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Functional Significance of Lysine 1423 of Neurofibromin and Characterization of a Second Site Suppressor Which Rescues Mutations at This Residue and Suppresses *RAS2*$^{Val-19}$-Activated Phenotypes

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Lysine 1423 of neurofibromin (neurofibromatosis type I gene product [NF1]) plays a crucial role in the function of NF1. Mutations of this lysine were detected in samples from a neurofibromatosis patient as well as from cancer patients. To further understand the significance of this residue, we have mutated it to all possible amino acids. Functional assays using yeast *ira* complementation have revealed that lysine is the only amino acid that produced functional NF1. Quantitative analyses of different mutant proteins have suggested that their GTPase-activating protein (GAP) activity is drastically reduced as a result of a decrease in their Ras affinity. Such a requirement for a specific residue is not observed in the case of other conserved residues within the GAP-related domain. We also report that another residue, phenylalanine 1434, plays an important role in NF1 function. This was first indicated by the finding that defective NF1s due to an alteration of lysine 1423 to other amino acids can be rescued by a second site intragenic mutation at residue 1434. The mutation partially restored GAP activity in the lysine mutant. When the mutation phenylalanine 1434 to serine was introduced into a wild-type NF1 protein, the resulting protein acquired the ability to suppress activated phenotypes of *RAS2*$^{Val-19}$ cells. This suppression, however, does not involve Ras interaction, since the phenylalanine mutant does not stimulate the intrinsic GTPase activity of RAS2^{Val-19} protein and does not have an increased affinity for Ras proteins.

Neurofibromatosis type I is a genetic disease of the peripheral nervous system that afflicts one in 3,500 people worldwide (14). The *NF1* gene encodes a protein of 2,818 residues (9). Alternatively spliced messages have been detected (1, 3, 13). An established biochemical activity of neurofibromin is to stimulate intrinsic GTPase activity of Ras proteins (GTPase-activating protein [GAP] activity) (2, 10, 20). This activity cannot stimulate the intrinsic GTPase activity of activated Ras proteins. The region responsible for this activity is an approximately 360-residue region called the GAP-related domain (GRD) which is present in all Ras-GAP proteins identified. An extended region of homology outside the GRD is detected between neurofibromin and its *Saccharomyces cerevisiae* counterparts, IRA1 and IRA2 proteins (4, 21). We have previously shown that the IRA2 protein exhibits GAP activity and functions to downregulate yeast RAS proteins (17). Furthermore, NF1 can complement the heat shock sensitivity of yeast *ira* mutants, suggesting functional similarities between the two proteins.

Within the GRD, four regions, termed boxes I to IV, contain residues that are conserved among different Ras-GAP proteins, which include NF1, p120-GAP, *S. cerevisiae* IRA1 and IRA2 proteins, and *Schizosaccharomyces pombe* Sar1 protein (2, 18). *Drosophila* Gap1 protein also contains these boxes (5). Among these, two regions, boxes III and IV, located toward the C-terminal side of the GRD, contain a high percentage of conserved residues. In fact, there are 14 perfectly conserved amino acids within the GRD, and 10 out of the 14 are found in these two boxes. A sequence, FLR...PA, located between residues 1389 and 1396 in box III, is highly conserved. The significance of these boxes has been investigated (6).

The importance of GAP activity for the function of NF1 has been underscored by the finding of mutations within the GRD in samples obtained from a neurofibromatosis patient as well as from tumors of several cancer patients (8). These mutations changed a conserved lysine at residue 1423 of neurofibromin to glutamic acid or glutamine. The authors reported that the GAP activity was drastically reduced by these mutations but the affinity for Ras protein was unaffected. An alteration of this lysine residue to methionine has been reported to affect the stability of the protein (19).

To gain further understanding of the significance of the lysine 1423 residue and to investigate the GRD domain, we have addressed the specificity of lysine 1423 by replacing this residue with all possible amino acids. Our results point to the importance of the lysine residue for the NF1 function. We also report the detection of a second site suppressor which rescued the loss of function in the lysine mutant. This mutation, in wild-type NF1, suppressed the activated phenotype of *RAS2*$^{Val-19}$ mutants.

MATERIALS AND METHODS

Materials and strains. [γ-^{32}P]GTP (5,000 Ci/mmol; 1 mCi = 37 MBq) and deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate (1,200 Ci/mmol) were purchased from Amersham. A nonhy-

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drolyzable analog of GTP, GMP-PCP (guanylyl (β,γ-methylene)-diphosphonate), was purchased from Boehringer Mannheim. The AmpliTaq DNA polymerase from Perkin Elmer Cetus was used for PCRs. The sequencing reactions were performed by using the Sequenase version 2.0 kit from United States Biochemical Corp. *S. cerevisiae* KT27-2D (*MATα ura3 leu2 his3 trp1 ira1::LEU2*), *S. cerevisiae* TK161-R2V (*MATa leu2 ura3 trp1 his3 ade8 can1 RAS2*$^{Val-19}$), and *Escherichia coli* HB101 were used.

Mutagenesis of the *NF1* gene. Site-specific mutagenesis was carried out by overlap extension, using the PCR technique (7). For this purpose, a DNA fragment encoding the 3′ half of the GRD (codons 1354 to 1538 of the *NF1* gene) was amplified. The 5′ sense primer, TCCTCAGAATTCCAGT GTGTGTATC, contains an *Eco*RI site. Two different 3′ antisense primers, ATCAATCACAGCTGCCAGTGTGTA TC and ATCAATAAGCTTCCAGTGTGTTATC, containing *Pvu*II and *Hin*dIII sites were used for yeast and *E. coli* constructs, respectively. The overlapping oligonucleotides harboring the appropriate mutation contained the sequence AGTTAATGTCNNNGATACTTCAGAG and its complementary sequence. The codon NNN varied with the mutations. It was GAG and CAG for the substitutions of lysine 1423 to glutamic acid and lysine 1423 to glutamine, respectively. For the alteration of lysine 1423 to all possible amino acid residues, a random sequence was used for this codon. In the case of the single (F1434S) and double (K1423R/F1434S) mutant proteins, this codon encoded the lysine (AAG) and the arginine (AGG) residues, respectively, and the DNA from the double mutant K1423S/F1434S was used as a template. The mutated fragments were cloned in the *Eco*RI-*Pvu*II and *Eco*RI-*Hin*dIII sites of pKP22 and pGNF1, respectively, for yeast and *E. coli* expression (20). The pKP22 vector contains the yeast glyceraldehyde 3-phosphate dehydrogenase promoter. All of these constructs express the GRD peptide corresponding to the region between residues 1172 and 1538 of NF1. pGNF1 constructs express glutathione *S*-transferase (GST) fusion proteins in *E. coli*.

Screen using the heat shock sensitivity of an *ira1* mutant. The mutant NF1-GRD library was transformed into *ira1* mutant KT27-2D. Transformants were transferred to a new plate and subjected to heat shock treatment (53°C, 30 min). Heat shock-resistant transformants were subjected to another cycle of heat shock treatment. Positive clones were further analyzed by growth on a plate containing 5-fluoroorotic acid. Cells growing on this plate should have lost their plasmids and become heat shock sensitive. Plasmid DNAs from the heat shock-resistant and heat shock-sensitive groups were recovered by transfection into *E. coli*, and their sequences were determined.

Production and purification of GST-NF1 and Ras proteins. *E. coli* HB101 cells harboring the appropriate NF1-GRD constructs were induced for 16 h at 25°C. The low temperature was used to improve solubility of mutant NF1 proteins. The cells were collected and lysed with lysozyme, lubrol, and DNase I. The fusion proteins were then purified to near homogeneity by using glutathione beads as described previously (20) except that the elution was carried out for 5 min twice at room temperature. The purified proteins were stored in 50% glycerol at −20°C. No significant loss of activity was detected during the course of the experiments (2 months). H-ras and RAS2^{Val-19} proteins were obtained as previously described (20).

GAP assay and determination of Ras affinity. GAP activity against H-ras and RAS2^{Val-19} proteins was determined by using a filter assay as described previously (20). Briefly, 25 nM Ras protein prebound with [γ-^{32}P]GTP was incubated at 30°C with the indicated concentrations of NF1 protein, and the radioactivity which remained bound to Ras was determined by using nitrocellulose filters.

Two methods were used to determine Ras affinity. In the first method, H-ras protein complexed with a nonhydrolyzable GTP analog, GMP-PCP, was used to competitively inhibit the GAP activity of NF1 essentially as described previously (15). Briefly, 5 nM H-ras protein with prebound [γ-^{32}P]GTP was incubated with NF1 protein in the presence of increasing concentrations of H-ras complexed with GMP-PCP. When indicated, RAS2^{Val-19} protein bound to GTP was also used as a competitor. The GAP activity was measured by using nitrocellulose filters after a 15-min incubation at 30°C. For the K1423E mutant protein, which did not have detectable GAP activity, we used an alternative method. In this case, increasing concentrations of K1423E mutant protein were added to the wild-type NF1 GAP assay to determine whether the mutant protein inhibited the GAP activity of the wild-type NF1 by binding to the H-ras protein.

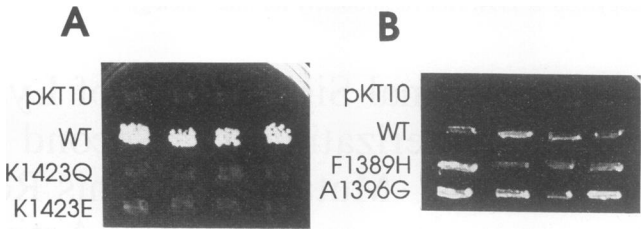
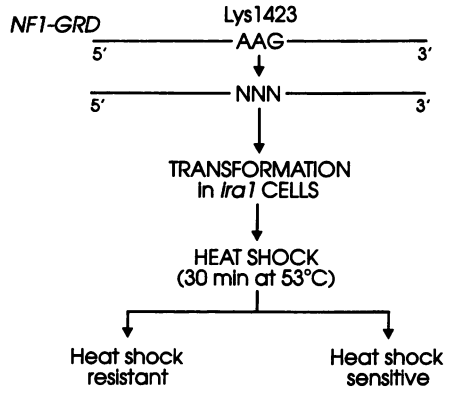


FIG. 1. Suppression of the heat shock sensitivity of *ira1* cells by NF1-GRD mutations. *ira1* yeast cells (KT27-2D) were transformed with various constructs containing the indicated NF1-GRD. pKT10 is a control vector with no NF1. The transformants were selected on uracil-deficient synthetic complete medium plates (17) and heat shocked for 30 min at 53°C. The cells were then allowed to grow for 3 days at 30°C. (A) Mutations at lysine 1423; (B) mutations in the FLR...PA region. WT, wild type.

RESULTS

Complementation of *ira1* mutants. To address the specificity for lysine 1423, we randomly mutagenized this residue and tested for function in the yeast system. The test was based on complementation of the heat shock-sensitive phenotype of yeast *ira* mutants. Figure 1 shows that a functional test can be performed. In this experiment, we prepared constructs of NF1-GRD (residues 1172 to 1538 of NF1) under the control of the glyceraldehyde 3-phosphate dehydrogenase promoter. Lysine 1423 was changed to glutamic acid or glutamine by site-directed mutagenesis. These mutations have been detected in samples from a neurofibromatosis patient as well as in tumors from cancer patients and are known to reduce GAP activity significantly (8). These constructs were transformed into yeast *ira1* mutant cells, and the transformants were subjected to heat shock treatment. As can be seen in Fig. 1A, both K1423Q (substitution of lysine 1423 with glutamine) and K1423E mutations were incapable of suppressing the heat shock sensitivity of the *ira1* mutant. In contrast, wild-type NF1 efficiently suppressed the heat shock sensitivity. The negative control was pKT10, the vector which contained no NF1. Western blotting (immunoblotting) with an anti-NF1 antibody revealed



Functional	Nonfunctional
K	R, E, D, Q, G, C, S, T, Y, A, L, P, V, F, I, M*, N*, H*, W*

FIG. 2. Strategy used for the search of functional residues at position 1423. See Materials and Methods for details. Plasmid DNAs from approximately 60 clones were sequenced. Methionine, asparagine, histidine, and tryptophan mutations, not found during the screening, were created in vitro, and their functionality was assayed by heat shock treatment.

that the levels of K1423Q and K1423E proteins were comparable to that of the wild-type protein (data not shown).

The lack of complementation by these NF1 mutants is in striking contrast to the effect of changing other residues which are also conserved in the GRD. Residues within the stretch of amino acids FLR...PA were changed by site-directed mutagenesis and tested for the ability to complement the heat shock sensitivity of an *ira1* mutant. As shown in Fig. 1B, a construct with phenylalanine 1389 changed to histidine as well as a construct with alanine 1396 changed to glycine still complemented the heat shock sensitivity of the *ira1* mutant. In fact, most amino acid changes of the conserved residues within this stretch did not affect their ability to complement the *ira1* mutant (data not shown) when a strong promoter, the yeast glyceraldehyde 3-phosphate dehydrogenase promoter, was used for the expression of these mutant constructs.

Random mutagenesis of lysine 1423. Using the assay described above, we carried out random mutagenesis of lysine 1423. The experimental scheme is outlined in Fig. 2. Briefly, oligonucleotides with random sequences at the lysine 1423 codon were used to prepare an NF1 plasmid library. The library was transformed into *ira1* cells, and the transformants were separated into two groups, a heat shock-sensitive group and a heat shock-resistant group. The heat shock-sensitive group contains nonfunctional NF1, whereas the heat shock-resistant group contains functional NF1. Heat shock-resistant transformants were further tested by another cycle of heat shock treatment. In addition, treatment with 5-fluoro-orotic acid was carried out to confirm that the complementation was due to the presence of the plasmids. Plasmid DNAs from these groups were recovered, and their sequences were determined. This resulted in the detection of 16 different amino acids. Four mutant constructs which were not found through this process, methionine, histidine, asparagine, and tryptophan, were made in vitro, and their abilities to suppress the heat shock sensitivity of the *ira1* mutant were examined. The results of these experiments are summarized in Fig. 2. It is clear that lysine is the only amino acid that produced a functional NF1.

TABLE 1. Biochemical properties of NF1-GRD proteins mutated at conserved residues

NF1 protein	GAP activity (nM)[a]	Affinity (K_d, nM)[b]
Wild type	1.3	40
K1423Q	520	>5,000
K1423E	>1,000	>5,000
K1423S	850	>5,000
K1423R	160	>5,000
F1389H	16.3	500
A1396G	5.5	140

[a] Concentration of GST-NF1-GRD required for hydrolysis of 50% of the bound GTP in 15 min. GAP assays were carried out as described in Materials and Methods for 15 min, using increasing concentrations of NF1. The intrinsic GTPase of H-ras was subtracted, and the concentration of NF1 required for hydrolysis of 50% of the bound GTP was determined graphically (see Fig. 4 for an example).

[b] Concentration of competitor (H-ras–GMP-PCP) necessary for 50% inhibition of NF1 activity.

Quantitative comparison of the biochemical activities of lysine 1423 mutant proteins. To quantitate the GAP activities of various lysine 1423 mutant proteins, the proteins were fused with GST and purified after their expression in *E. coli*. Their GAP activities against H-ras protein are summarized in Table 1. Although all these mutant proteins exhibited greatly reduced GAP activity compared with the wild-type protein, significant differences were still observed. The GAP activity of the arginine mutant was reduced the least (125-fold compared with the wild-type protein), whereas that of the glutamic acid mutant was reduced the most. The GAP activities of the glutamine and serine mutants were between these two values.

We observed a correlation between the decrease in the GAP activities of these mutant proteins and a decrease in their affinities for Ras proteins. Ras affinity was determined by addition of Ras protein complexed with a nonhydrolyzable GTP analog to the reaction. This complex acts as a competitive inhibitor with a K_i identical to the K_d of the GAP reaction (see Materials and Methods). As shown in Table 1, the Ras affinities of all of these mutants were more than 100-fold lower than that of wild-type NF1. Because of the limitation for the highest concentration of competitor added, a minimum K_d value is presented here.

In contrast to the mutants of lysine 1423, the mutants of FLR...PA still retained a significant level of GAP activity. As shown in Table 1, these mutant NF1s had GAP activities and Ras affinities 4- to 12-fold lower than those of the wild-type protein, consistent with the yeast complementation results.

Detection of a second site mutation at residue 1434 which rescues defective NF1. In the course of the analyses of amino acid changes at lysine 1423, we noticed that there were second site suppressors included within the functional group. These suppressors still contained mutations at lysine 1423 but had an additional mutation. We identified three such suppressors: K1423S/F1434S (changes of lysine 1423 to serine and phenylalanine 1434 to serine), K1423V/F1434S, and K1423G/F1434L. It is interesting to note that all three

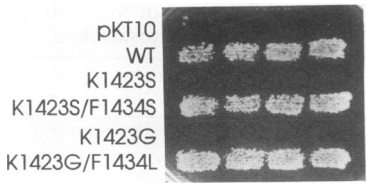


FIG. 3. Rescue of lysine 1423 substitutions by mutations at phenylalanine 1434. *ira1* yeast cells (KT27-2D) were transformed with plasmids containing the indicated NF1-GRD constructs and tested for the ability to survive a heat shock treatment (30 min at 53°C) as described for Fig. 1. WT, wild type.

suppressors contain an additional mutation at residue 1434. The additional mutations are likely to be caused by the PCR used during the library construction. Complementation of the heat shock sensitivity of the *ira1* mutant by two of these suppressors is shown in Fig. 3. As can be seen, the K1423S single-mutant construct was incapable of complementing the heat shock sensitivity of the *ira1* mutant, whereas the K1423S/F1434S double-mutant construct provided complementation. Similarly, we observed no complementation with K1423G, whereas K1423G/F1434L complemented the heat shock sensitivity of the *ira1* mutant.

To understand the biochemical basis of this second site suppression, we compared GAP activities of the single- and

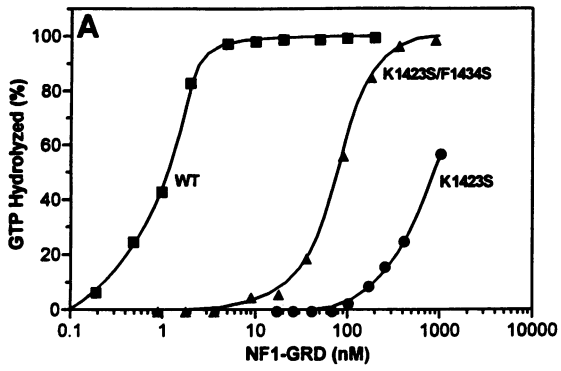


FIG. 4. Comparison of GAP activities of various NF1 single- and double-mutant proteins. GAP assays were performed as described in Materials and Methods for 15 min with the indicated NF1 proteins. The amount of GTP remaining on H-ras in a control with no NF1 was taken as the reference (100%). WT, wild type.

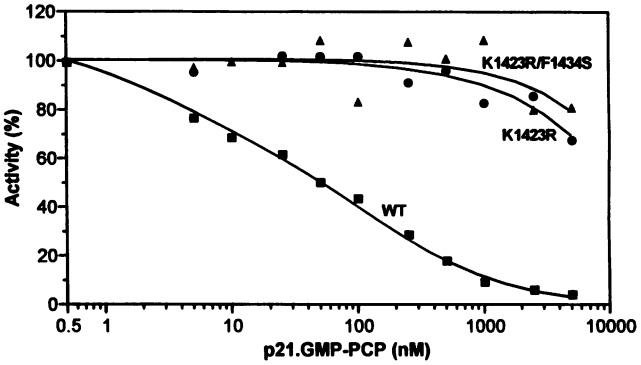


FIG. 5. Affinities of NF1-GRD single-mutant (K1423R) and double-mutant (K1423R/F1434S) proteins for H-ras. The assays were performed as described in Materials and Methods. The concentrations of NF1 proteins were chosen according to their relative activities to allow no more than 30% of GTP hydrolysis. Under this condition, the percentage of GTP hydrolyzed in 15 min stays linearly proportional to the concentration of active NF1 protein in solution. Concentrations of NF1 protein used are as follows: wild-type NF1 (WT), 0.7 nM; K1423R, 90 nM; and K1423R/F1434S, 15 nM. H-ras complexed with GMP-PCP was added to the reaction at the indicated concentrations, and the inhibition of NF1-GAP activity was monitored.

double-mutant proteins. Wild-type NF1 as well as K1423S and K1423S/F1434S were fused with GST and purified after their expression in *E. coli*. Their GAP activities against H-Ras protein were compared. Results are shown in Fig. 4A. The K1423S mutation resulted in an approximately 600-fold reduction in the GAP activity of the wild-type protein. The additional F1434S mutation increased the GAP activity approximately 13-fold. Thus, the second site mutation results in partial restoration of the GAP activity of NF1. Similar results were obtained when another set of mutants was examined (Fig. 4B). Mutation of lysine 1423 to arginine resulted in a 125-fold decrease in GAP activity. Introducing an additional mutation of phenylalanine 1434 to serine resulted in an approximately 6-fold increase of GAP activity, so that the GAP activity of the double mutant was about 20-fold lower than that of the wild-type protein. Although the GAP activity is not fully restored to the wild-type level, this is nonetheless sufficient for the complementation of the heat shock sensitivity of *ira1* mutant cells (see Discussion).

Ras affinities of these mutant proteins were also examined, using H-Ras protein complexed with GMP-PCP. Figure 5 shows the results obtained with K1423R and K1423R/F1434S. The Ras affinity of the K1423R protein was greatly reduced compared with that of the wild-type protein. This decreased affinity was not rescued by the additional mutation at phenylalanine 1434. Similar results were obtained with the K1423S/F1434S double-mutant protein (data not shown). These results suggest that the mutations at phenylalanine 1434 affect GAP activity rather than Ras affinity.

Mutations at phenylalanine 1434 suppress activated *RAS2* phenotypes. Next we examined whether mutations at residue 1434 affect the function of wild-type NF1. Phenylalanine 1434 was changed to serine by in vitro mutagenesis, and the resulting NF1-GRD construct was transformed into yeast cells expressing the wild-type *RAS2* gene. F1434S transformants grew more slowly than the transformants with wild-type NF1 (Fig. 6A). Thus, the expression of F1434S protein is growth inhibitory.

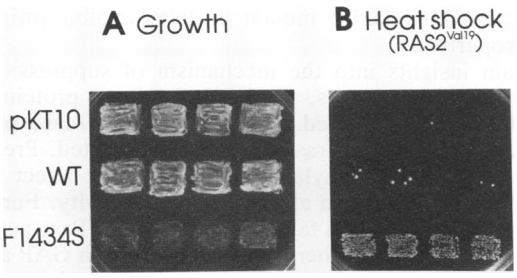


FIG. 6. Biological effects of the F1434S mutation. (A) Growth inhibition on a yeast strain containing a wild-type *RAS2* gene. Yeast strain KT27-2D was transformed with the indicated plasmids, and the transformants were directly plated on uracil-deficient synthetic complete medium plates and allowed to grow for 3 days. WT, wild type. (B) Suppression of the $RAS2^{Val-19}$-activated phenotype. Yeast strain TK161-R2-V containing an activated *RAS2* gene ($RAS2^{Val-19}$) was transformed with various NF1 constructs and heat shock treated as described for Fig. 1.

Yeast cells expressing the $RAS2^{Val-19}$ gene exhibit activated *ras* phenotypes which include heat shock sensitivity. Interestingly, the NF1-F1434S mutation can suppress the activated phenotype of $RAS2^{Val-19}$ cells (Fig. 6B). $RAS2^{Val-19}$ cells transformed with the wild-type NF1 construct or the vector pKT10 failed to survive a 15-min treatment at 53°C. In contrast, $RAS2^{Val-19}$ cells transformed with the F1434S construct survived 30 min of the treatment. Although this NF1 mutant shows a growth-inhibitory effect on wild-type cells, no significant inhibition of growth was seen with $RAS2^{Val-19}$ cells.

Effects of the mutations at phenylalanine 1434 on GAP activity and Ras affinity were examined after purification of the F1434S mutant protein. No significant difference of GAP activity was detected between the wild-type and F1434S mutant proteins. Furthermore, the two proteins showed similar affinities for the wild-type H-ras protein (data not shown). We also examined GAP activity and affinity for $RAS2^{Val-19}$ protein. Figure 7A shows the results of the GAP activity on $RAS2^{Val-19}$ protein. As can be seen, no significant stimulation of the low intrinsic GTPase activity of $RAS2^{Val-19}$ protein was detected when either wild-type or mutant NF1 protein was added. In addition, no significant difference of affinity for $RAS2^{Val-19}$ protein was observed between the two NF1 proteins (Fig. 7B). Thus, the K1434S mutation does not affect NF1-Ras interaction in vitro.

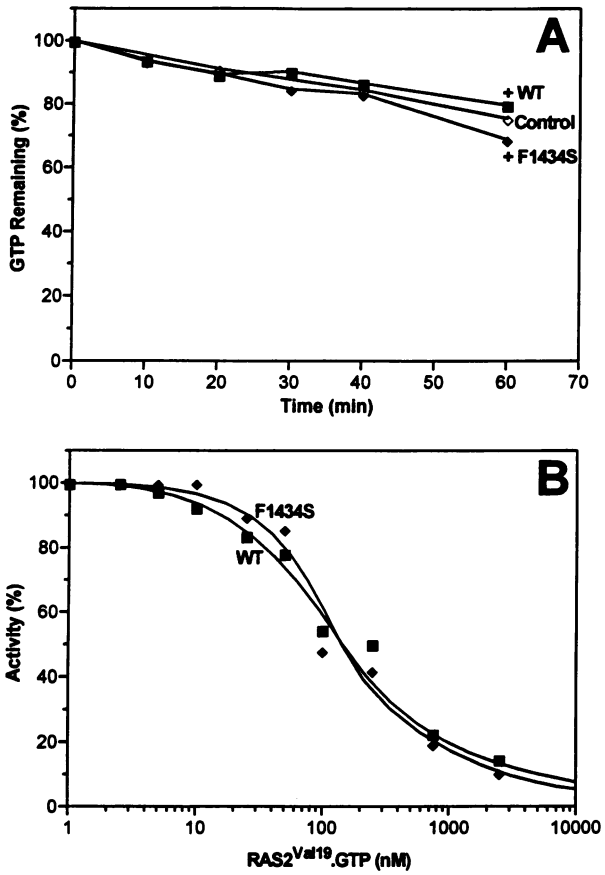


FIG. 7. Effect of F1434S on $RAS2^{Val-19}$. (A) GAP activity. The assay was carried out as described in Materials and Methods, using $RAS2^{Val-19}$-GTP as a substrate. No NF1 (control) or the indicated NF1 proteins (73 nM) were used for the assay. The radioactivity remaining on RAS2 was determined by using nitrocellulose filters. WT, wild type. (B) Comparison of affinities of wild-type NF1 (WT) and F1434S for $RAS2^{Val-19}$-GTP. The competition assay was performed essentially as described for Fig. 5 except that $RAS2^{Val-19}$-GTP was used as a competitor. The concentration of the wild type and of F1434S was 0.7 nM.

DISCUSSION

Significance of lysine 1423 for the function of NF1. The mutations found at lysine 1423 in a neurofibromatosis type I patient are of particular interest since they occur in a region responsible for the GAP activity of NF1. Our results with NF1-GRD constructs show that the alteration of this residue to glutamic acid or to glutamine results in a dramatic decrease of the GAP activity. Furthermore, Ras affinity is drastically affected by the mutations. These results are in contrast to the results obtained by mutating residues in the FLR...PA sequence. Although this sequence is perfectly conserved in all Ras-GAP proteins, alterations of these residues resulted in only a modest effect on GAP activity as well as on Ras affinity. This finding suggests that conserved residues within the GRD are important for the function of NF1 and that lysine 1423 plays a more critical role than the FLR...PA residues in GAP activity and Ras interaction. These results confirm and expand previous findings on the significance of conserved residues in the GRD (6, 8). However, our results differ from those of Li et al. (8), who reported that the lysine residue is important for GAP activity but not for Ras affinity. The reason for the discrepancy between our Ras affinity results and theirs is unclear. It has also been suggested that the lysine residue is important for the stability of the NF1 protein (19). This does not appear to be the case for the glutamine and glutamic acid changes.

Further insights into the importance of lysine 1423 for the function of NF1 were obtained by changing it to all 20 possible amino acids. Our results clearly show that the lysine is the only residue that can function at this position in our assays. Methionine was not detected as a functional substitution. This may be due to the instability of the methionine substitution (19), although we detected the protein by Western blotting (unpublished data). The strong specificity for a particular amino acid at this position is reminiscent of glycine 12 in Ras proteins. Substitution of glycine by any other amino acid (with the exception of proline) resulted in oncogenic activation of Ras protein (16). One of the reasons

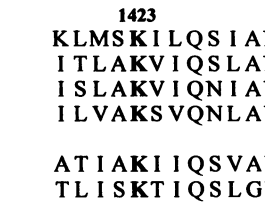


FIG. 8. Sequences of residues around phenylalanine 1434 for the six known Ras-GAPs (2, 5, 18). Alignment shows conservation of lysine 1423 and phenylalanine 1434 among NF1, IRA, and p120-GAP proteins. Sar1 (18) and Gap1 (5) correspond to *S. pombe* and *Drosophila* gene products, respectively.

for the requirement of lysine at residue 1423 of NF1 is likely its basicity because, among different substitutions, arginine gave the highest GAP activity. However, the basicity is not sufficient, since the arginine mutant still has reduced GAP activity and Ras affinity compared with the wild-type protein. Lysine may be required for the correct interaction with the corresponding residues in Ras.

Mutations of phenylalanine 1434 partially restore NF1 function. Defective NF1 proteins due to the alterations at lysine 1423 can be partially rescued by having an additional mutation at phenylalanine 1434. The second site suppressors were picked up because of their ability to complement the heat shock sensitivity of *ira1* mutant cells. The GAP activity of the suppressors is increased approximately 10-fold compared with the single lysine mutants. Although this activity is still about 40-fold lower than that of the wild type, this level of GAP activity is sufficient to complement the *ira1* mutant. Using NF1-GRDs with various levels of GAP activity, we find that the complementation occurs as long as the GAP activity is decreased by less than 80-fold (unpublished observation). We did not detect an increase of Ras affinity in the suppressors, which suggests that the mutations at phenylalanine 1434 affect catalytic activity rather than Ras affinity. This might suggest that lysine 1423 and phenylalanine 1434 serve distinct functions in NF1.

It is striking that all three suppressors had a mutation at the same residue. Since an alteration of phenylalanine to either serine or leucine restores NF1 function, it appears that the suppression does not depend on the presence of a particular amino acid at residue 1434. Furthermore, a change of phenylalanine to serine at residue 1434 can suppress the effects of both lysine-to-serine and lysine-to-glycine changes at 1423. As shown in Fig. 8, one can observe the conservation of the phenylalanine 1434 among GAP, NF1, and IRA proteins. However, this residue is not conserved in *S. pombe* Sar1 and *Drosophila* Gap1 proteins. It will be interesting to further characterize the properties of these proteins.

Mutations at phenylalanine 1434 can suppress activated *ras* phenotypes. Surprisingly, when a mutation at phenylalanine 1434 was introduced into a wild-type NF1, the resulting protein was capable of suppressing the heat shock sensitivity of $RAS2^{Val-19}$ cells. In fact, the same mutation was also identified by Nakafuku et al. (12) as a mutation which rendered NF1 capable of suppressing activated yeast *RAS2* phenotypes. They identified two such residues, one of which was a phenylalanine-to-leucine change at residue 1434. It is intriguing that the same mutation was identified by two different methods. These authors further found that expression of the mutant NF1 construct in *ras*-transformed mammalian cells induced morphological reversion of these cells. Thus, the phenylalanine mutant proteins exhibit antioncogenic properties.

To gain insights into the mechanism of suppression of activated *ras* phenotypes, the F1434S mutant protein was purified and characterized. No significant increase of the GAP activity against H-ras protein was detected. Presumably, mutations of phenylalanine 1434 do not affect GAP activity when the protein already has full activity. Furthermore, the mutant protein failed to stimulate GTPase activity of $RAS2^{Val-19}$ protein. Therefore, an increase in GAP activity appears not to be the mechanism for the antioncogenic property of the NF1 mutant protein. An alternative possibility is that the mutant NF1 protein has an increased affinity for the activated Ras protein, interfering with its binding to the effector. However, no significant increase in Ras affinity was detected with the mutant protein (Fig. 7B). These observations lead us to suggest that the mutant NF1 protein influences *ras*-activated phenotypes by a mechanism not involving NF1-Ras interaction. Although various proteins could affect NF1 activity in vivo, one obvious possibility is that the mutant NF1 protein directly interacts with the effector protein, adenylate cyclase in this case. In fact, such an interaction has been reported by Mitts et al. (11), who showed that adenylate cyclase is anchored to the membrane through the interaction with IRA proteins (11). The mutant NF1 protein might interfere with the interaction of these molecules, resulting in the mislocalization of the cyclase to the cytosol. Alternatively, the mutant protein could disrupt a ternary complex consisting of IRA, Ras, and adenylate cyclase. These possibilities are being investigated.

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