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Role of Neurofibromin in Modulation of Expression of the Tyrosinase-Related Protein 2 Gene[1]

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Tyrosinase-related protein 2 (TRP-2)/DOPAchrome tautomerase is an enzyme involved in melanin biosynthesis and plays an important role in cytoprotection by preventing the production of a toxic melanin precursor, 5,6-dihydroxyindole. Neurofibromin is the protein product of a gene linked to neurofibromatosis type 1 (NF1), which is characterized by multiple neurofibromas and abnormalities in skin pigmentation. To explore the pathogenesis of NF1, we studied the role of neurofibromin in the regulation of TRP-2 gene expression. By means of transient cotransfection assays, we show that the expression of a reporter gene under the control of the TRP-2 gene promoter was increased by a neurofibromin-dependent signal through the 71-bp region (positions −415 to −345). A Lys-to-Glu substitution at position 1425 in neurofibromin abrogated this activating function. A dominant negative Ki-ras inhibitor mimics neurofibromin's function, and additively increases TRP-2 promoter activity when coexpressed with neurofibromin. Therefore, we suggest that neurofibromin is involved in the regulation of TRP-2 gene expression. Moreover, we found a single case of a glioblastoma multiforme that expresses TRP-2 mRNA but not tyrosinase mRNA, suggesting that TRP-2 may function in human neural tissues under certain conditions.

Key words: gene transcription, melanin, melanocyte, neurofibromatosis, tyrosinase-related protein-2.

Tyrosinase [EC 1.14.18.1], tyrosinase-related protein 1 (TRP-1), and TRP-2 [EC 5.3.2.3] are enzymes involved in melanin biosynthesis, and they exhibit 40% amino acid identity. Tyrosinase is the rate-limiting enzyme in melanin biosynthesis that catalyzes the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and that of DOPA to DOPAquinone (*1*). DOPAquinone is spontaneously converted to DOPAchrome, which is subsequently converted to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by TRP-2, also termed DOPAchrome tautomerase (*2-5*). DHICA is then converted to indole-quinone-carboxylic acid by TRP-1, also known as DHICA oxidase (*6, 7*). These three enzymes are associated with melanosomes, a special organelle for melanin production, and form large melanogenic complexes within melanosomes (*8*).

DOPAchrome is spontaneously converted to 5,6-dihydroxyindole under certain conditions (*9*), which is more toxic than DHICA to melanoma and non-melanocytic cells (*10*). On the other hand, DHICA has been shown to stimulate the production of nitric oxide by macrophages (*11*). Thus, TRP-2/DOPAchrome tautomerase may play an important role in cytoprotection in the skin by regulating the levels of DHICA and 5,6-dihydroxyindole. Moreover, the TRP-2 gene is expressed in migratory melanoblasts, the telencephalon and the endolymphatic duct in developing mouse embryos (*12*), in which the tyrosinase and TRP-1 genes are not expressed. It was therefore suggested that the TRP-2 expressed in these tissues is involved in detoxification of the metabolites derived from DOPA, an intermediate product in catecholamine biosynthesis.

Neurofibromatosis type 1 (NF1), a common autosomal dominant disorder, primarily affects cells of neural crest origin, and is characterized by multiple *café-au-lait* skin spots, Lisch nodules on the iris, and multiple neurofibromas (*13*). *Café-au-lait* spots are present at birth or completed by the first few years in all affected children, and are characterized by increased numbers of epidermal melanocytes. Lisch nodules consist of masses of melanocytes. These clinical observations indicate that a mutant neuro-

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Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; TRP-1, tyrosinase-related protein 1; TRP-2, tyrosinase-related protein 2; NF1, neurofibromatosis type 1; GAP, GTPase-activating protein; GRD, GAP-related domain; kb, kilobase pairs; bp, base pairs.

fibromin allele results in the overgrowth of melanocytes. A gene linked to NF1 encodes neurofibromin that possesses a domain related to GTPase-activating protein (GAP) (*14-17*) and functions as a negative regulator of ras protein by stimulating its GTPase activity (*18-20*). It has been reported that expression of the stably integrated neurofibromin gene in a melanoma cell line inhibits its growth and increases the amount of immunoreactive tyrosinase (*21*). Subsequently, we have shown that the overexpression of the full-length rat neurofibromin cDNA increased the transient expression of the reporter gene linked to the human tyrosinase gene promoter (*22*). The activation of the tyrosinase gene promoter may be due to an indirect effect of neurofibromin on the promoter activity, because neurofibromin is not localized in the nucleus (*23, 24*). Neurofibromin may therefore modulate an intracellular signal, which triggers activation of transcription of the tyrosinase gene.

Here we provide evidence that neurofibromin may modulate the regulation of TRP-2 gene expression. Considering the above mentioned properties of TRP-2, we suggest that TRP-2 may be a key protein in melanogenesis that is subject to regulation by neurofibromin.

MATERIALS AND METHODS

Plasmid Construction—The rat full-length neurofibromin cDNA, representing type I mRNA (*25, 26*), was cloned into pRc/CMV (Invitrogen), a mammalian expression vector containing the cytomegalovirus promoter, yielding pRc/CMV-RNF (*22, 27*). The human GAP cDNA, PUC101A (*28*), was a gift from Dr. F. McCormick (Onyx Pharmaceuticals), and its *Eco*RI/*Eco*RI fragment (positions −119 to 3863), containing the entire protein-coding region, was recloned into the *Hin*dIII site of pRc/CMV, yielding pRc/CMV-GAP. The 5′ *Eco*RI site represents the linker sequence, and the ends of both DNA fragments had been blunt-ended prior to ligation. The three c-Ki-ras expression plasmids, a wild type construct, pCEV4-Ki-ras (*29*), an activated mutant (V12-ras), pCEV4-Ki-ras^{Val12}, and a dominant negative mutant (N17-ras) construct, pCEV4-Ki-ras^{Asn17} (*30*), were gifts from Dr. K. Kaibuchi (Nara Institute of Science and Technology). For some experiments, each of the full-length ras cDNAs was recloned into the *Hin*dIII site of the pRc/CMV vector by blunt-end ligation, yielding pRc/CMV-Ki-ras, pRc/CMV-Ki-ras^{Val12}, and pRc/CMV-Ki-ras^{Asn17}, respectively.

Transient Coexpression Assays—MeWo human melanoma cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The MeWo cells ($1\text{-}2\times10^5$) in a 3-cm dish were transfected by the calcium phosphate method as described previously (*22, 31*). Fusion genes, comprising the 5′-flanking region of the human tyrosinase or TRP-2 gene upstream from the firefly luciferase reporter gene (*32*), were constructed as described previously (*31, 33*). The amounts of DNA used for cotransfection were 2 μg of either neurofibromin, GAP or ras cDNA, 2 μg of a test fusion gene, and 0.8 μg of β-galactosidase expression vector pCH110 containing the simian virus 40 early promoter (Pharmacia), unless otherwise stated. The optimum plasmid DNA concentrations were determined in each series of experiments. Soluble extracts of transfected cells were prepared, and assayed for luciferase and β-galactosidase activity as described previously (*34, 35*). The luciferase activity was normalized as to β-galactosidase activity (an internal control) to correct for the variability in transfection efficiency.

Site-Directed Mutagenesis of Neurofibromin cDNA—To construct an expression plasmid coding for a mutant neurofibromin, E1425-RNF, which has the substitution of Glu (GAG) for Lys (AAG) at position 1425, an *Sac*I/*Sac*I fragment (3669/6688) containing the GAP-related domain (GRD) was isolated from pRc/CMV-RNF and then cloned into the *Sac*I site of pBluescript (Stratagene). The A residue at position 4273 was changed to a G residue in this subclone using a Transformer™ site-directed mutagenesis kit (Clontech) according to the method reported by Deng and Nickoloff (*36*). The mutagenic primer used was the oligonucleotide, RNF•A→G (5′-CTGAAGGACCTCTGACATTAACTTC-3′, complementary to positions 4260 to 4284, except for the residue, C, underlined). The *Aor*51HI/*Nde*I fragment containing this point mutation was then isolated from a mutant subclone and ligated to pRc/CMV-RNF, which had been linearized with *Aor*51HI and partially digested with *Nde*I, yielding pRc/CMV-E1425RNF. The nucleotide residues shown were numbered from the A residue of the ATG codon for the initiation Met of rat neurofibromin cDNA (*27*).

Northern Blot Analysis—Total RNAs of human brain and brain tumors were derived from the same preparations as used in a previous study (*37*). RNA (15 μg) was denatured with formaldehyde and then electrophoresed in an agarose gel (1.0%) with MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer (pH 7.0). RNAs were transferred to a Zeta-Probe blotting membrane (Bio-Rad), and then fixed with UV Stratalinker 1800 (Stratagene). The blot was hybridized with a radiolabeled probe under the conditions of 50% formamide-5×SSC-1% SDS-5×Denhardt-0.1 mg/ml heat-denatured salmon sperm DNA at 42°C. After hybridization, the membrane was initially washed in 2× SSC-0.1% SDS at 65°C and then in 0.1×SSC-0.1% SDS at 65°C, dried, and autoradiographed at −70°C. The hybridization probes used were the *Sac*I/*Eco*RV fragment (positions 358 to 2225) derived from a human TRP-2 cDNA (*4*), and the *Nae*I/*Apa*LI fragment (positions 99 to 1751) derived from a human β-actin cDNA provided by Tokuo Yamamoto (Tohoku University Gene Research Center). Both probes were labeled with [α-^{32}P]dCTP by the random priming method (*38*).

RESULTS AND DISCUSSION

Transactivation of the TRP-2 Gene Promoter by a Neurofibromin-Dependent Signal—We studied the effect of neurofibromin on the TRP-2 gene promoter activity, by means of transient coexpression assays, in MeWo melanoma cells, which are deficient in neurofibromin (*22, 39*). The 447-bp 5′-flanking region of the TRP-2 gene, carried by pHDTL12, has been shown to be sufficient for pigment cell-specific transcription of the reporter gene (*33, 40*). Overexpression of neurofibromin cDNA increased the expression of pHDTL12 about 3-fold, but rather reduced the expression of pHDTL1 containing the 56-bp TRP-2 promoter region (Fig. 1). The basal expression level of pHDTL1 is lower than that of pHDTL12 by more than 4-fold (data not shown; see Fig. 3). As reported previously (*22*), neurofibro-

min increased the expression of pHTL14, containing the 2.7-kb 5′-flanking region of the tyrosinase gene, about 2.5-fold. A small level of activation was detected with pHTL8 containing the 83-bp 5′-flanking region. We also analyzed the effects of GAP, Ki-ras, and a dominant negative Ki-ras mutant (N17-ras). Like neurofibromin, overexpression of GAP cDNA led to a small increase in the expression of pHDTL12 and reduced the expression of pHDTL1. In contrast, no statistically significant effects of GAP were detected on the expression of pHTL14 and pHTL8. The expression of pHDTL12 or pHTL14 was remarkably enhanced by overexpression of N17-ras, but not significantly affected by ras. Thus, N17-ras could mimic the function of neurofibromin. It is noteworthy that no noticeable effects of neurofibromin, GAP and ras constructs were detected on the expression of a TRP-1-luciferase construct containing the 3.5-kb 5′-flanking region of the human TRP-1 gene (data not shown).

Presence of a Neurofibromin-Regulatory Element in the TRP-2 Promoter—We then attempted to localize the *cis*-acting element of the TRP-2 gene responsible for the activation by a neurofibromin-dependent signal. The *cis*-regulatory elements of pHDTL12 required for melanocyte-specific transcription consist of a 32-bp element (positions −447 to −416) and a proximal region (positions −268 to −56). Deletion studies on the TRP-2 gene promoter revealed that overexpression of neurofibromin causes statistically significant increases in the expression of

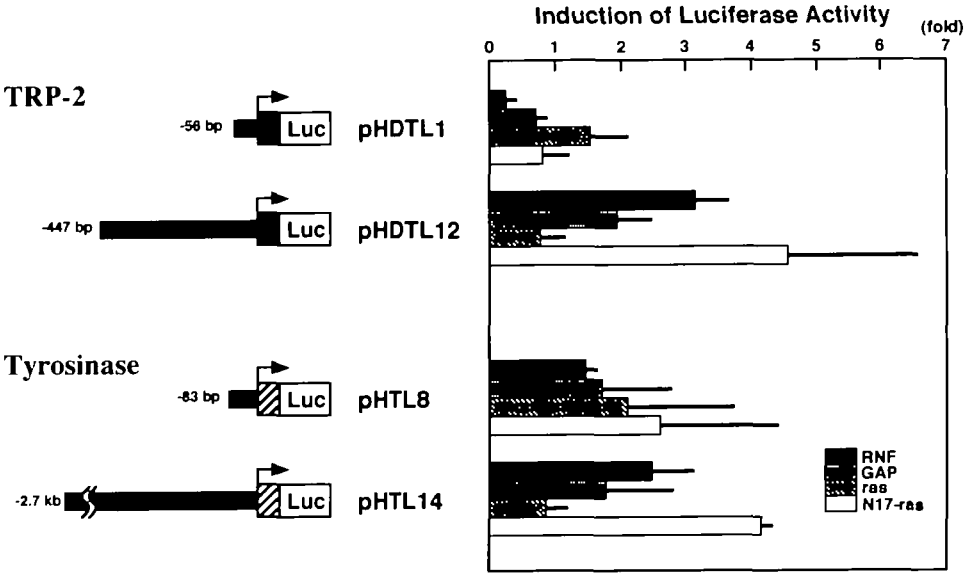
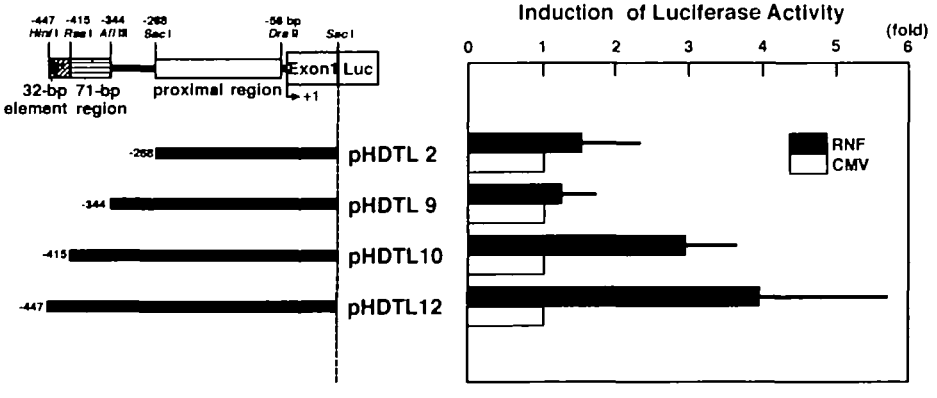


Fig. 1. **Effects of neurofibromin and ras on the promoter activity of the human TRP-2 gene.** MeWo melanoma cells were transiently cotransfected with each fusion gene together with a neurofibromin construct, pRc/CMV-RNF, a GAP construct (GAP), a c-Ki-ras construct (ras), its dominant negative inhibitor (N17-ras), or a vector DNA (pRc/CMV or pCEV4). The promoter regions used for the construction of fusion genes are schematically shown to the left. The direction of transcription is from left to right, as indicated by arrows, and the number shown at the 5′-end of each upstream region represents the position from the transcription initiation site. The level of activation is presented as the ratio of normalized luciferase activity obtained with a neurofibromin construct (RNF) or GAP to that with pRc/CMV vector DNA (CMV). For the data for ras constructs, the level of activation was calculated using the value obtained with pCEV4. The data shown are the means with standard deviations (narrow bars) for at least three independent experiments.



71-bp region:

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-415 ACCCTGGTTCTACATAAATGTTTCTTTATTTTTGAAGTTGCATGACCCCCTGTTGTGGTCTTTTTTGCACA -345
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Fig. 2. **Presence of the *cis*-acting element required for activation of the TRP-2 gene promoter by neurofibromin.** The 32-bp element and the proximal region are indicated by slashed and open boxes, respectively. Each fusion gene was transiently coexpressed in MeWo cells as described in Fig. 1, and the data are presented as the ratio of normalized luciferase activity obtained with a neurofibromin construct (RNF) to that with pRc/CMV vector (CMV). The results of at least three independent experiments are shown with standard deviations. The nucleotide sequence of the 71-bp region is shown at the bottom.

pHDTL12 and pHDTL10 containing the 415-bp 5′-flanking region, but not the expression of pHDTL9 containing the 344-bp region (Fig. 2). Thus, the 71-bp region, comprising positions −415 and −345, is required for the activation mediated by neurofibromin, and may contain a neurofibromin-regulatory element. It is noteworthy that the basal expression level of pHDTL10 lacking the 32-bp element was lower than that of pHDTL12 by 2-3-fold (data not shown), as reported previously (*33, 40*).

Localization of the cis-Regulatory Region That Is Responsive to N17-ras—We then looked for the *cis*-regulatory elements that are responsive to N17-ras (Fig. 3). Expression of pHDTL2, lacking the 71-bp region, was not noticeably affected by neurofibromin, but was significantly increased by N17-ras. The expression of pHDTL1 was rather reduced by either neurofibromin or N17-ras. Thus, the *cis*-acting element that is required for the activation by N17-ras is located in the proximal region (positions −268 to −56), and is separable from the neurofibromin-regulatory element located in the 71-bp region. The basal expression level of pHDTL12M, containing the point mutation in the M box, was lowest and was similar to that of pHDTL1. Neurofibromin did not increase the expression of pHDTL12M, but N17-ras appeared to slightly increase it. These results suggest that the 71-bp region by itself is not sufficient for promoter activation in response to a neurofibromin-dependent signal, and that the cooperation of the 71-bp region with the intact M box is required for the activation of the TRP-2 promoter by neurofibromin.

Antagonistic Effects of ras Proteins and Neurofibromin on the TRP-2 Promoter—We then determined whether or not the activation caused by neurofibromin was modulated through the coexpression of a ras construct (Fig. 4). In this series of experiments, the amounts of total DNA were adjusted to 4 μg with the vector DNA, in which the magnitude of activation obtained with N17-ras was relatively lower compared to that in other series of experiments. The increased expression of pHDTL12 mediated by neurofibromin was partially and completely inhibited by ras and an activated Ki-ras (V12-ras), respectively, whereas expression of pHDTL12 was not noticeably affected by ras, V12-ras, or both ras and V12-ras under the conditions used. On the other hand, statistically significant additive effects of neurofibromin and N17-ras were detected, and the increased expression of pHDTL12 caused by N17-ras was inhibited by V12-ras. All these results also indicate that a neurofibromin-dependent signal enhances the TRP-2 promoter function.

Neurofibromin as a Regulator of TRP-2 Gene Expression—To confirm the specificity of the observed neurofibromin function as a regulator of TRP-2 gene expression, we also analyzed the function of a mutant neurofibromin, E1425-RNF, with a Lys-to-Glu substitution at position 1425 (Fig. 5). This amino acid substitution was chosen, because mutations at Lys 1423 in human neurofibromin, which is equivalent to Lys 1425 in rat neurofibromin, were detected in one family with NF1 and in sporadic tumors

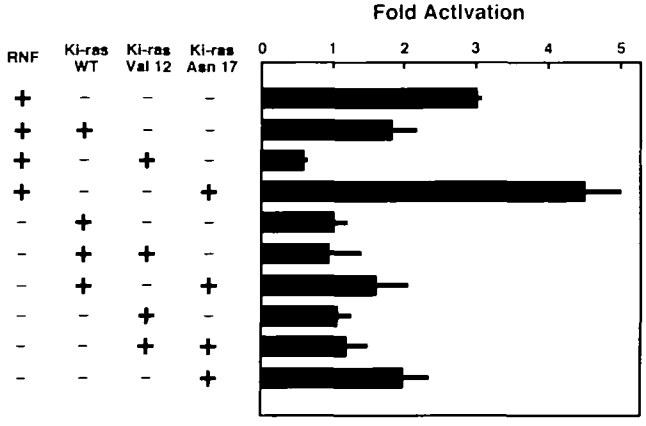

Fig. 4. **Effects of coexpression of neurofibromin and ras proteins on the TRP-2 promoter function.** MeWo cells were cotransfected with pHDTL12 and the indicated combination of test plasmids: neurofibromin (RNF), a wild-type c-Ki-ras (WT), an activated Ki-ras (Val 12), and a dominant negative Ki-ras (Asn 17). In this series of experiments, each ras cDNA was recloned into pRc/CMV as described under "MATERIALS AND METHODS." Equal amounts of a neurofibromin construct and each ras construct were used (2 μg each), and the total amounts of plasmid DNA were maintained at 4 μg with the vector DNA (pRc/CMV). The data are presented as the ratio of normalized luciferase activity obtained with each combination to that with vector DNA. The results of at least three independent experiments are shown with standard deviations.

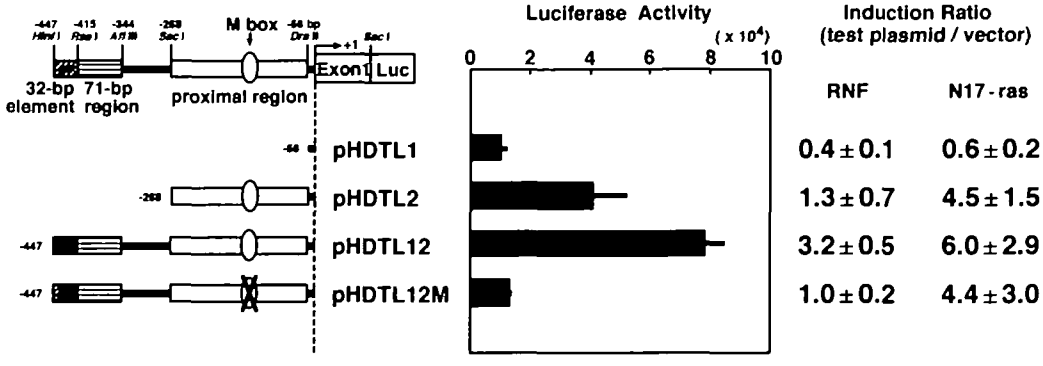

Fig. 3. **Localization of the *cis*-acting region required for activation of the TRP-2 gene promoter by a dominant negative ras inhibitor.** The data shown represent the relative luciferase activity in cells transfected with pRc/CMV. The level of activation is also presented as the ratio of normalized luciferase activity obtained with either a neurofibromin construct (RNF) or a dominant negative ras (N17-ras) to that with the respective vector DNA (pRc/CMV or pCEV4). Similar expression patterns as to the relative luciferase activity were obtained with pCEV4 (data not shown), the values of which were used to calculate the level of activation by N17-ras. The results of at least three independent experiments are shown with standard deviations.

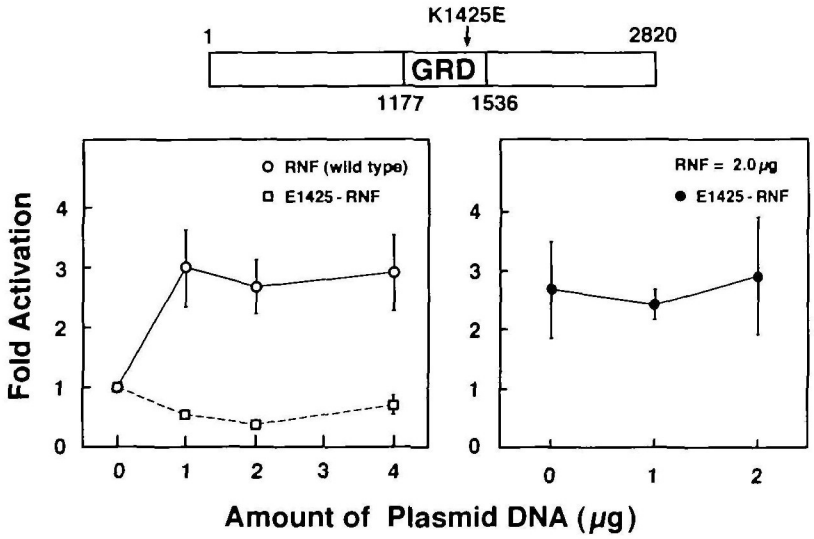

Fig. 5. **Functional analysis of the neurofibromin mutant containing an amino acid substitution, altering the conserved Lys residue.** The structures of neurofibromin and its mutant, E1425-RNF, are schematically shown at the top. The GAP-related domain is denoted as GRD. The effects of neurofibromin and E1425-RNF were compared by means of transient coexpression assays involving pHDTL12. Dose-response experiments are shown to the left. Various amounts of the indicated plasmids were used for transfection. The data are presented as the ratio of normalized luciferase activity obtained with each neurofibromin construct to that with vector DNA (pRc/CMV). The results of three independent experiments are shown with standard deviations (vertical lines). For some points, the vertical lines are not visible due to the small values. In the right panel, the indicated amounts of a construct for E1425-RNF were cotransfected with a constant amount (2 μg) of a wild type construct (RNF).

(*41*). Human GRD, with a Lys-to-Glu substitution at 1423, showed about 200-fold lower GAP activity compared to the activity of the wild type (*41, 42*). Transient coexpression assays demonstrated that E1425-RNF is unable to increase the expression of pHDTL12 (Fig. 5). Furthermore, co-expression of E1425-RNF and neurofibromin showed that E1425-RNF does not affect the level of activation caused by neurofibromin, suggesting that the substitution of Glu for the Lys at position 1425 may lead to a loss of neurofibromin's function. Alternatively, E1425-RNF may be more labile than the wild-type neurofibromin. In any case, these results suggest the lack of a dominant negative effect of E1425-RNF, which is of interest in view of the pathogenesis of dominantly inherited NF1.

Café-au-lait spots are characterized by increased melanogenesis (*13, 43*), and increased numbers of epidermal melanocytes which contain abnormally large pigment granules, known as macromelanosomes (*44*). It has been reported that immunoreactive neurofibromin is reduced in cultured melanocytes of NF1 patients, but alteration of the level of ras-GTP was not detected in these melanocytes (*45*). In addition, no loss of the wild-type allele has been detected in melanocytes of *café-au-lait* skin spots. Thus, the expression of both neurofibromin alleles is essential for the normal differentiation and migration of melanoblasts and melanocytes. In this context, the TRP-2 gene is expressed in migratory melanoblasts shortly after they have left the neural crest in mice (*12*), and thus its expression precedes that of the tyrosinase and TRP-1 genes. Therefore, we suggest that TRP-2 rather than tyrosinase may be the key protein subject to regulation by neurofibromin during melanocyte differentiation.

Differential Regulation of Expression of the TRP-2 and Tyrosinase Genes—To obtain *in vivo* evidence for the functional significance of TRP-2 in human neural tissues, we analyzed the expression of TRP-2 mRNA in human brains and primary brain tumors (Fig. 6). In such brain tumors, neurofibromin mRNAs were expressed (*37*). TRP-2 mRNA of 4.3 kilobases was expressed in a glioblastoma multiforme arising from the frontal cortex (Fig. 6, lane 5), but was not detectable in anaplastic astrocytomas (lanes 1–4) or in various brain regions (data not shown). A

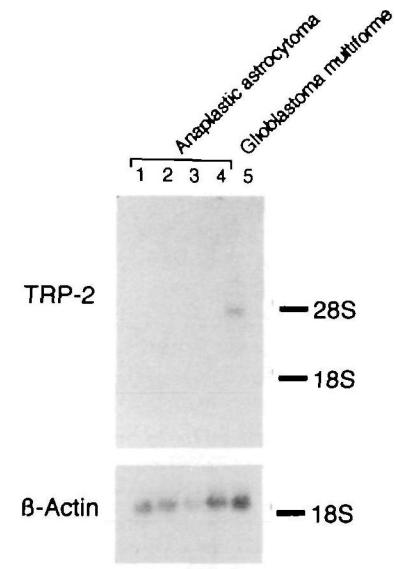

Fig. 6. **Expression of TRP-2 mRNA in a human brain tumor.** Each lane contained total cellular RNA (15 μg) prepared from excised human brain tumors, *i.e.* anaplastic astrocytomas (lanes 1–4) and a glioblastoma multiforme (lane 5). The RNA samples were the same as those used in a previous study (*37*). The bottom panel shows the expression of β-actin mRNA as an internal control.

glioblastoma multiforme is the most malignant brain tumor comprising undifferentiated cells. The expression of tyrosinase and TRP-1 mRNAs was not detectable in such brain tumors (data not shown), indicating that the detected TRP-2 mRNA expression is not due to the presence of melanocytes in the excised tumor tissue. These results suggest that TRP-2 may function in the brain under certain conditions. In this context, TRP-2 is expressed in the telencephalon of developing mouse embryos, and was proposed to be involved in the detoxification of the metabolites derived from DOPA (*12*). NF1 patients often suffer from mild mental retardation and learning disability (*13*), which might be related to the altered expression of the TRP-2 gene at some critical period of neuronal maturation.

It remains to be determined whether or not NF1 patients are associated with decreased expression of TRP-2.

The above observations also suggest that TRP-2 gene expression is regulated in a different manner from the regulation of tyrosinase and TRP-1 gene expression. Consistent with this notion, we have shown by means of transient cotransfection assays that the melanocyte-specific microphthalmia-associated transcription factor transactivates the tyrosinase and TRP-1 gene promoters (*40, 46*), but not the TRP-2 gene promoter (*40*). It is therefore of significance to determine the neurofibromin-regulatory element in the 71-bp region and the *cis*-regulatory element in the proximal region (positions −268 to −56) required for the activation by N17-ras. Such studies will help us identify the factors regulating TRP-2 gene transcription.

Implications—A crucial question remaining to be answered is how the reduced expression of neurofibromin leads to increased melanogenesis in NF1 patients. Neurofibromin by itself is dispensable for the transcription of the tyrosinase, TRP-1 and TRP-2 genes, because these genes are expressed in MeWo melanoma cells, which are deficient in neurofibromin (*22, 39*). It should be noted that reduced neurofibromin level is associated with the formation of macromelanosomes (*44, 45*), which may represent the altered protein-protein interactions within melanosomes. We therefore suggest that a haploinsufficiency of neurofibromin may disturb the formation of melanogenic complexes, containing tyrosinase, TRP-1 and TRP-2, by changing the level of a certain regulatory factor or a certain melanosomal component. However, such an alteration in the melanosomal structure appears to favor the catalytic function of tyrosinase in the melanocytes of NF1 patients (*13, 43*), which could be accompanied by the increased production of DOPAchrome, a substrate for TRP-2. Under these conditions, the spontaneous conversion of DOPAchrome to 5,6-dihydroxyindole is likely to be increased, and the production of DHICA may vary depending on the expression level of TRP-2. Altered levels of these melanin precursors may impair the immune system in the skin of NF1 patients. These notions will help us answer the question of why the abnormal phenotype of NF1 is mainly related to the skin in spite of the ubiquitous expression of the neurofibromin gene (*25, 27*).

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