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# Suppression of oncogenic Ras by mutant neurofibromatosis type 1 genes with single amino acid substitutions

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**ABSTRACT** ***NF1* was first identified as the gene responsible for the pathogenesis of the human genetic disorder neurofibromatosis type 1. cDNA cloning revealed that its putative protein product has a domain showing significant sequence homology with the mammalian Ras GTPase activating protein and two yeast *Saccharomyces cerevisiae* proteins, Ira1 and Ira2. The Ras GTPase activating protein-related domain of the *NF1* gene product (NF1-GRD) stimulates GTPase activity of normal Ras proteins but not of oncogenic mutant Ras from both mammalian and yeast cells. Thus, in yeast, NF1-GRD can suppress the heat-shock-sensitive phenotype of *ira*$^-$ cells but not the same phenotype of activated *RAS* such as *RAS2*$^{Val19}$ and *RAS2*$^{Leu68}$. We have screened a pool of mutagenized NF1 expression plasmids and obtained two mutant NF1 cDNA clones that can suppress the heat-shock-sensitive phenotype of *RAS2*$^{Val19}$ cells. One clone (NF201) suppressed *RAS2*$^{Leu68}$, *RAS2*$^{Ser41}$, and *RAS2*$^{Val19}$, whereas another clone (NF204) preferentially suppressed *RAS2*$^{Val19}$. When expressed in mammalian cells, these mutant NF1-GRDs were able to induce the morphological reversion of v-*ras*-transformed NIH 3T3 cells. Both wild-type and mutant NF1-GRDs can stimulate the GTPase activity of normal but not transforming Ras. We suggest that mutant NF1-GRDs may bind tightly to transforming Ras, which stays in GTP-bound conformation, thus preventing the interaction with the putative effector molecule. On the other hand, normal Ras cannot be sequestered since the bound GTP is rapidly hydrolyzed upon interaction with mutant NF1-GRD to yield Ras·GDP, which is readily released from the NF1-GRD and recycled.**

Recent progress in molecular biology of human genetic disorders has identified a gene named *NF1* that is involved in neurofibromatosis type 1 disease (1–4). The *NF1* gene encodes a protein of 2818 aa sharing significant sequence homology with the members of Ras GTPase-activating protein (GAP) family (1–4), and the GAP-related domain of the *NF1* gene product (NF1-GRD) can stimulate the GTPase activity of Ras proteins (5–7).

Ras is a GTP-binding protein and serves as a molecular switch in signal transduction controlling the proliferation and differentiation of cells (8–10). Somatic mutations that activate Ras may contribute to tumorigenesis in up to 30% of human tumors (11, 12). Ras is active when it binds GTP, and the active Ras·GTP complex is converted to an inactive Ras·GDP complex by intrinsic GTPase activity (9, 10). The members of the GAP family, including NF1, stimulate the conversion from Ras·GTP to Ras·GDP, and negatively regulate the activity of Ras (13).

Ras is ubiquitously present among eukaryotic cells. Yeast *Saccharomyces cerevisiae* possesses two members of the Ras family (Ras1 and Ras2) that play an important role in cell growth through the regulation of adenylate cyclase (14, 15). Thus, yeast cells carrying activated mutations in Ras (such as [Val$^{19}$]Ras2 and [Leu$^{68}$]Ras2) are defective in responding to environmental conditions and show a variety of phenotypes including a heat-shock-sensitive phenotype (14, 15).

*S. cerevisiae* also possesses two NF1 homologs, Ira1 and Ira2, and human NF1 is structurally closer to yeast Ira than to human GAP (1–4). NF1 and Ira are also functionally related since NF1-GRD expressed in yeast cells can complement *ira*-deficient yeast. In *ira*$^-$ cells, the conversion of Ras·GTP to Ras·GDP is defective, and hence the cells show a heat-shock-sensitive phenotype that is very similar to that of activated Ras mutants (16, 17). NF1-GRD can suppress the heat-sensitive phenotype of *ira*$^-$, but not of *RAS2*$^{Val19}$ or *RAS2*$^{Leu68}$ (5–7). This is consistent with the fact that NF1-GRD stimulates GTPase activity of normal but not mutant Ras proteins.

In this study, we took advantage of the yeast Ras system to isolate NF1-GRD mutants that can act as antioncogenes specific for oncogenic Ras. Suppressors of the heat-shock phenotype of *RAS2*$^{Val19}$ were selected from mutagenized NF1-GRD plasmids in yeast cells, and two mutants with single amino acid substitutions were obtained. We demonstrate that these mutant NF1-GRDs, when expressed in mammalian cells, are able to induce the morphological reversion of v-*ras*-transformed NIH 3T3 cells.

## MATERIALS AND METHODS

**Yeast Strains and Medium.** The wild-type *S. cerevisiae* RAY-3A-D (*MAT***a**/*MATα ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade8/ade8*) and its isogenic strains, TK161-R2V-D (*RAS2*$^{Val19}$/*RAS2*$^{Val19}$) and KT63-2B-D (*ira2*$^-$/*ira2*$^-$) (16, 17), were used in this study. Transformants of NF1-GRD plasmids were grown in SDC medium, which contains 2% (wt/vol) dextrose, 0.67% yeast nitrogen base without amino acids (Difco), 0.5% Casamino acids (Difco), 50 mg of adenine sulfate per liter, and 50 mg of L-tryptophan per liter.

**Mammalian Cell Lines.** DT is a NIH 3T3-derived cell line transformed by two copies of a v-Ki-*ras* gene that carries both Ser-12 and Thr-59 mutations (18) and was obtained from M. Noda (Cancer Institute, Tokyo). COS-7 cells are simian cells expressing the simian virus 40 tumor antigen (19). Both cell lines were cultured in Dulbecco's modified Eagle's medium containing high glucose (4.5 g/liter) supplemented with 10% (vol/vol) fetal bovine serum (JRH Biosciences,

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Abbreviations: GAP, Ras GTPase activating protein; NF1-GRD, GAP-related domain of the *NF1* gene product.

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Lenexa, KS), 100 units of penicillin per ml, and 100 μg of streptomycin per ml.

**Plasmids.** Construction of pKP11 was as described (5), in which wild-type NF1-GRD was cloned into the yeast expression vector pKT10 containing the glyceraldehyde-3-phosphate dehydrogenase promoter (16). RAS2 plasmids, YCp-RAS2$^{Val19}$, YCp-RAS2$^{Leu68}$, and YCp-RAS2$^{Ser41}$, were as described (20). For the expression of NF1-GRD in mammalian cells, 1.7-kb cDNA fragments of wild-type and two mutant NF1-GRDs, named NF201 and NF204 (see *Results and Discussions*), were cloned into pEF-BOS, which is a mammalian expression vector containing human EF1-α promoter (21), and named pEF-NF1, pEF-NF201, and pEF-NF204, respectively. A rat cDNA encoding full-length GAP was also cloned into pEF-BOS to yield pEF-GAP (T. Satoh, K. Saitoh, and Y.K., unpublished material). pKrev-1 carries K*rev-1* cDNA and the neomycin-resistance gene under the control of the simian virus 40 early promoter (18).

**Mutagenesis and Screening of Mutant NF1-GRD in Yeast Cells.** pKP11 DNA (100 μg) was mutagenized by hydroxylamine *in vitro* as described (22) and transformed into the *RAS2$^{Val19}$* strain, TK161-R2V-D. About 2 × 10$^5$ colonies were grown on selection plates, and the plates were incubated at 57°C for 15 min. The resultant plates were incubated at 30°C for 4 days, and growing colonies were picked. The heat-shock sensitivity of these colonies was checked, and 12 clones were selected at this stage. Plasmid DNAs were recovered from these cells and retransformed into TK161-R2V-D. Phenotypic reversion was examined, and four plasmids were found to be positive. Among them, two clones, NF201 and NF204, were characterized in this study.

**Transfection of Plasmid DNAs into Mammalian Cells.** NF1-GRD plasmids were introduced into COS-7 cells by electroporation (23). DT cells were transfected by the calcium precipitation method exactly as described (18).

**Western Blot Analysis of NF1-GRD Proteins.** The NF1-GRD expression plasmid (20 μg) was transfected into 2 × 10$^6$ COS-7 cells by electroporation. After 3 days of culture, the cells were harvested and lysed in Laemmli's sample buffer. Lysates from 3.0 × 10$^5$ cells were subjected to Western blot analysis with an anti-NF1-GRD rabbit serum (24) by using an peroxidase-conjugated goat anti-rabbit IgG and the enhanced chemiluminescence system (ECL, Amersham; RPN2108).

**GTPase-Stimulating Activity of Wild-Type and Mutant NF1-GRDs.** Cell extracts were prepared from wild-type yeast cells, RAY-3A-D, carrying various NF1-GRD plasmids. The cells grown to the stationary phase were collected and disrupted with acid-washed glass beads (0.5-mm diameter) in buffer A [50 mM Tris·HCl, pH 7.4/100 mM KCl/5 mM $MgCl_2$/2 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/pepstatin A (10 μg/ml)/aprotinin (10 μg/ml)/leupeptin (10 μg/ml)]. The crude extract was clarified twice by centrifugation at 2000 × *g* for 20 min. Resultant supernatants were used for GAP assay. Recombinant [Gly$^{12}$]c-Ha-Ras or [Val$^{12}$]c-Ha-Ras proteins (25) were loaded with [γ-$^{32}$P]GTP (30 Ci/mmol; 1 Ci = 37 GBq) in a buffer [50 mM Tris·HCl, pH 7.4/50 mM KCl/1 mM $MgCl_2$/2.5 mM EDTA/bovine serum albumin (0.2 mg/ml)] at 30°C for 10 min. The reaction was stopped by the addition of $MgCl_2$ to the final concentration of 7 mM. Yeast cell extract was then mixed with an aliquot of the Ras·[γ-$^{32}$P]GTP mixture and incubated at 30°C. At the indicated times, an aliquot was filtered through a nitrocellulose membrane, and radioactivity retained on the membrane was measured. The final concentrations of proteins and Ras·[γ-$^{32}$P]GTP were 1 mg/ml and 11.5 nM, respectively.

## RESULTS AND DISCUSSION

To obtain mutant NF1-GRDs that can suppress the phenotype of activated Ras, we used a plasmid, pKP11, that expresses a domain of NF1 (aa 1063–1651; the numbers of amino acid residues are from ref. 26) and a yeast strain carrying the *RAS2$^{Val19}$* mutation. In a previous study, this plasmid was shown to suppress *ira2*$^-$ but not *RAS2$^{Val19}$* (5). The plasmid was randomly mutagenized by treatment with hydroxylamine *in vitro*, and a pool of mutagenized DNAs was transformed into *RAS2$^{Val19}$* cells. Subsequently, 2 × 10$^5$ colonies were screened for heat-shock resistance. Twelve positive colonies were obtained in the initial screening. Subsequently, two clones, NF201 and NF204, that had a relatively strong suppression activity for *RAS2$^{Val19}$* were selected and subjected to further analysis as described below.

Table 1. Suppression of the heat-sensitive phenotypes of various activated alleles of *RAS2* by mutant NF1-GRD

| NF1-GRD type | Suppression of heat-sensitive phenotype | | | |
|---|---|---|---|---|
| | *RAS2$^{Val19}$* | *RAS2$^{Leu68}$* | *RAS2$^{Ser41}$* | *RAS2$^{wt}$* |
| NF201 | +++ | +++ | ++ | +++ |
| NF204 | +++ | + | − | +++ |
| NF1 (wild-type) | − | + | − | +++ |

Wild-type *S. cerevisiae* RAY-3A-D, harboring a combination of RAS2 plasmids (YCp-RAS2$^{Val19}$, -RAS2$^{Leu68}$, and -RAS2$^{Ser41}$; ref. 20) and NF1-GRD plasmids, was subjected to heat-shock assay as described in Fig. 1. The ability of each NF1-GRD plasmid to suppress the heat-sensitive phenotype was scored as follows: +++, strong suppression; ++, intermediate suppression; +, weak suppression; −, no detectable suppression. The complementation activity in *ira2*$^-$ cells (KT63-2B-D; ref. 17), which reflects the activity of these NF1-GRDs on wild-type *RAS2* (*RAS2$^{wt}$*), was also scored and is shown.

We examined the effect of NF201 and NF204 on different alleles of activated *RAS2* in yeast cells (Table 1 and Fig. 1). [Val$^{19}$]Ras2 and [Leu$^{68}$]Ras2 mutants possess decreased GTPase activity (14, 15), and their slow GTPase cannot be stimulated by NF1-GRD (5–7). The [Ser$^{41}$]Ras2 mutation has been shown to abolish the stimulation of the GTPase activity of the mutant Ras by Ira or NF1-GRD (20). Wild-type NF1-GRD could weakly revert the phenotype of *RAS2$^{Leu68}$* but was totally ineffective on *RAS2$^{Val19}$* and *RAS2$^{Ser41}$*. Mutant NF201 suppressed the heat-shock-sensitive phenotype of all three alleles of *RAS2* examined in this study, including *RAS2$^{Val19}$*, *RAS2$^{Leu68}$*, and *RAS2$^{Ser41}$*. On the other hand, NF204 preferentially suppressed *RAS2$^{Val19}$* but not the other two alleles. These results indicate that NF201 and NF204 possess distinct properties as suppressors of activated Ras. Interestingly, these two mutant NF1-GRDs could suppress *ira2*$^-$ cells, in which the GTP-bound form of normal Ras is increased, to the same extent as wild-type NF1-GRD, suggesting that NF201 and NF204 retain the ability to stimulate GTPase activity of normal Ras (described below).

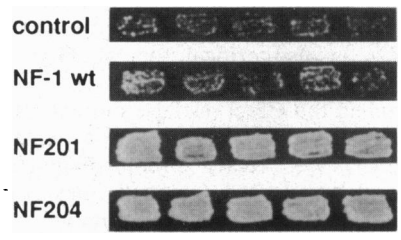


FIG. 1. Suppression of the heat-shock-sensitive phenotype of *RAS2$^{Val19}$* by mutant NF1-GRD. pKT10 (control vector), pKP11 (expressing wild-type NF1-GRD), and two mutant plasmids, NF201 and NF204, were transformed into the *RAS2$^{Val19}$* strain, TK161-R2V-D. Five transformants carrying each plasmid were freshly grown at 30°C for 24 h, and replica plated on a new plate. The plate was incubated at 52.5°C for 20 min, and subsequently, incubated at 30°C for 2 days. Heat-shock-resistant cells survived and grew on the plate. NF-1 wt, wild-type NF1-GRD.

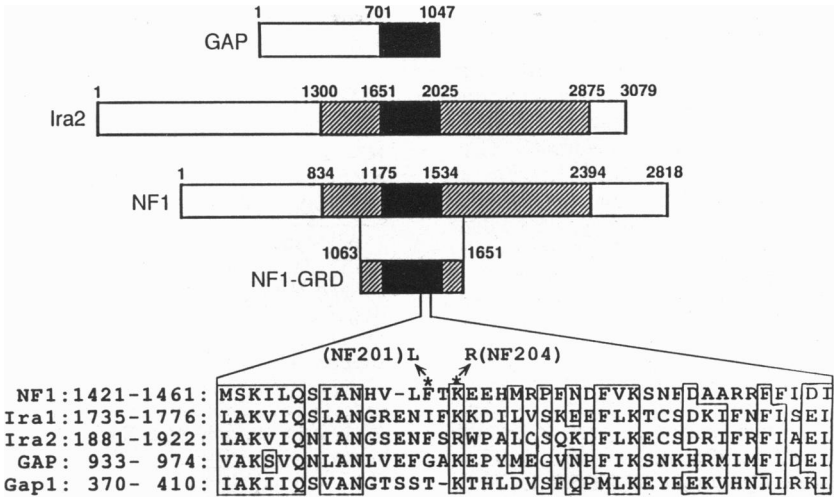


FIG. 2. Amino acid substitutions identified in mutant NF1-GRD. (*Upper*) Schematic representation of the structure of NF1 protein and NF1-GRD used in this study. The yeast plasmid pKP11 was designed to express a domain encompassing aa 1063–1651 of NF1. The solid box represents the region highly conserved among the members of the GAP family, and the flanking hatched boxes are the region showing extended homology between NF1 and Ira (5). (*Lower*) The amino acid sequence of a small region of NF1 that includes mutation sites of NF201 and NF204 is aligned with the homologous sequences of *S. cerevisiae* Ira1 and Ira2 (16, 17), human GAP (27), and a *Schizosaccharomyces pombe* GAP homolog, Gap1 (28). The positions of amino acid changes in NF201 and NF204 are marked with asterisks, and the residues found in the mutants are shown (see text for detail). Amino acid residues identical or conservatively changed in at least four out of five members of GAP family are boxed (29).

To identify mutations in NF201 and NF204, we sequenced the entire region of mutant NF1-GRDs and compared the sequences with that of wild-type NF1-GRD. In both NF201 and NF204, we found a single nucleotide change in the DNA sequences. In NF201, the codon TTC for Phe at residue 1434 was changed to TTA coding for Leu, and in NF204, the codon AAA for Lys at residue 1436 was replaced by AGA coding for Arg (Fig. 2). Although both mutation sites are located in one of the most conserved regions of the GAP-related domain (5–7), the amino acid residues at these sites (Phe 1434 and Lys 1436) are not strictly conserved among the members of the GAP family. Phe-1434 in NF1 is conserved in yeast Ira2 protein but is replaced by other residues in Ira1, GAP, and Gap1. On the other hand, Lys 1436 is conserved among NF1, Ira1, GAP, and Gap1, but Ira2 contains Arg at the corresponding site. It is noteworthy that two studies have demonstrated that Lys 1423 in NF1-GRD, which is located just 11 and 13 aa upstream of the mutation sites of NF201 and NF204, respectively, is also important for the structure and function of NF1. The Lys-1423 → Glu substitution has been identified in some human tumors and in a family of neurofibromatosis patients (30); the GAP activity of this mutant NF1-GRD was 200- to 400-fold lower than that of the wild-type NF1-GRD. A Lys-1423 → Met substitution in NF1-GRD resulted in decreased stability of the protein (31). Furthermore, Gln-1426 is also critical for the GAP activity of NF1-GRD (32).

We next studied the effect of these mutant NF1-GRDs in mammalian cells. The cDNA fragments of the wild-type and mutant NF1-GRDs were recloned into a mammalian expression vector and transfected into cell lines. We used an expression vector with human EF-1$\alpha$ promoter, which is one of the strongest promotors in mammalian cells, to express NF1-GRD at high levels. We checked the size of the NF1-GRD protein transiently expressed in COS-7 cells. Western blot analysis with an anti-NF1-GRD antiserum (24) identified a protein band with an apparent molecular mass of 67–68 kDa in the cells transfected with wild-type and mutant NF1-GRD plasmids but not with the control vector (Fig. 3). This indicates that a protein of ≈67 kDa was translated starting from the internal Met 1073 of NF1 cDNA and that the size of the mutant protein is indistinguishable from that of the wild-type protein.

To study the anti-Ras activity of mutant NF1-GRDs, we examined their effect on v-Ras-induced transformation. The above plasmids expressing NF1-GRD were cotransfected with pSV2neo into DT cells, a v-Ki-*ras*-transformed. NIH 3T3 derivative, and the ability to induce morphological reversion of the cells was examined. As shown in Table 2, transfection of the plasmids expressing NF201 and NF204 could induce flat reversion at considerably high frequencies (8–9% of total G418-resistant colonies). The frequency was even higher than that obtained by transfection of a K*rev-1* plasmid, which has been shown to possess antioncogenic activity in DT cells (18). Under the same conditions, the wild-type NF1-GRD could also induce flat reversion of DT

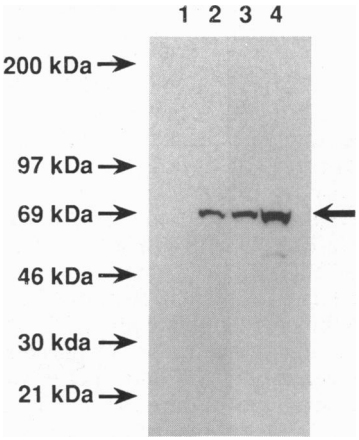


FIG. 3. Expression of wild-type and mutant NF1-GRDs in COS-7 cells. Each of NF1-GRD expression plasmids (20 $\mu$g) was transfected into COS-7 cells, and lysates from $3.0 \times 10^5$ cells were subjected to Western blot analysis with an anti-NF1-GRD antiserum (24). The samples were from the cells transfected with the following plasmids. Lanes: 1, pEF-BOS; 2, pEF-NF1; 3, pEF-NF201; 4, pEF-NF204. The 68-kDa band of NF1-GRD protein is indicated by an arrow on the right, and the positions of molecular size markers are shown on the left.

Table 2. Induction of morphological reversion of v-*ras*-transformed cells by mutant NF1-GRD

| Transfected DNA | No. flat colonies/no. G418-resistant colonies | | | Ratio, % |
|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 | |
| pEF-BOS | 0/1155 (<0.1) | 2/1279 (0.1) | 3/878 (0.4) | 0.1 |
| pEF-NF1 | 20/1522 (1.3) | 26/1151 (2.3) | 15/1004 (1.5) | 1.7 |
| pEF-NF201 | 86/1190 (7.2) | 61/691 (8.8) | 34/356 (9.6) | 8.0 |
| pEF-NF204 | 40/448 (8.9) | 46/426 (10.8) | 24/350 (6.9) | 9.0 |
| pEF-GAP | ND | 0/856 (<0.1) | 0/561 (<0.2) | <0.1 |
| pKrev-1 | ND | 26/1385 (1.9) | 15/736 (2.0) | 1.9 |

DT cells were cotransfected with 20 μg of NF1-GRD plasmids and 2 μg of pSV2neo as described (18), and transfectants were selected in a medium containing G418 (0.5 mg/ml). Since pKrev-1 plasmid (18) itself contained the neomycin-resistance gene, 2 μg of the plasmid was cotransfected with 20 μg of pEF-BOS (the vector for NF1-GRD). pEF-GAP contained rat full-length GAP cDNA in pEF-BOS. Frequency of reversion in DT cells is defined as the ratio (%) of morphologically flat cell colonies to total G418-resistant colonies. ND, not determined. Numbers in parentheses are percentages of the flat colonies in each experiment.

cells, although it was 5–6 times less potent than mutant clones. This is particularly interesting since a previous study has shown that overexpression of GAP inhibited normal c-Ha-*ras*- but not v-Ha-*ras*-induced transformation (33). We confirmed that no revertant of DT cells could be obtained from transfectants of the GAP plasmid (Table 2). This difference may be due to the fact that NF1-GRD possesses a much higher affinity for Ras proteins than GAP (6, 34) (discussed below). In any case, our results clearly demonstrate that mutant NF1-GRDs possess transformation-suppressor activity against oncogenic Ras.

To understand the molecular mechanism of the antioncogenic activity of mutant NF1-GRDs, we analyzed the biochemical properties of the mutant NF1-GRDs. Extracts were prepared from yeast cells expressing wild-type and mutant NF1-GRDs, and the GTPase-stimulating activity was measured *in vitro* by using recombinant c-Ha-Ras proteins as substrates. Two mutant NF1-GRDs, NF201 and NF204, stimulated the GTPase activity of [Gly$^{12}$]c-Ha-Ras to the same extent as wild-type NF1-GRD (Fig. 4). This is consistent with the observation that NF201 and NF204 can effectively complement *ira2*$^-$ in yeast (see Table 1). On the other hand, the same extracts were not able to stimulate the GTPase activity of [Val$^{12}$]c-Ha-Ras under our experimental conditions. This suggests that the antioncogenic activity of the mutant NF1-GRD is not due to the stimulation of the slow GTPase of oncogenic Ras proteins.

For a possible mechanism of antioncogenic activity of mutant NF1-GRD, we hypothesize that the mutant NF1-GRD may have higher affinity for Ras·GTP compared to the wild-type NF1-GRD. The so-called "effector region" of Ras, which is supposed to bind to putative downstream effector molecules, is also involved in the interaction between NF1-GRD and Ras (5, 6). Thus, if mutant NF1-GRD tightly binds to Ras, it would interfere with the interaction of Ras with its effectors, and therefore, Ras would be sequestered from signal transduction pathways. The following two observations support this hypothesis. As shown in Table 1, we observed weak but significant phenotypic reversion of *RAS2*$^{Leu68}$ by wild-type NF1-GRD. In a previous study (34), it was shown that the mammalian [Leu$^{61}$]Ras protein (corresponding to yeast [Leu$^{68}$]Ras2) has a much higher affinity for NF1-GRD than wild-type or [Val$^{12}$]Ras. The high-affinity binding between [Leu$^{68}$]Ras2 and wild-type NF1-GRD can explain the phenotypic suppression. Likewise, our hypothesis can also explain the differences in transformation-suppressor activities among GAP, wild-type NF1-GRD, and mutant NF1-GRDs. As shown in Table 2, wild-type NF1-GRD, but not GAP, can weakly suppress transformation by v-Ras; two mutant NF1-GRDs are more potent suppressors than wild-type NF1-GRD. This order of potency as transformation suppressors may reflect the relative affinity for Ras proteins; that is, wild-type NF1-GRD has 20 times higher affinity for Ras than does GAP (6); mutant NF1-GRDs may

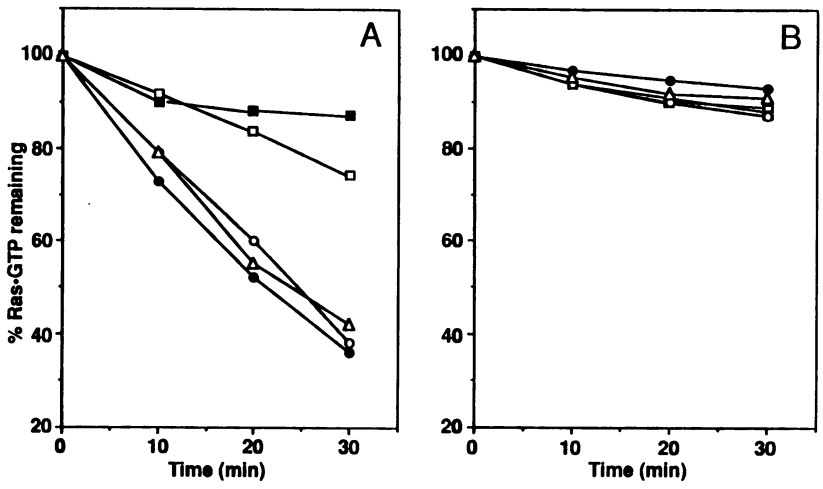


FIG. 4. Stimulation of GTPase activity of [Gly$^{12}$]c-Ha-Ras and [Val$^{12}$]c-Ha-Ras proteins by wild-type and mutant NF1-GRDs. Cell extracts were prepared from the wild-type yeast cells, RAY-3A-D, carrying various NF1-GRD plasmids and incubated with recombinant [Gly$^{12}$]c-Ha-Ras (*A*) or [Val$^{12}$]c-Ha-Ras (*B*) proteins loaded with [$\gamma$-$^{32}$P]GTP. At the indicated times, an aliquot was filtered through a nitrocellulose membrane, and radioactivity retained on the membrane was measured. The cell extracts assayed were from the cell carrying the following plasmids: ●, wild-type NF1-GRD; ○, NF201; △, NF204; ■, vector (pKT10) alone; □, buffer A plus bovine serum albumin (1 mg/ml).

have even greater affinities. In relation to this, it is noteworthy that Ballester *et al.* (7) observed the inhibitory effect of wild-type NF1-GRD but not of GAP on [Val$^{12}$]c-Ha-Ras expressed in yeast cells. This is consistent with our observation that wild-type NF1-GRD, but not GAP, can weakly suppress v-Ras-transformation in mammalian cells (Table 2).

However, a recent study has reported that a C-terminal fragment of GAP inhibited the transactivation of a Ras-responsive promoter by [Val$^{12}$]c-Ha-Ras in Chinese hamster ovary cells (35). In the present study, we compared only the full-length GAP and a truncated NF1 (NF1-GRD). Thus, it may be also possible that the difference in transformation-suppressor activity between GAP and NF1 is partly due to the effect of the truncated *NF1* gene. In any case, it is clear that the two mutant NF1-GRDs, which possess only single amino acid substitutions, are more potent suppressors of v-*ras*-induced transformation than wild-type NF1-GRD. The second observation supporting our hypothesis is that NF201 can suppress the activity of not only $RAS2^{Val19}$ and $RAS2^{Leu68}$ but also $RAS2^{Ser41}$. The [Ser$^{41}$]RAS2 mutation (corresponding to Ser-34 of human Ras), which is located in the effector region, disrupts the effective binding of Ras2 proteins to yeast Ira proteins as well as NF1-GRD and GAP (20). Thus, the fact that NF201 can suppress the phenotype of $RAS2^{Ser41}$ suggests that the mutation in NF201 may restore the interaction between [Ser$^{41}$]Ras2 and NF1-GRD.

In summary, we have shown that NF1-GRDs with single amino acid substitutions can suppress the biological activity of oncogenic Ras. These mutant NF1-GRDs appear to inhibit specifically oncogenic Ras. For normal Ras·GTP, bound GTP is rapidly hydrolyzed to GDP upon interaction with NF1-GRD; the mutant NF1-GRD would be readily released from Ras·GDP, which can go to the normal cycling; and the interaction with the effector molecule can take place. In this study, we expressed the mutant NF1-GRD as a protein of 578 aa, which is still a substantially large protein. By using the yeast screening system described above, it should be possible to determine the minimum fragment length of NF1-GRD that still retains the antioncogenic activity. This line of study should provide useful information for a better understanding of the molecular mechanism of the Ras–NF1 interaction and for developing Ras-specific antioncogenic compounds.

**Note Added in Proof.** Patrick Poullet and Fuyuhiko Tamanoi (personal communication) have recently isolated several intragenic suppressor mutants to the NF1 Lys-1423 mutant by using a yeast assay system similar to ours. One of their mutants had the same Phe-1434 → Leu substitution as in our NF1 mutant.

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