The GTPase-activating NF1 Fragment of 91 Amino Acids Reverses v-Ha-Ras-induced Malignant Phenotype*

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The human neurofibromatosis type 1 gene encodes a Ras GAP (GTPase-activating protein) of 2818 amino acids called NF1. This GAP contains a domain of 338 amino acids (residues 1194-1531) called NF1-GRD, which shares 26% sequence identity with the C-terminal domain (GAPlC, residues 709-1044) of another Ras GAP called GAP1. Both NF1-GRD and GAPlC activate normal Ras GTPases but not oncogenic mutants such as v-Ha-Ras. Any attempt to reverse the v-Ha-Ras-induced malignant transformation by the GAPlC has, so far, been unsuccessful. However, we have found that when the NF1-GRD is overexpressed in v-Ha-Ras-transformed NIH/3T3 cells, it greatly reduces their ability to form colonies in a soft agar, the property tightly associated with their malignancy. This is, so far, the first demonstration that a Ras-binding protein can act as a potent antagonist of the oncogenic Ras mutants in mammalian cells. In an attempt to further screen the smallest anti-oncogenic fragment derived from the NF1-GRD, we have prepared a series of its deletion mutants and examined their interaction with Ras first by monitoring their GAP activity (ability to activate the normal Ras GTPase). The deletion analysis has revealed that the N-terminal 247 amino acids (residues 1194-1440) of NF1-GRD are not required for its GAP activity, suggesting that its remaining domain of 91 amino acids (NF91, residues 1441-1531) is sufficient to bind the v-Ha-Ras, although its GAP activity is 20 times lower than the NF1-GRD. The NF91 is, so far, the smallest among the tumor suppressor proteins that show the anti-v-Ha-Ras action in vivo. Thus, the NF91 appears to be a good starting material from which a smaller and more potent v-Ha-Ras antagonist could be devised to be used as a potential cure for the human cancers caused by the Ras mutants.

Monomeric GTP/GDP-binding (G) proteins of 188/189 amino acids encoded by Ras protooncogenes are GTP-activated signal transducers that are involved in the regulation of cell proliferation or differentiation, depending on the cell types (1). Their transducing activity is attenuated when their bound GTP is hydrolyzed to GDP by their low intrinsic GTPase activity. The majority of oncogenically mutated Ras shows much lower intrinsic GTPase activity than the normal Ras. Furthermore, the normal Ras GTPases, but not oncogenic mutants, are activated by two distinct GAPs, i.e. GAPl of 1044 amino acids (also called Ras GAP of 120 kDa) and NF1 of 2818 amino acids (also called neurofibromatosis type 1 gene product or neurofibromin), in mammalian cells (2-7). Thus, the oncogenic Ras mutants such as v-Ha-Ras would remain in the GTP-bound (active) forms for much longer periods than the normal Ras in the cells. This appears to be the major reason why the Ras mutants are much more oncogenic than the normal Ras for the majority of the target cells, in particular pancreatic and colonic cells, where v-Ha-Ras and other Ras mutants most frequently cause development of malignant tumors in human (8).

For more than a decade since the first discovery of oncogenic Ras mutants in retroviruses (v-Ha-Ras and v-Ki-Ras) and human carcinomas (1), various attempts have been made to attenuate the oncogenic Ras signals in the tumor cell lines such as v-Ha-Ras/v-Ki-Ras-transformed NIH/3T3 cells with a hope to find a cure for Ras-associated cancers. The major approach in the field of molecular Ras oncology has been to screen the tumor suppressor genes that are able to reverse the v-Ha-Ras/v-Ki-Ras-induced malignant transformation by the DNA-mediated transfection and overexpression. Several distinct tumor suppressor genes have been reported to reverse the malignancy caused by the Ras oncogenes (9-15) as follows: the full-length human Rap1 (also called Krev1) encoding a Ras-related G protein of 184 amino acids (9), which tightly binds GAPl but without any stimulation of its intrinsic GTPase activity (16); a truncated v-Jun gene (10) encoding only the C-terminal domain of 150 amino acids (residues 147-296), which lacks the N-terminal transactivating domain; the full-length murine Thy-1 gene encoding a cell surface glycoprotein of 142 amino acids, which is covalently linked to a glycosphatidyl inositol (11); a human c-Ets-2 DNA (12) encoding the C-terminal DNA-binding domain of 133 amino acids (residues 333-466), which binds a Ras-responsive DNA element in enhancers of several Ras-transactivated genes; a rat b-Myc DNA (15) encoding the N-terminal DNA-binding domain of 120 amino acids, which acts as a c-Myc antagonist. Interestingly, none of these gene products binds directly Ras GTPases.

Any attempt with either the full-length GAPl or its C-terminal GTPase-activating domain (GAPlC, residues 709-1044) to reverse the malignant transformation caused by the oncogenic Ras mutants has been unsuccessful so far, although the GAPlC (but not the full-length GAPl) is able to reverse the malignant transformation caused by overexpression of normal Ras genes such as c-Ha-Ras (17). Shortly after the

* The abbreviation used is: GAP, GTPase-activating protein.
NF1-GRD of 338 amino acids, i.e. a GAP1C-related domain (GRD, residues 1194–1531) of NF1, which also activates the normal Ras GTPases (18, 19), was shown to bind Ras much more tightly than the GAP1C (6), and a similar, but slightly larger, NF1-GRD fragment was reported to reduce the v-Ha-Ras-induced heat-shock susceptibility of yeasts (20), we have started to examine the possible anti-v-Ha-Ras action of the NF1-GRD and its truncated mutants in mammalian cells.

In this paper, we demonstrate, for the first time, that either the NF1-GRD or its short fragment of 91 amino acids (NF91, residues 1441–1531) are overexpressed, each reverses the v-Ha-Ras-induced malignant transformation of NIH/3T3 cells. The NF91 is, so far, the smallest anti-tumor polypeptide that has ever shown the potent anti-v-Ha-Ras action in mammalian cells.

MATERIALS AND METHODS

Construction of the Plasmids Expressing NF1-GRD (in Sense and Anti-sense Orientations) and NF91 (in Sense Orientation)—For the NF1-GRD (NF338+ or NF338−) expression, a HindIII DNA fragment of 1.1 kilobases containing at 5′ end a Kozak sequence (GCC GCC ACC ATG) followed by codons 1194–1531 of human NF1 (type I) and at 3′ end a termination codon (TAA) was prepared by polymerase chain reaction and subcloned into the retroviral vector pMV7 (21). The orientation of the insert was determined by EcoRI digestion, as one EcoRI site is located 100 base pairs upstream of the HindIII insertion site and the other at the codon 1555 of the NF1 (for detail, see Fig. 1). For the NF91 expression, an EcoRI/HindIII polymerase chain reaction fragment of 0.5 kilobases containing at 5′ end the Kozak sequence followed by codon 1441–1531 of the human NF1 and at 3′ end the termination codon was prepared and inserted into the EcoRI/HindIII sites of the vector pMV7. The resultant plasmid called NF91 as well as the other two plasmids called NF338+ (sense) and NF338− (anti-sense) were purified by CsCl density gradient centrifugation and used for transfection.

Assay for Colony-Forming Ability in a Soft Agar of v-Ha-Ras-transformed Cells Expressing the NF1-GRD or v-Ha-Ras-transformed NIH/3T3 cells (22) were transfected with either NF338+, NF338−, or NF91 (1 μg) as complexes with liposomes (55 μg) as described previously (22). The resultant G418-resistant transfectants were cloned in the presence of 400 pg/ml G418 (a neomycin analogue) and screened for the presence of 50% formamide, and the filter was subsequently washed at 30 °C with 0.1 M formamide in the supernatant as described previously (22). The remaining probes on the filter were then scanned by a PhosphorImagerTM-400 series (Molecular Dynamics, California) or radioautographed.

Preparation of Recombinant NF1-GRD Mutants and Ras GTPase—By subcloning the corresponding polymerase chain reaction DNA fragments into the bacterial expression vector pGEX-2TH (22), a series of N- and C-terminal deletion mutants of the human type 1 NF1-GRD, human type II NF1-GRD (residues 1355–1531 plus the insertion of 21 amino acids), and murine c-Ha-Ras (GTPase (residues 1–189) were produced in Escherichia coli as glutathione S-transferase fusion proteins and affinity-purified as described previously (19, 22). The Assay for GAP (Ras GTPase-stimulating) Activity of the NF1-GRD Mutants—The hydrolysis of [γ-32P]GTP bound to the c-Ha-Ras GTPase was determined at 37 °C for 60 min in the presence or absence of each NF1-GRD mutant preparation in 100 μl of a buffer containing 50 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 2.5 mM EDTA, 3 mM ATP, 1 mM dithiothreitol, and 0.5 μg/ml bovine serum albumin, reciprocating the unhydrolyzed [γ-32P]GTP with charcoal, and, finally, measuring the radioactivity of the γ-32P inorganic phosphate in the supernatant as described previously (22). Protein concentrations were determined by the Bradford method with bovine serum albumin as standard (22).

RESULTS AND DISCUSSION

Anti-v-Ha-Ras Action of NF1-GRD in Mammalian Cells—To examine whether the NF1-GRD is able to reverse the oncogenic action of v-Ha-Ras in mammalian cells, we have subcloned a DNA encoding the NF1-GRD of 338 amino acids (residues 1194–1531) into the retroviral vector pMV7, in both sense (+) and anti-sense (−) orientations, which has the neomycin-resistant selectable marker. The detail of the resultant constructs called NF338+ and NF338− is shown in Fig. 1. The NF1-GRD was overexpressed under the control of the LTR promoter in the v-Ha-Ras-transformed NIH/3T3 cells in both orientations as described previously (22, 23). The transfectants were cloned in the presence of a neomycin analogue (G418), and the reversion of their transforming phenotypes, i.e. their morphological changes and loss or reduction of the soft agar colony-forming ability, were examined as described previously (22, 23). Unlike the normal NIH/3T3 cells, the majority of v-Ha-Ras-transformed cells expressing the sense NF1-GRD did not look very flat (data not shown).

However, overexpression of the sense NF1-GRD in the transfectants greatly reduced their soft agar colony-forming ability (Fig. 2 and Table I). The clones 22 and 8 formed only 3 (small size) and 37 (medium size) colonies, respectively, whereas the parental v-Ha-Ras-transformed cells formed 735 (large size) colonies. However, the clone 12, which overexpressed the anti-sense NF1-GRD, did not significantly differ from the parental cells in either their soft agar colony-forming ability (Table I) or morphology (data not shown). Interestingly, there appears
FIG. 2. NF1-GRD-dependent inhibition of v-Ha-Ras-induced colony formation in soft agar. i, nontransfected cells (clone 0); ii, NF1-GRD-expressing cells (clone 22). v-Ha-Ras-transformed NIH/3T3 cells were transfected with either NF338+ or NF338−, and the resultant G418-resistant transfectants were cloned as described under "Materials and Methods." Colony-forming ability of the parental clone and each transfectant was examined in a soft agar as described under "Materials and Methods."

TABLE I
Anti-oncogenic action of NF1-GRD and NF91 in v-Ha-ras-transformed NIH/3T3 cells

| Clone   | NF1-GRD or NF91* | Colonies/10^3 cells | SAC+ |
|---------|------------------|---------------------|------|
| 0       | None             | 0                   | 100  |
| 8       | GRD (sense)†     | 37 (medium)         | 5    |
| 22      | GRD (sense)‡     | 3 (small)           | 0.4  |
| 12      | Anti-sense‖      | 910 (large)         | 124  |
| 17      | NF91 (sense)§    | 0                   | 0    |
| 7       | NF91 (sense)‖    | 10 (small)          | 1.3  |

† The relatively high (H) or low (L) steady-state levels of NF1-GRD or NF91 mRNAs as judged by Northern blot (see Figs. 3A and 6A) are shown.
‡ The number of colonies formed in soft agar whose average size is indicated in parentheses (large, more than 100 cells; medium, approximately 25 cells; small, less than 10 cells/colony (see Fig. 2B).
§ The relative soft agar colony (SAC)-forming ability of each clone. Each presented value was the average of the data from three independent experiments, and the standard deviation in each case was less than 5%.

a good correlation between the sense NF1-GRD mRNA level and the extent of reduction in the soft agar colony-forming ability (Fig. 3A and Table I). As judged by Northern analysis, the NF1-GRD mRNA level of the clone 22 was much higher than that of the clone 8. Furthermore, Northern analysis of v-Ha-Ras mRNA confirmed that all transfectants still express the v-Ha-Ras as the parental cells (Fig. 3B), indicating that the reduction of their oncogenicity is not due to the loss of v-Ha-Ras expression but instead due to overexpression of the NF1-GRD. This is the first demonstration of the NF1-GRD's interference with the oncogenic action of v-Ha-Ras in mammalian cells.

How does the NF1-GRD interfere with the oncogenic action of v-Ha-Ras? Unlike the c-Ha-Ras GTPase, the v-Ha-Ras GTPase is not activated by the NF1-GRD. Thus, it is unlikely that the conversion of GTP/v-Ha-Ras to GDP/v-Ha-Ras is the basis for the anti-v-Ha-Ras action of the NF1-GRD. Instead, as the NF1-GRD binds Ras much more tightly than GAP1 (6), it is more likely that the NF1-GRD competitively inhibits the binding of GAP1 or other Ras targets to the v-Ha-Ras, and, therefore, blocks their oncogenic Ras-effector actions. It has been shown previously that either v-Ha-Ras-GAP1 complex or the N-terminal SH2 domain of GAP1 alone block the muscarinic K+ channels of atrial myocytes (26, 27), indicating that GAP1 serves as an effector of v-Ha-Ras, as well as an attenuator of the normal Ras signals.

FIG. 3. Expression of NF1-GRD (A) and v-Ha-Ras (B) judged by Northern blot. Lanes 1, nontransfected (clone 0); 2, sense NF338-transfected (clone 8); 3, sense NF338-transfected (clone 22); 4, anti-sense NF338-transfected (clone 12). The steady-state levels of both NF1-GRD and v-Ha-Ras mRNAs in the parental and transfectants were determined as described under "Materials and Methods" with Northern blots, which were monitored by a phosphorimager. kb, kilobases.

Interestingly, Arshavsky and Bownds (28) have recently demonstrated that another GTPase called transducin is activated by the γ-subunit of retinal rod cGMP phosphodiesterase, indicating that the phosphodiesterase-γ of only 87 amino acids serves as a transducin GTPase activating protein. This observation has raised a distinct possibility that, unlike the GAP1C, only a small region of the NF1-GRD might be sufficient to bind Ras GTPases and thereby stimulate the intrinsic GTPase activity of the normal Ras or interfere with the transforming ability of oncogenic Ras mutants. In an attempt to find the smallest Ras-binding fragment of the NF1-GRD, which would potentially be useful for treatment of Ras-associated cancers, as well as to more precisely understand the nature of Ras-NF1 interaction(s), we have recently begun to screen such a candidate among the short GTPase-activating fragments derived from the NF1-GRD.

The NF1 Residues Required for GTPase Activation—We have shown previously that the GAP1C, residues 720–1044 of bovine GAP1, is the minimal domain that is still able to activate Ras GTPases (22). Further deletion of either Tyr706 or the last 7 amino acids of the GAP1C completely abolished the Ras GTPase activation. Interestingly, the corresponding domain of NF1, residues 1205–1531 (NF327), is also able to activate Ras GTPases (18, 19). Neither GAP1 nor NF1 activates Rap1 or Rho GTPases. The specificity for both GAP1 and NF1 was predominantly determined by Glu60 of Ras GTPases, and the sensitivity to both GAP1 and NF1 was maximized by Glu60 (19).

2 H. Maruta, unpublished observation.
To determine the minimal GTPase-activating domain of the NF1 and further screen the smallest anti-oncogenic NF1 fragment, we have prepared a series of its N- or C-terminal deletion mutants (see Fig. 4) as glutathione S-transferase fusion proteins in E. coli as described previously (19, 22). As shown in Table II, the deletion of either its N-terminal 236 amino acids (residues 1205-1440) or its C-terminal 35 amino acids (residues 1497-1531) did not abolish the Ras GTPase activation, although these mutations significantly reduced the efficiency of the GTPase activation; the NF1 fragment of 91 amino acids (NF91, residues 1441-1531) still activates Ras GTPase but 20 times less efficiently than the NF1-GRD. However, the further deletion of the N-terminal 11 amino acids (residues 1441-1451) completely abolished the GTPase activation. These observations suggested that (i) the 56 amino acids (residues 1441-1496) of NF1 might be sufficient for GTPase activation, and (ii) some of the 11 amino acids (residues 1441-1451) are essential for its action.

Further, the glutathione S-transferase fusion protein containing only these 56 amino acids of NF1 (residues 1441-1496, NF56) was no longer able to activate the Ras GTPases (Fig. 4 and Table II). Instead, the slightly larger NF1 fragment of 78 amino acids (residues 1441-1518) linked to glutathione S-transferase was able to activate the Ras GTPase (Fig. 4 and Table II). These observations indicate that (i) the 78 amino acids, but not the 56 amino acids, of NF1 are sufficient for GTPase activation, and (ii) some of the 22 amino acids (residues 1497-1518) are functionally replaceable with the N-terminal 236 amino acids (residues 1205-1440) for the proper folding of NF56 or stability of its active site. This N-terminal domain alone did not show any GTPase activation (Table II), suggesting that its possible role is the structural rather than the functional.

Since the deletion of the N-terminal 236 amino acids significantly reduced the efficiency of the GTPase activation, we have been identifying the residue(s) that play the key role in maximizing the GTPase activation. Interestingly, deletion of Phe^{1206} (or its replacement by Ser) alone significantly reduced the GTPase activation of the NF327 (Fig. 4 and Table II), suggesting that the Phe^{1206} plays an important role in maximizing the GTPase activation. In this context, it is of interest to note that the corresponding residue (Tyr^{207}) of the GAP1C also appears to play the key role in the GTPase activation; although replacement of Tyr^{207} by either Phe or Leu does not affect the GTPase activation, either Glu^{207} or Gly^{207} mutations almost completely abolish the GTPase activation. These observations suggest that a hydrophobic residue (Tyr, Phe, or Leu) is required at position 720 of the GAP1C or at position 1205 of the NF1 for the full activation of the Ras GTPases. Further deletion of 235 amino acids (residues 1206-1440) from the NF326 also significantly reduced the GTPase activation (Fig. 4 and Table II), indicating that some of these amino acids are involved in stimulation of the NF91 and activation of the NF56, which alone is inactive.

It is of interest to note that the GTPase-activating NF1 fragment of 78 amino acids (NF78) shares only 19% sequence identity with the corresponding domain of GAP1, which is totally inactive. Surprisingly, this sequence identity is even lower than that (26%) between the active GAP1C (residues 720-1044) and the corresponding NF1-GRD. Furthermore, although the GAP1C is completely inactivated by either freezing/thawing or storage at 4 °C for 6 months, the GAP activity of both the corresponding NF1-GRD and even the NF78 is not significantly affected by these treatment/storage conditions. These observations suggest that the NF1-GRD and NF78 are significantly different from the corresponding domains of the GAP1 in either the overall conformation or stability of the active site.

**Anti-v-Ha-Ras Action of 91-Amino Acid Fragment Derived from NF1-GRD**—We have shown that deletion of the N-terminal 247 amino acids (residues 1194-1440) from NF1-GRD still allows the remaining domain of 91 amino acids (NF91) to activate the normal Ras GTPases, although it significantly reduced (20-fold) its GAP activity. This observation indicates that the NF91 still binds to the Ras GTPases. Thus, to examine whether the N-terminal 247 amino acids are required for the anti-oncogenicity of NF1-GRD against the v-Ha-Ras, we have overexpressed its Ras-binding domain of 91 amino acids (NF91) in the v-Ha-Ras-transformed NIH/3T3 cells by means of the vector pMV7. Unlike the parental cells, many NF91-transfected clones became morphologically

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**Table II**

| Name | Construct | Activation | EC_{50} | Name | Construct | Activation | EC_{50} |
|------|-----------|------------|--------|------|-----------|------------|--------|
| NF1-GRD | 1175-1531 | 14 | 0.50 | NF56 | 1441-1496 | 11 | 7.5 |
| NF342 | 1290 | 14 | 0.52 | NF292 | 1205-1496 | 11 | 7.5 |
| NF327 | 1205 | 14 | 0.40 | NF266 | 1175-1440 | 0 | |
| NF326 | 1206 | 11 | 1.5 | NF266 | 1175-1440 | 0 | |
| NF91 | 1441-1531 | 10 | 0.40 | NF266 | 1175-1440 | 0 | |
| NF80 | 1452-1518 | 10 | 0.40 | NF266 | 1175-1440 | 0 | |
| NF78 | 1441-1531 | 10 | 0.40 | NF266 | 1175-1440 | 0 | |
| NF56 | 1441-1496 | 11 | 7.5 | NF266 | 1175-1440 | 0 | |
| NF266 | 1175-1440 | 0 | | | | | |

* For detail of the constructs, see Fig. 4.

* Activation of Ras GTPase by 20 μg/ml/ml NF1-GRD mutants.

[For further details, please refer to the original paper.]
flat as the normal NIH/3T3 fibroblasts (Fig. 5). Some flat revertants, such as clone 17, were no longer able to form any colony in soft agar, while other flat revertants such as clone 7 formed only a few small colonies in soft agar (see Table I). These observations suggest that, like the NF1-GRD, the NF91 is anti-oncogenic toward the v-Ha-Ras-transformed NIH/3T3 cells. By Northern blot analysis, we have compared the steady-state levels of v-Ha-Ras and NF91 mRNAs between the two flat revertants and the parental cell line. As shown in Fig. 6, both flat revertants still expressed the v-Ha-Ras gene as the parental cell line, whereas only the flat revertants overexpressed the NF91 gene (slightly higher expression in clone 17 than clone 7). These observations clearly indicate that their loss or reduction of the malignant phenotype is due to overexpression of NF91 gene and not due to a loss of the v-Ha-Ras gene expression. Thus, the N-terminal 247 amino acids of NF1-GRD are not essential for its anti-oncogenicity toward the v-Ha-Ras. It is of interest to note that the NF91 is significantly smaller than the b-Myc N-terminal domain of 120 amino acids, which, so far, has been the smallest among the proteins that reverse v-Ha-Ras-induced malignancy (15).

Relationship between Anti-oncogenicity and GAP Activity of NF1—It is worth noting that, unlike the NF91 revertants, none of the NF1-GRD revertants is flat, although both revertants form little colony in soft agar, suggesting that the NF91 is more potent than the NF1-GRD as a v-Ha-Ras antagonist in this sense. Conversely, as a GAP (Ras GTPase-activating protein), the NF1-GRD is 20 times more active than the NF91 (see Table II). These observations have suggested, if not proved as yet, that the poorer as a GAP, the more potent as an anti-oncogene, as long as it still binds the v-Ha-Ras. Interestingly, deletion of the C-terminal 13 amino acids from the NF91 reduces its GAP activity by 2.5-fold (see Table II). Thus, it is of interest to examine whether the remaining domain of 78 amino acids (NF78) is more potent as a v-Ha-Ras antagonist than the NF91. More importantly, deletion of the C-terminal 35 amino acids (residues 1497–1531) from NF1-GRD still allows the remaining domain to activate the normal Ras GTPases (see Table II), suggesting that this C-terminal domain is not essential for the Ras binding of NF1-GRD. Unfortunately, the remaining domain of 56 amino acids (NF56) alone, produced by deletion of nonessential N- and C-terminal domains (residues 1194–1440 and 1497–1531), is no longer able to activate the Ras GTPases (see Table II). However, it is still conceivable that the NF56 does bind tightly the v-Ha-Ras and blocks the Ras oncogenicity, perhaps more strongly than the NF91 and NF78. Our preliminary results indicate that both NF78 and NF56 are anti-oncogenic as NF91 at the cell culture levels, suggesting that NF56 still binds v-Ha-Ras. We are currently examining whether these NF91/NF78/NF56-induced flat revertants develop tumors in nude mice or not.

The Role of Lys$^{1423}$ and the Type II-specific Insert of 21 Amino Acids Located Outside the GTPase-activating Domain of the NF1—It has been reported previously that replacement of Lys$^{1423}$ by either Glu or Gln in the NF1 almost completely abolishes its GTPase activation (29), suggesting that this residue plays an important role in GTPase activation. However, the Met$^{1423}$ mutation only converts the NF1 to a temperature-sensitive (ts) molecule but does not significantly affect the GTPase activation (18), suggesting that Lys$^{1423}$ may be involved in stabilization of the NF1, but is not essential for GTPase activation. We have demonstrated here that either NF78 or NF91 (residues 1441–1518 or 1531), which lack Lys$^{1423}$, are still able to activate Ras GTPases, clearly indicating that this Lys residue is located outside the GTPase-activating domain of the NF1.

It has been shown recently that there are at least two different NF1 isoforms (types I and II) in human cell lines (30). The type I is identical to that previously reported (4, 7), whereas the type II contains an additional insertion of 21 amino acids between residues 1370 and 1371 of the type I. Interestingly, the expression of the type II is associated with neuronal differentiation (30). In SH-SY5Y neuroblastoma cells, the type I is predominantly expressed. However, when the cells are treated with retinoic acid, the type I expression

\[ M. Varga, M. S. A. Nur-E-Kamal, and H. Maruta, unpublished observations. \]
is suppressed, and the type II expression is highly induced along the neuronal differentiation. These observations have implied the distinct roles of the types I and II isoforms of the NF1 in cell proliferation and neuronal differentiation, respectively. Our deletion analysis has revealed that this insertion in the type II occurs outside the GTPase-activating domain (NF78).

To examine further whether the insertion of 21 amino acids affects the GTPase activation, we have assayed the GAP activity of the NF1 fragment (residues 1355–1531) in the presence or absence of the insert. As shown in Fig. 7, the insertion resulted in only 50% reduction of the NF1-dependent c-Ha-Ras GTPase activation. Interestingly, like the type I NF1-GRD, the type II NF1-GRD is able to reduce the v-Ha-Ras-induced susceptibility of yeast to heat shock when it is overexpressed (31). Thus, it is not clear whether this insert alone is sufficient for playing any important role in its regulation of Ras GTPase activation or its anti-v-Ha-Ras action in vivo. It is also conceivable that its interaction with the N-terminal non-GTPase-activating domain of the NF1 or with other molecule(s) plays an essential role in the induction of neuronal differentiation. We are currently identifying the possible target(s) of the type II-specific insert.

Another NF1 cDNA has recently been cloned from rat, which encodes a third isoform of the NF1 (32). This isoform, called type III, is identical to both types I and II in the N-terminal half (residues 1–1370) but differs from type I, as it contains the additional insert of 21 amino acids as type II. The type III differs from type II as it lacks entirely the C-terminal half (residues 1371–2918) including the GTPase-activating domain (NF78). Thus, it is most likely that the type III is unable to activate Ras GTPases. It is intriguing to identify the possible function of the N-terminal non-GTPase-activating half of the NF1 (type III) in mammalian cells.

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FIG. 6. Expression of NF91 (A and C) and v-Ha-Ras (B and D) judged by Northern blot. T, v-Ha-Ras-transformed parental cells (clone D); R, 17, and T, flat revertants (clones 17 and T) derived from v-Ha-Ras-transformed cells transfected with the NF91. The steady-state levels of both NF91 and v-Ha-Ras mRNAs in the parental and revertant cells were determined as described under “Materials and Methods” with Northern blots, which were radioautographed. kb, kilobases.

FIG. 7. GAP activity of the NF1-GRD in the presence (O) or absence (●) of the type II-specific insert. The NF1-GRDs (residues 1355–1531) derived from type I (minus insert) or type II (plus insert) were affinity-purified as glutathione S-transferase fusion proteins, and their GAP activities were compared at indicated concentrations as described under “Materials and Methods.”
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