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Analysis of the neurofibromatosis type 1 (NF1) GAP-related domain by site-directed mutagenesis

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The gene for von Recklinghausen neurofibromatosis type 1 (NF1) was recently identified by positional cloning and found to encode a protein with sequence similarity to a family of eucaryotic GTPase-activating proteins (GAPs). Expression of the NF1-GAP-related domain (NF1GRD) has been shown to complement yeast strains deficient in the yeast GAP homologs, IRA1 and IRA2, to interact with human RAS proteins and to accelerate the conversion of *ras*-GTP to *ras*-GDP. Further analysis of this region has revealed a number of residues that are highly conserved between members of the GAP family. Mutational analysis of a representative number of these residues produced one of three effects: (1) no change in NF1GRD function, (2) complete disruption of NF1GRD function and (3) intermediate retention of NF1GRD function. One of these mutations at residue 1423 was shown to have reduced ability to negatively regulate *ras* in yeast, which is interesting in light of a recent report demonstrating a similar naturally occurring mutation in human malignancies.

Introduction

von Recklinghausen neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder affecting approximately 1 in 3000 individuals (Riccardi & Eichner, 1986). The disease has a high spontaneous mutation rate with 40–50% of NF1 cases representing new mutations. Clinically, patients present with abnormalities predominantly but not exclusively of neural crest-derived tissues, such as *café-au-lait* spots and peripheral neurofibromas. The *NF1* locus, which was identified by positional cloning, spans ~300 kb of genomic DNA with a ubiquitously expressed transcript of 11–13 kb and an open reading frame of 8.8 kb (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990; Marchuk *et al.*, 1991). The protein product, neurofibromin, has been identified using antibodies directed against fusion proteins and synthetic peptides (DeClue *et al.*, 1991; Gutmann *et al.*, 1991). Neurofibromin migrates as a 250-kDa protein, and is expressed predominantly in brain, spleen and kidney. It appears to be a cytoplasmic protein by indirect immunofluorescence and subcellular fractionation (DeClue *et al.*, 1991; P.E. Gregory *et al.*, manuscript submitted).

Analysis of the *NF1* gene revealed sequence similarity between neurofibromin and members of the GTPase-activating protein family, including mammalian GAP as well as yeast IRA1, IRA2 and sar1 (Xu *et al.*, 1990a; Wang *et al.*, 1991). The region of greatest sequence similarity spans approximately 1.26 kb of coding region and has been termed the NF1-GAP-related domain (NF1GRD). Although the role of GAP in mammalian cells is not well understood, the two *Saccharomyces cerevisiae* proteins, IRA1 and IRA2, regulate RAS signal transduction pathways that control cell growth by regulating adenylate cyclase (Tanaka *et al.*, 1989; 1990a,b). Expression of the NF1GRD in yeast strains deficient in IRA1 and IRA2 demonstrates that this portion of neurofibromin can functionally substitute for yeast GAP molecules (Ballester *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990b). Further demonstration that the NF1GRD can physically associate with RAS protein to accelerate the conversion of RAS-GTP to RAS-GDP *in vivo* and *in vitro* supports the contention that the GRD of neurofibromin is a functional GAP domain (Martin *et al.*, 1990). Recent studies using antibodies against neurofibromin have shown that the full-length molecule has GAP catalytic properties (Basu *et al.*, 1992).

Alignment of the amino acid sequences of all five reported members of the GAP family reveals a limited number of highly conserved residues (Wang *et al.*, 1991). Computer-generated models based on these conserved residues suggest that subregions of the NF1GRD may have different effects on neurofibromin's ability to interact with and regulate $p21^{ras}$. In an effort to determine which residues might be critical for $p21^{ras}$ interaction, site-directed mutagenesis experiments were undertaken to dissect this functional domain of the *NF1* gene.

Results

Comparative sequence analysis

Previous quantitative alignment studies among mammalian and fungal *ras*-GAPs (Wang *et al.*, 1991) have shown that the most highly conserved regions of the 'catalytic' domain are limited to several homology blocks that contain only 15 invariant residues. Subsequent cloning of the *Drosophila Gap1* gene (Gaul *et al.*,

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1992) showed that 14/15 of these residues remain invariant (the single difference, glycine to alanine, represents a conservative substitution) (Figure 1). This observation supports the hypothesis that these conserved residues are important for GAP structure or activity. Thus some of these residues were targets for our *in vitro* mutagenesis studies. Because a splicing variant of neurofibromin contains a 21 amino acid insertion that disrupts the integrity of block 2 (Figure 1) without adversely affecting biological activity (Andersen *et al.*, 1993; Nishi *et al.*, 1991), attention was primarily focused on blocks 1, 3A and 3B.

Because the sequence databases have grown considerably in size since our previous motif analyses (Wang *et al.*, 1991), we repeated the searches with catalytic domain motifs on the current database releases. The pattern **FLR...PA...P** from block 3A remains absolutely diagnostic for *ras*-GAPs out of the approximately 75 000 known protein sequences. In contrast, the block 3B pattern **K..Q..AN** is found in more than 250 sequences unrelated to GAP (data not shown). The discriminating ability of this pattern could be improved by specifying additional limitations among the invariant residues. For example, the pattern **K**[IVST][LIV]**Q** [SN][LV][AG]**N** (where residues in brackets indicate allowed alternatives at each position) is specific for *ras*-GAP sequences only.

Generation of NF1GRD mutants

In order to study the conserved residues described above, the NF1GRD (residues 1125–1537) was sub-

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                      Block 1
1251  HLLYQLLWNMFSK | EVELADSMQTLFRGNSLASKIMTFCFK | -VYGATYLQKLLD    NF1GRD
                    | Y           R               |                  Mutations
 764  KLESLLLCTLNDR | EISMEDEATTLFRATTLASTLMEQYMK | -ATATQFVHHALK    p120-GAP
 520  ERIAPIIKALADH | EISHLTDPTTIFRGNTLVSKMMDEAMR | -LSGLHYLHQTLR    Drosophila Gap1
1571  NASHILVTELLKQ | EIKRAARSDDILRRNSCATRALSLYTR | -SRGNKYLIKTLR    Ira1
1717  NATHIVVAQLIKN | EIEKSSRPTDILRRNSCATRSLSMLAR | -SKGNEYLIRTLQ    Ira2
 170  HLLLSLFQMVLTT | EFEATSDVLSLLRANTPVSRMLTTYTR | RGPGQAYLRSILY    sar1

                EIE.......LLR.NS.ASR.L..Y.R                        Consensus

1303  PLLRIVITSSDWQHV-------SFEVDPTRLEPSES-----------------LE    NF1GRD
 816  DSILKIMESKQ-----------SCELSPSKLEKNED-----------------VN    p120-GAP
 572  PVLSQIVAEKK-----------PCEIDPSKIKDRSA-----------------VD    Drosophila Gap1
1623  PVLQGIVDNKE-----------SFEID--KMKPGSE-----------------NS    Ira1
1769  PLLKKIIQNRD-----------FFEIE--KLKPEDS-----------------DA    Ira2
 223  QCINDVAIHPDLQLDIHPLSVYRYLVNTGQLSPSEDDNLLTNEEVSEFPAVKNAIQ    sar1

                     Block 2
                     ------------ 21-residue insertion --
1334  ENQRNLL-QMTE | KFFHAIISSSSEFPPQLRSVCHCLYQ^VVSQRFPQNS | ----IG    NF1GRD
 843  TNLTHLL-NILS | ELVEKIFMASEILPPTLRYIYGCLQK SVQHKWPTNT | TMR-TR    p120-GAP
 599  TNLHNLQ-DYVE | RVFEAITKSADRCPKVLCQIFHDLRE CAGEHFPSNR | EVR-YS    Drosophila Gap1
1648  EKMLDLFEKYMT | RLIDAITSSIDDFPIELVDICKTIYN AASVNFPEYA | ----YI    Ira1
1794  ERQIELFVKYMN | ELLESISNSVSYFPPPLFYICQNIYK VACEKFPDHA | ----II    Ira2
 279  ERSAQLL-LLTK | RFLDAVLNSIDEIPYGIRWVCKLIRN LTNRLFPSIS | DSTICS    sar1

               .LL.AI..S...FPP.LR.IC..IY. .....FP.....                Consensus

      Block 3A                                 Block 3B
1383  | AVGSAMFLRFINPAIVSPYEAGILD | KKPPPRI | ERGLKLMSKILQSIAN | HVLF    NF1GRD
      |         S   I    R        |         |         S  R   M |         Mutations
 895  | VVSGFVFLRLICPAILNPRMFNIIS | DSPSPIA | ARTLILVAKSVQNLAN | LVEF    p120-GAP
 651  | VVSGFIFLRFFAPAILGPKLFDLTT | ERLDAQT | SRTLTLISKTIQSLGN | LVSS    Drosophila Gap1
1698  | AVGSFVFLRFIGPALVSPDSENIII | VTHAHD- | RKPFITLAKVIQSLAN | GREN    Ira1
1844  | AAGSFVFLRFFCPALVSPDSENIID | ISHLSE- | KRTFISLAKVIQNIAN | GSEN    Ira2
 332  | LIGGFFFLRFVNPAIISPQTSMLLD | SCPSDNV | RKTLATIAKIIQSVAN | GTSS    sar1

        AVGSFVFLRFI.PAIVSP...NIID             RRTLI..AK.IQS.AN            Consensus
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Figure 1 Multiple sequence alignment of known GAP-related domains. Optimal alignments were computed using software tool kits as previously described (Boguski *et al.* 1992) on a Sun (Unix) workstation. The sources of the sequence data used were essentially as previously described (Wang *et al.*, 1991; Boguski *et al.*, 1992) except for the inclusion of the *Drosophila Gap1* sequence in the current analysis (GenBank/GenPept accession number M86655). Residue numbers along the left margin identify the locations of the GAP-related domains within their parent sequences. Rectangles are drawn around the highly significant 'homology blocks' (Wang *et al.* 1991); a dashed line is used for block 2 to indicate that this region is disrupted by a naturally occurring insertion (Andersen *et al.*, 1993; Nishi *et al.*, 1991). Consensus sequences for these highly conserved blocks are given with bold-faced characters representing invariant residues and dots indicating the most variable positions. Mutations in NF1GRD are indicated as italicized residues immediately below their wild-type counterparts

cloned into pSELECT-1 and specific mutations were made by oligonucleotide-directed mismatch. The mutants used in this study are listed in Table 1. These nucleotide changes were engineered so that each new mutation created a novel restriction site that was then used as a convenient initial screen for the presence of the desired mutant NF1GRD. Each mutant was then sequenced to confirm the desired mutation and subcloned into pAD54 in the same reading frame as the hemagglutinin peptide sequence. In this vector, the desired sequences are expressed in yeast under the control of the strong *ADH1* promoter as a fusion protein with an N-terminal peptide epitope (see Materials and methods). The specific mutations listed in Table 1 were chosen because they represent radical changes in charge or structure from the wild-type amino acid residue.

Complementation of ira⁻ strains by NF1GRD mutants

Disruption of either IRA gene leads to a phenotype that resembles that seen in cells containing the mutationally activated $RAS2^{Val\text{-}19}$. In particular, ira1⁻ or ira2⁻ strains are exquisitively sensitive to heat shock. We and others have previously shown that NF1GRD and GAP can complement loss of IRA function by suppressing heat shock sensitivity of an ira⁻ strain.

To determine the effect of the different mutations on the function of the NF1GRD, we transformed the ira1⁻ ira2⁻ strain IR2.53 (see Materials and methods) with the plasmids expressing the different NF1GRD mutants and tested for the ability of transformants to withstand heat shock. Typical results are shown in Figure 2. Based on their ability to suppress heat shock sensitivity, the different mutations can be grouped into three categories. The first category includes mutations that do not alter the suppressor activity since their ability to suppress the heat shock sensitivity is indistinguishable from the wild-type NF1GRD (E1264Y, A1281R, P1395I, P1400R and N1430M). The second group includes mutations that completely abolish suppressor function. These mutants are unable to suppress the heat shock sensitivity of the ira1⁻ ira2⁻ strains (Δ53, Q1426R). The third category includes mutations with reduced suppressor activity (K1423S, R1391S). This group shows a decreased ability to suppress the heat shock-sensitive phenotype of the ira⁻ cells. As can be seen in Figure 2, these mutants are able to suppress the heat shock sensitivity at time 0 (plates have been preheated to 55°C for 1 h), but in longer periods of incubation at 55°C this ability decreases (5 min) or is absent (10 min).

Complementation of pde2⁻ strains expressing human H-ras

We have previously shown that the NF1GRD is capable of inhibiting the human wild-type H-*ras* protein when expressed in yeast (Ballester *et al.*, 1990). To determine if the NF1GRD mutations had an effect on

Table 1 Generation of NF1GRD catalytic domain mutations

*Residue**	*Nucleotide alteration*	*Resulting amino acid*	*Restriction site created*	*Description*	*Block*
Glutamic acid 1264	GAA to TAC	Tyrosine	SnaBI	E1264Y	1
Alanine 1281	TTGGCC to TTACGT	Arginine	SnaBI	A1281R	1
Arginine 1391	CCTAGA to CTAAGC	Serine	HindIII	R1391S	3a
Proline 1395	CCT to ATT	Isoleucine	SspI	P1395I	3a
Proline 1400	TCACCG to TCTAGA	Arginine	XbaI	P1400R	3a
Lysine 1423	AAG to TCG	Serine	ClaI	K1423S	3b
Glutamine 1426	CTTCAG to CTACGT	Arginine	SnaBI	Q1426R	3b
Asparagine 1430	AAT to ATG	Methionine	NsiI	N1430M	3b

*The amino acid residue is specified according to the numbering of Marchuk *et al.* (1991) using the entire neurofibromin amino acid sequence

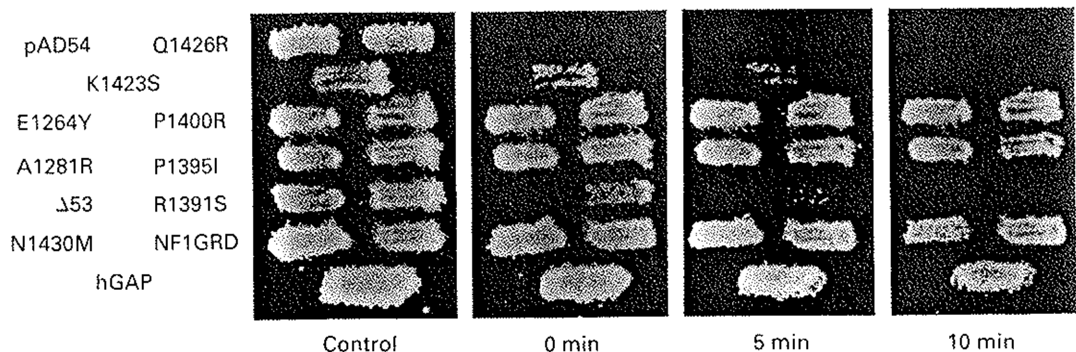

Figure 2 Effect of the NF1GRD mutations on the suppression of the heat shock sensitivity of an ira1⁻ira2⁻ strain. The ira1⁻ira2⁻ strain (IR2.53) was transformed with the various plasmids containing the NF1GRD mutants (see Table 1) and selected on SC-Leu plates. Independent transformants were patched onto SC-Leu plates, incubated at 30°C for 2 days, replica plated onto SC-Leu plates that had been preheated for 1 h at 55°C and further heat shocked for 0, 5 or 10 min. After heat shock, the plates were incubated for 2 days at 30°C. The various plasmids used are depicted in the first plate. pAD54 is the control plasmid. NF1GRD is the plasmid containing the wild-type NF1GRD construct and hGAP represents a plasmid expressing the full-length GAP cDNA

the ability to inhibit the function of wild-type H-*ras* in yeast cells, we transformed a strain (J1041F7) that lacks the *PDE2* gene but expresses the wild-type H-*ras* under the control of the strong *PGK* promoter (Banroques *et al.*, 1986) integrated in the *trp1* locus. The strain J1041F7 is derived from the strain J104 that has a disruption in the phosphodiesterase gene *PDE2*. This strain is not sensitive to heat shock. When human wild-type H-*ras* is integrated in the *trp1* locus under the control of the *PGK* promoter, this strain becomes heat shock sensitive. The different mutant NF1GRD constructs were tested for the ability to suppress the heat shock sensitivity of this strain. As can be seen in Figure 3, the effect of the NF1GRD mutants on human H-*ras* is similar to that observed with the yeast RAS. The mutations can again be grouped into those that have no effect on the suppressor activity (E1264Y, A1281R, P1395I, P1400R, and N1430M), those that completely abolish suppression (Δ53, Q1426R, K1423S) and those that have ability to suppress the heat shock sensitivity of the J1041F7 strain (R1391S). The only relative differences between the previous assay using yeast RAS and this experiment using human H-*ras* are seen with the K1423S mutation. In the ira1⁻ heat shock suppression assay, the K1423S muation has reduced activity, but behaves more like a null mutant with human H-*ras*.

Ability of NF1GRD mutants to increase the GTPase activity of H-ras

To determine whether the differences in heat shock sensitivity were due to effects on the ability of these mutants to induce *ras*-GTP to hydrolyze GTP, the GAP activity of the NF1GRD mutants was assayed *in vitro*. Cell lysates prepared from yeast strains expressing the different NF1GRD mutants were incubated with purified H-*ras* prelabeled with [^{32}P]GTP. H-*ras* was then immunoprecipitated and the eluted nucleotides were resolved by thin-layer chromatography as described in the Materials and methods section. The results of two representative experiments are shown in Table 2. These mutations can be categorized into two groups: (1) mutations that eliminate GAP activity (Δ53, E1264Y, A1281R, Q1426R, K14235) and (2) mutations that do not alter GAP activity (R1391S, P1400R, P1395I, N1430M). The Δ53 mutation that eliminates catalytic activity of the NF1GRD also abolishes its ability to inhibit RAS activity in intact cells. The mutations E1264Y and A1281R, on the other hand, show no GAP activity *in vitro*, but in intact cells they are indistinguishable from wild-type NF1GRD in their ability to inhibit the activity of both yeast and human H-*ras*. These differences are not the result of varying amounts of NF1GRD protein expressed, since Western immunoblotting of lysates dem-

Table 2 Properties of the NF1 GRD mutants

	Suppression of heat shock sensitivity		GAP activity		
			In vitro‡ GTP (%)		In vivo**
Mutant	*Yeast RAS**	*H-ras†*	*A*	*B*	
Vector alone	–	–	62.5	59.2	–
Wild-type NF1GRD	+	+	9.3	9.6	+
Δ53	–	–	51.9	57	–
E1264Y	+	+	50.5	51.7	±
A1281R	+	+	51.8	49.1	±
R1391S	±	±	7.5	8.7	+
P1395I	+	+	9.7	7.2	+
P1400R	+	+	8.5	7.9	+
K1423S	±	–	44.6	42.2	±
Q1426R	–	–	57.1	55.3	–
N1430M	+	+	12.3	11.7	+

*Yeast RAS refers to the heat shock sensitivity assay performed using a yeast strain (IR2.53) deficient in IRA1 and IRA2 as described in Figure 2 and Materials and methods
†H-*ras* refers to the heat shock sensitivity assay performed using a yeast strain deficient in PDE2 but overexpressing wild-type H-*ras* as described in Figure 3 and in Materials and methods
‡Determinations of percentage GTP bound to RAS were performed in two representative experiments designated A and B. Cell lysates were prepared from yeast strains expressing the different NF1GRD mutants. The cell lysates were incubated with purified H-*ras* prelabeled with [α-^{32}P]GTP. The samples were then incubated with the Y13-259 antibody. Immunoprecipitates were washed and the nucleotides were eluted and resolved by chromatography. The labeled nucleotides were scraped off the plates and counted as described in Materials and methods
**Yeast strains expressing wild-type H-*ras* under the control of the *PGK* promoter were labeled with [^{32}P]orthophosphate and RAS proteins were immunoprecipitated. Labeled nucleotides were eluted and resolved by thin-layer chromatography as described in Figure 4 and Materials and methods

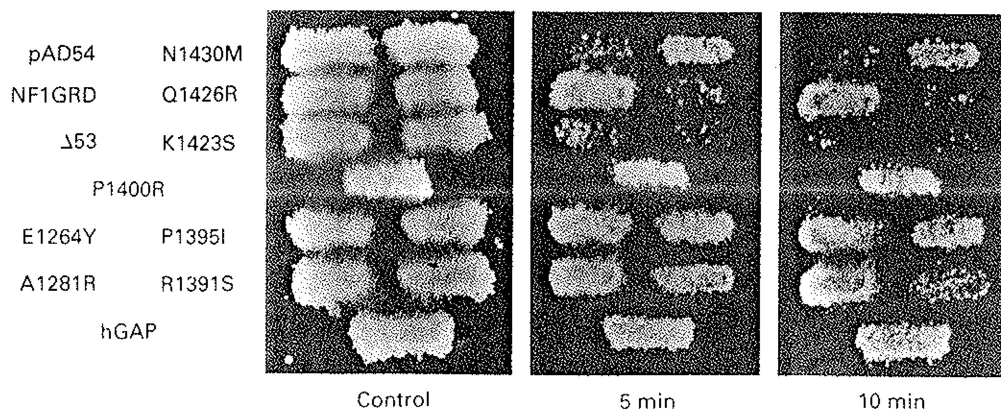

Figure 3 Effect of the NF1GRD mutations on the suppression of the heat shock sensitivity of strains expressing wild-type human H-*ras*. The yeast strain J1041F7 that contains a deletion in the PDE2 gene and expresses human H-*ras* was transformed with the various mutant NF1GRD plasmids and tested for heat shock sensitivity as described in Figure 2. The constructs used are depicted in the first plate

onstrates equivalent amounts of protein (data not shown). The mutations that do not alter GAP activity *in vitro* (R1391S, P1400R, P1395I, N1430M) also show wild-type ability to inhibit RAS function in intact cells with the exception of R1391S, which is a weak inhibitor of yeast and human H-*ras* in yeast. The mutation K1423S reduces the NF1GRD GAP activity *in vitro* as well as its ability to inhibit RAS function in intact cells. Therefore, from the combination of analysis of the suppression of the heat shock sensitivity and the GAP assays *in vitro*, we can further group the mutations into those that behave like wild-type NF1GRD (P1395I, P1400R, N1430M), those that show no activity in intact cells or *in vitro* (Δ53, Q1426R), those that show wild-type activity in intact cells but no activity *in vitro* (E1264Y, A1281R) and those that show weak activity in intact cells but wild-type activity *in vitro* (R1391S).

*Determination of the amount of H-*ras *bound to GTP in yeast cells expressing NF1GRD mutants*

In order to determine whether the GAP assay *in vitro* reflects the activity of these mutants in intact cells, we determined the amount of H-*ras* bound to guanine nucleotides in strains expressing the wild-type H-*ras* under the control of the *PGK* promoter (J1041F7) and transformed with the plasmid expressing the different NF1GRD mutants (Figure 4). Yeast strains were grown in SC-Leu to an early exponential growth phase, inoculated in SD-P medium with [^{32}P]orthophosphate and incubated for 3 h. Cell extracts were prepared and *ras* proteins were immunoprecipitated as described in the Materials and methods section. The antibody Y13-259 immunoprecipitates the endogenous RAS1 and RAS2 proteins as well as expressed H-*ras* protein. The guanine nucleotide bound to H-*ras* was analysed by chromatography on polyethyleneimine–cellulose plates followed by autoradiography or direct quantitative measurement of the radioactivity of [^{32}P]-GTP or [^{32}P]GDP spots using a Fujix BAS200 bioimaging analyser. The autoradiogram in Figure 4 shows a representative experiment, and Table 2 summarizes the results obtained for all the mutants. As shown in Figure 4, in the parental strain J104, which expresses endogenous RAS1 and RAS2, most of the RAS is bound to GDP. When this strain expresses human H-*ras* under the control of the *PGK* promoter (J1041F7, see Materials and methods) and is also transformed with a control vector plasmid, a significant fraction of the H-*ras* immunoprecipitated is bound to GTP, as has been previously reported (Tanaka *et al.*, 1990a). In yeast, the H-*ras* expressed is in a GTP-bound state because of the inability of the *IRA1* and *IRA2* genes to increase the GTPase activity of human H-*ras* (Tanaka *et al.*, 1990b; 1992). Expression of the wild-type NF1GRD results in a reduction of the amount of H-*ras* bound to GTP in intact cells. As shown in Figure 4 and Table 2, in intact cells the mutants Δ53 and Q1426R, which eliminate the ability to inhibit RAS activity in intact cells and *in vitro*, fail to decrease the amount of GTP-bound H-*ras*. All the other mutants tested were able to decrease the amount of GTP-bound H-*ras* to some extent. Of particular interest are the mutants E1264Y and A1281R. These do not shown GAP activity *in vitro*, but can increase

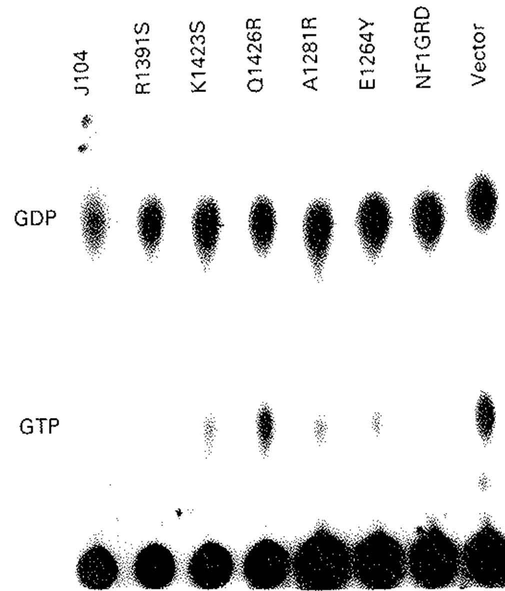

Figure 4 Autoradiogram of the guanine nucleotide bound to H-*ras* in intact cells expressing NF1GRD mutants. Yeast strains expressing wild-type H-*ras* under the control of the *PGK* promoter (strain J1041F7) integrated into the *trp1* locus were transformed with control plasmid or the various NF1GRD mutants as shown, labeled with [^{32}P]orthophosphate for 3 h and H-*ras* was immunoprecipitated as described in the Materials and methods section. The labeled nucleotides were eluted and resolved by thin-layer chromatography. J104 represents the parental strain that does not express human H-*ras* protein. The figure shows the autoradiograph of plates exposed to X-ray film

the amount of GDP-bound H-*ras* in intact cells, suggesting that these mutations retain some of the catalytic activity of the NF1GRD.

Discussion

The gene product of the *NF1* locus, denoted neurofibromin, is a member of the GAP family of proteins by sequence similarity and functional analysis. Since little information is known about the function of neurofibromin aside from its GAP-like properties, initial studies to determine more about the role of neurofibromin have focused on the GAP-related domain. Previous work by ourselves and others (Ballester *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990b; Wiesmuller & Wittinghofer, 1992) has demonstrated that this region of neurofibromin can substitute for the IRA1 and IRA2 GAP-like proteins in yeast and can accelerate *ras*-GTP hydrolysis. Deletion analysis has demonstrated that the GAP activity of this domain resides between residues 1175 and 1524 (Xu *et al.*, 1990b). Alignment of the sequences of the various related GAP catalytic domains clearly identified a number of conserved residues that may have functional significance. Of particular interest were the residues contained in blocks 3A and 3B (as defined in Wang *et al.*, 1991), which by computer modeling may represent the *ras* binding pocket. To address the functional significance of these conserved residues, a limited number of NF1GRD mutants were generated and assayed for their ability to accelerate *ras*-GTP hydrolysis and complement yeast strains deficient in IRA1

and IRA2. These mutants can be classified into three groups based on these experiments: (1) mutants that have normal GAP activity, (2) mutants with no GAP activity and (3) mutants with reduced GAP activity (Table 2). In addition, there does not appear to be a general preference of the NF1GRD mutants for yeast RAS versus human H-*ras*. These mutants suppress the heat shock sensitivity of strains expressing either yeast RAS or H-*ras* in a similar fashion.

The first group of NF1GRD mutations, which did not alter NF1GRD activity, includes P1395I, P1400R and N1430M. The resulting changes in the protein might have been predicted to be dramatic in that a proline is replaced in two of these mutants. However, none of these residues appear to be critical for wild-type NF1GRD function. The second group of mutations, which virtually eliminate GAP-like activity, included Q1426R and Δ53. The Δ53 mutation removed 53 amino acids from the region just 5′ to and including some of block 1 while still preserving the reading frame. The lack of catalytic activity is most likely the result of a structural change in the protein rather than the result of reduced protein stability, as immunoblotting using the antibody to the common hemagglutinin epitope demonstrated that equivalent amounts of this mutant were expressed (data not shown). The Q1426R mutation completely destroys GAP activity, and Q1426 probably represents a residue critical for RAS interaction.

The most interesting group of mutations are those that are neither wild-type nor null. The NF1GRD mutant K1423S shows reduced function both *in vivo* and *in vitro*. Recently, certain tumors and patients with NF1 have been shown to have mutations affecting residue 1423 (Li *et al.*, 1992). In three different tumor types, including colon adenocarcinoma, myelodysplastic syndrome and anaplastic astrocytoma, and in one family with NF1, nucleotide mutations resulted in non-conservative amino acid substitutions from lysine to either glutamic acid or glutamine. When the NF1GRD containing the patient mutations was expressed in baculovirus Sf9 cells, the GAP activity was reduced 200- to 400-fold, while the binding affinity for $p21^{ras}$ was not affected. In addition, expression of the NF1GRD (residues 1198–1529 of the full-length neurofibromin protein) in *Escherichia coli* demonstrated that alteration of this K1423 residue results in diminished GAP activity, which may be related to decreased thermal stability of the mutant protein (Weismuller & Wittinghofer, 1992). The mutation described herein (K1423S) has reduced GAP activity in yeast cells, which is in agreement with its reduced GAP function *in vitro*. These results strongly argue that this residue is critical for GAP function.

The NF1GRD mutants E1264Y, A1281R and R1391S show reduced function in at least one of the assays performed. The mutant E1264Y and A1281R show wild-type activity in their ability to suppress heat shock sensitivity when transformed into strains lacking the IRA genes or overexpressing the human H-*ras*, but their ability to increase the GTPase activity of H-*ras* cannot be measured *in vitro*. When the amount of GTP-bound H-*ras* in strains transformed with these mutants is measured, the GTP-bound H-*ras* is somewhat reduced, but not to the extent seen in strains expressing the wild-type NF1GRD. Thus these two mutants indeed have reduced GTPase-activating activity. On the other hand, the mutant R1391S is only weakly active as a suppressor yet shows nearly wild-type GTPase-activating activity both *in vitro* and *in vivo*. Taken together, these results suggest that the *in vivo* yeast assay measures a property of NF1GRD other than its catalytic properties. The simplest explanation is that the *in vivo* yeast assay reflects binding to RAS, and hence interference with RAS effector function. This is in keeping with the observation that wild-type NF1GRD can suppress the heat shock sensitivity of yeast strains expressing the mutant, activated H-$ras^{val\text{-}12}$ protein even though the GTP hydrolysis of the latter is completely resistant to NF1GRD (Ballester *et al.*, 1990). Hence, mutations at positions E1264 and A1281 may affect predominantly the GTP hydrolysis of associated RAS without affecting affinity, while mutations at R1391 diminish affinity without affecting hydrolysis.

In summary, our studies indicate that mutation at position Q1426 abolishes the biological and biochemical activity of NF1GRD, and mutation at position K1423 greatly reduces both. Mutations at positions E1264 and A1261 as well as R1391 reduce one but not the other. Mutations at the completely conserved residues P1395, P1400 and N1430 do neither. It is unexpected, in a protein from a family so diverged in sequence, that alterations at highly conserved residues are without consequence. It is thus altogether likely that these alterations produce changes that we cannot properly observe, either by our current biochemical assays or by expression in yeast. It would therefore be of great interest to develop assays for neurofibromin function in mammalian cells.

Materials and methods

Media, genetic manipulations and nomenclature

Yeast was grown in YPD (2% peptone, 1% yeast extract, 2% glucose) or in synthetic medium (0.67 g l^{-1} yeast nitrogen base, 2% glucose and appropriate auxotrophic supplements). Standard yeast genetic methods were followed as described previously (Sherman *et al.*, 1986). The lithium acetate method was used for transformation of yeast cells (Ito *et al.*, 1983).

Wild-type alleles and dominant mutations are denoted by capital letters, recessive mutations by lower-case italicized letters, and gene disruptions by lower-case letters, which represent the disrupted gene, followed by two colons and the auxotrophic gene marker used for the disruption. For example, *ira1::HIS3* indicates the *IRA1* gene was disrupted by the HIS3 marker. In the text, gene disruptions are abbreviated by lower-case italicized letters representing the gene followed by a superscript minus sign, such as *ira1*$^{-}$.

DNA manipulation

DNA manipulations were performed by standard methods (Maniatis *et al.*, 1982). DNA restriction endonucleases, polymerase and ligases were used under conditions recommended by suppliers (New England Biolabs, Bethesda Research Laboratories or Perkin-Elmer Cetus). For gene disuption experiments, suitable linear DNA fragments were isolated and used for transformation of yeast cells as previously described (Rothstein, 1983).

Plasmids

The plasmid pAD54-NF1 was generated by cloning the NF1GRD into the yeast expression vector, pAD54. The pAD54 vector derives from the pADNS plasmid (Collicelli *et al.*, 1989), which is an expression vector designed to express cDNA genes from the alcohol dehydrogenase (ADH1) promoter. This vector contains a sequence 5′ to the neurofibromin GAP-related domain that specifies a hemagglutinin epitope (Field *et al.*, 1988), the LEU2 marker and the 2μ circle origin of replication. SalI–HindIII and SacII–SacI linkers were generated to adapt the amino acid and carboxy termini, respectively, of the NF1GRD (amino acid residues 1125–1537 of the full-length cDNA translated sequence; Marchuk *et al.*, 1991) to pAD54. The accuracy of the clone was confirmed by dideoxy sequencing (Sequenase kit, US Biochemicals, version 2.0) of the PCR insert encoding the catalytic domain.

The plasmid pADGAP contains the entire coding region of the human GAP gene on a high-copy *LEU2* plasmid containing the *ADH1* promoter and terminator sequences (Ballester *et al.*, 1989). The YepPDE2 plasmid contains the yeast *PDE2* gene on the high-copy *LEU2* plasmid, YEp13 (Sass *et al.*, 1986). The *PDE2* gene encodes a high-affinity cAMP phosphodiesterase.

The plasmid p1F7 was constructed by inserting a BamHI fragment that contains the human H-*ras* cDNA into the vector PGK2F5. The BamHI fragment containing the H-*ras* cDNA was obtained by digesting the plasmid pAHRG-H1 (Ballester *et al.*, 1989) with the HindIII enzyme, adding BamHI linkers and cloning into the BglII site of the PGK2F5 plasmid. The PGK2F5 plasmid contains the *TRP1* gene, the *URA3* gene and the phosphoglycerokinase (*PGK*) promoter followed by a BglII site used for cloning purposes and also contains the terminator sequences of the *PGK* gene. The PGK2F5 plasmid was constructed as follows: a BglII–SphI fragment from plasmid Yrp7 (Tschumper & Carbon, 1980) containing the *TRP1* gene without the adjacent autonomously replicating sequence (ARS) was cloned into the BamHI–SphI sites of PGKYi2, creating PGK2F5. The PGKYi2 was constructed by inserting the HindIII fragment of plasmid pEMBLYe30/2 containing a PGK promoter-terminator cassette (Banroques *et al.*, 1986) in the HindIII site of plasmid pEMBLYi31 (Baldari & Cesareni, 1985).

Yeast strains

The yeast strain IR2.53 (*MATa his3 leu2 ura3 trp1 ade8 ira1::HIS3 ira2::ADE8*) containing disruptions in the *IRA1* and *IRA2* genes was constructed in two steps. First, the *IRA2* gene was disrupted as previously described (Ballester *et al.*, 1990) using a BamHI fragment containing the *ADE8* gene as an auxotrophic marker. This results in a deletion of the 5′ coding sequences of the *IRA2* gene as well as the catalytic domain. To carry out gene replacement experiments, the yeast haploid auxotrophic SP1 was transformed with a SalI-digested plasmid containing the deleted *IRA2* gene, and transformants were selected for adenine prototrophy. The resulting strain, IRA2.5 (*MATa his3 leu2 ura3 trp1 ade8 ira2::ADE8*), was transformed with a XbaI fragment containing a deletion in the *IRA1* gene. In this plasmid (described in Ballester *et al.*, 1989), the HIS3 gene is inserted into the BglII sites of the *IRA1* gene. This results in a 3.2-kb deletion that includes the catalytic domain of the *IRA1* gene, leaving the 5′ coding sequences intact. The resulting transformants were selected for histidine prototrophy. Southern hybridization analysis was used to verify that both the *IRA1* and the *IRA2* genes were replaced by the disrupted genes.

The yeast strain J1041F7 (*MATa his3 leu2 ura3 trp1 ade8 pde2::HIS3 TRP1::pTRP1-URA3 Ha-ras*) was constructed by transforming the strain J104 (*MATa his3 leu2 ura3 trp1 ade8 pde2::HIS3*) with the plasmid p1F7 digested with the enzyme XbaI. This leads to the integration of the plasmid into the *trp1* locus. Transformants were then selected for uracil prototrophy.

Site-directed mutagenesis

Initially, the neurofibromin catalytic domain was subcloned into the altered sites vector, pSELECT-1 (Promega), to generate pSELECT-CAT. Specific oligonucleotides (27–30 nucleotides in length) complementary to the region to be altered were annealed to single-stranded pSELECT-CAT DNA along with an oligonucleotide necessary to correct a mutation in the β-lactamase gene and double-stranded DNA was synthesized. The oligonucleotides synthesized for these mutagenesis reactions were:

TGCCAATTCTACGTATTTAGAAAACAT (glutamic acid 1264)
CATTATTTTACTACGTAAGCTGTTGCC (alanine 1281)
GAAGAGAACATGCATGGCAATACTCTGAAG (asparagine 1430)
GATTGGCAATACTACGTAGTATCTTTGACA (glutamine 1426)
CTGAAGTATCGATGACATTAACTTCAAGCC (lysine 1423)
AATCCCTGCTTCATATCTAGAGACAATGGC (proline 1400)
TGAGACAATGGCAATATTGATAAATCTGAG (proline 1395) and
AGGATTGATAAAGCTTAGGAACATGGCACT (arginine 1391)

Each new mutation was created so that a new endonuclease restriction site was introduced, thus facilitating the screening of mutant colonies (Table 1). After verifying by restriction enzyme digestion that the correct mutation was generated, each mutated NF1GRD was subcloned into pAD54 and sequenced. The Δ53 deletion mutation was generated entirely within the pAD54 vector by digestion with BalI, resulting in a 53 amino acid deletion (residues 1227–1281) with maintenance of the reading frame. The Δ53 mutant protein could be demonstrated as a faster migrating species by Western blot and immunoprecipitation (data not shown) using a monoclonal antibody directed against the hemagglutinin epitope.

Heat shock sensitivity assay

Heat shock sensitivity was determined as described previously (Toda *et al.*, 1985; Sass *et al.*, 1986). Yeast strains were transformed with various plasmids containing the *LEU2* gene and plated onto SC-Leu plates. Independent transformants were patched onto SC-Leu plates and incubated at 30°C for 2 days. Heat shock was performed by replica plating cells to a plate preheated for 1 h at 55°C followed by 10-min incubation at 55°C. After heat shock treatment, the plates were transferred to 30°C for 2 days and photographed.

GAP assays and quantitation

Yeast strains were grown to saturation in 5 ml of selective media and then diluted in 200 ml of YPD media. Cultures were grown for 12 h and cells were harvested by centrifugation. Cell pellets were resuspended in buffer A (20 mM Tris–HCl pH 7.5, 50 mM sodium chloride, 5 mM magnesium chloride) with 1% NP-40, 1 μg ml^{-1} each of aprotinin and pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed by vortexing in Eppendorf tubes containing glass beads. The lysates were centrifuged at 4°C for 2 min in an Eppendorf centrifuge, and the supernatant was used in the GAP assays.

GAP assays were performed according to Trahey & McCormick (1987). Purified H-*ras* p21 (0.07 μM) (Gross *et al.*, 1985) was prebound to GTP by incubation for 30 min at 30°C in buffer B (20 mM Tris–HCl pH 7.5, 20 mM sodium chloride, 2.5 mM magnesium chloride), 0.025 μM [^{32}P]GTP (3000 mCi $mmol^{-1}$, New England Nuclear), 1 mM ATP and 1 mg ml^{-1} bovine serum albumin in a volume of 40 μl. The cell lysates, 0.4 mg in 20 μl, were incubated with 1 μl of p21 prebound to GTP for 30 min at room temperature. The samples were then diluted to 200 μl with buffer A and incu-

bated for 30 min at 4°C with 2 μg of monoclonal antibody Y13-259 (Furth et al., 1982) followed by incubation with rabbit anti-rat immunoglobulin and protein A–Sepharose for 1 h at 4°C. Immunoprecipitates were washed with buffer A, and the nucleotides were eluted from p21 by incubating with 10 μl of 1% SDS and 20 mM EDTA for 5 min at 65°C. Two microliters of eluted nucleotides was resolved by chromatography on polyethyleneimine-impregnated cellulose plates in 0.75 M potassium dihydrogen phosphate (pH 4.0). The chromatography plates were exposed for autoradiography and the labeled nucleotides were scraped off the plates and counted in scintillation fluid.

Analysis of the guanine nucleotide bound to RAS

The analysis was performed essentially as described in Gibbs et al. (1987). Cells expressing human H-*ras* under the control of the *PGK* promoter (strain J1041F7) were grown to A_{600} of 0.6–1.0 in selective media. Cells were collected and incubated in 10 ml of SD-P media with 1 mCi of [^{32}P]orthophosphate (NEN-Dupont) for 3 h. The cultures were collected by centrifugation and washed once with 40 ml of water. The pellets were resuspended in 1 ml of cold lysis buffer containing 50 mM Tris–HCl pH 7.4, 20 mM magnesium chloride, 100 mM sodium chloride, 0.5% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and 1 μg ml^{-1} each of leupeptin and aprotinin and disrupted by vortexing with glass beads. The lysates were centrifuged for 15 min at 4°C in an Eppendorf centrifuge and the lysates containing 1.0×10^8 c.p.m. of trichloracetic acid-insoluble radioactivity were subjected to immunoprecipitation as described above for the GAP assays. The nucleotides were eluted and resolved as described above and the chromatography plates were exposed to X-ray film for direct quantitative measurements with a Fujix BAS2000 bio-imaging analyser. The percentage GTP of total guanine nucleotides was calculated from these analyses by assuming uniform ^{32}P labeling of all phosphates and by correcting for the moles of phosphate per mole of guanosine.

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