outside the previously identified effector region (residues 32–40). The presence of such functional residues in the region of residues 41–60 has been predicted from the finding that a chimeric Ras protein containing these 20 residues from the Krev-1 protein did not have transforming activity [13]. The Val^{45}→Glu and Gly^{48}→Cys mutations impair the signal-transducing activity of both the normal Ras protein (Gly^{12}-type) and the activated Ras protein (Val^{12}-type). But these mutations did not affect the guanine nucleotide-binding activity or the intrinsic GTPase activity (Table II).

There was a possibility that Val^{45} and Gly^{48} were required for interaction of the Ras protein with the guanine nucleotide-exchange factor [23,24]. However, micro-injections of the Val^{45}→Glu and Gly^{48}→Cys mutant Ras proteins in the GMPPNP-bound form did not induce morphological change of PC12 cells, indicating that these two residues are probably required for interaction with the target effector. Therefore, the effector region or effector-binding region should be extended by definition to cover the Val^{45} and Gly^{48} residues. The Gly^{48} residue is located at the turn between the two strands (residues 38–46 and 49–57) constituting the an-

tiparallel β-sheet [25,26], suggesting that the Gly^{48}→Cys mutation may affect the conformation of this antiparallel β-sheet.

Residues 32–40 of the Ras protein are also required for GAP sensitivity [14,15], although the problem of whether GAP is the target effector or a negative regulator of the Ras protein has not yet been resolved. Some residues other than residues 32–40 have been anticipated also to be required for GAP sensitivity [17,18]. In fact, residues 21–31 (or some of them) are required for the signal-transducing activity and/or GAP sensitivity of the Ras protein ([11], unpublished results by M. Shirouzu, J. Fujita-Yoshigaki and S. Yokoyama). However, the present analyses showed that the region of residues 45–54 does not contain any residue that is important for the GAP sensitivity of Ras.

Val^{45}→Glu and Gly^{48}→Cys mutations of the Ras protein did not affect the GAP sensitivity, but resulted in loss of signal-transducing activity. GAP sensitivity has been shown not to be correlated with signal-transducing activity in some mutants at position 35 or 40 [16]. Therefore, the effector region is longer than the previously defined region of residues 32–40, and a subset of residues in the extended effector region constitutes the determinant for GAP sensitivity.

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