Neuron, Vol. 10, 335-343, March, 1993, Copyright © 1993 by Cell Press

The Neurofibromatosis Type 1 Gene and Its Protein Product, Neurofibromin

Review

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Introduction

Von Recklinghausen neurofibromatosis, or neurofibromatosis type 1 (NF1), affects approximately 1 in 3500 individuals of all ethnic backgrounds. It is inherited as an autosomal dominant disease and is manifested clinically by abnormalities that predominantly affect tissues which derive from the neural crest (Riccardi, 1981, 1991; Riccardi and Eichner, 1986). Affected individuals are often noted to have multiple cafe-au-lait spots during the first year of life. These pigmented birthmarks contain melanocytes harboring macromelanosomes, but have no clinical significance other than as a diagnostic clue. Similarly, another important clinical feature, the presence of Lisch nodules of the iris, has no associated morbidity. However, these hamartomas appear during childhood and are eventually present in close to 100% of affected adults. Neurofibromas, which give the disease its name and usually make their appearance just before or during adolescence, are benign cutaneous tumors consisting of Schwann cells, fibroblasts, and other cellular elements. They increase in size and number with age, but at an unpredictable rate. More deeply placed neurofibromas, called plexiform lesions, usually appear in childhood and can lead to significant complications due to associated overgrowth of nearby tissues. Furthermore, such plexiform lesions have a modest but significant risk of degenerating into malignant neurofibrosarcoma, a highly invasive soft tissue tumor that is frequently fatal. This potential, plus the risk of optic glioma (which affects 2%-5% of all individuals with NF1) justifies placing this disease on the list of familial cancer syndromes (Bader, 1986).

Other features of NF1 are variable but can be significant in a given patient. Approximately half of affected individuals have at least some degree of learning disability, and a small percentage have frank mental retardation (Riccardi, 1981). Seizures are present in about 5% of individuals, and megalencephaly is a typical finding. The diagnosis is usually not difficult to make in an adolescent or adult, but can occasionally present difficulties in a very young child. NF1 is characterized by extreme variability, even among individuals within the same family who carry the same mutation.

Approximately two-thirds of individuals with NF1 lead relatively normal lives, with occasional interruptions for surgical management of their disease, often to remove neurofibromas that are causing cosmetic or physical distress. About one-third of individuals suffer a severe complication sometime during their lifetime. NF1 has occasionally been erroneously referred to as "the elephant man disease"; while the gross facial distortion and other deformities present in Joseph Merrick, the Elephant Man, in some ways resemble the most severe end of the spectrum of NF1, subsequent evaluation of Merrick's skeleton indicates that he probably had another disorder known as Proteus syndrome (Tibbles and Cohen, 1986).

Cloning of the *NF1* Gene

The gene for NF1 was identified in 1990 by positional cloning, an approach that relies on isolating an unknown gene through knowledge of its chromosomal location (Figure 1) (Viskochil et al., 1990; Cawthon et al., 1990b; Wallace et al., 1990). The *NF1* gene spans over 300 kb of genomic DNA and encodes an mRNA of 11-13 kb found in all tissues examined (Figure 2) (Wallace et al., 1990; Marchuk et al., 1991). This rather large gene contains approximately 50 exons and produces a protein of 2818 amino acids with a predicted molecular mass of 327 kd (Marchuk et al., 1991). Of particular interest are three genes found buried within one intron on the DNA strand opposite the strand coding for the *NF1* gene (Viskochil et al., 1990). Two of these genes are human homologs of genes postulated to be activated by retroviral insertion in murine leukemia (*EVI2A* and *EVI2B*), and the third gene (*OMgP*) encodes the oligodendrocyte myelin glycoprotein expressed in myelinating Schwann cells and oligodendrocytes (Cawthon et al., 1990a, 1990b; O'Connell et al., 1990; Viskochil et al., 1991). The relationship between these embedded genes and NF1 remains to be elucidated.

Based on the presence of multiple tumors in affected patients and the fact that the first few mutations in the *NF1* gene appeared to inactivate it, *NF1* was hypothesized to represent one member of the growing family of tumor suppressor genes (Stanbridge, 1990; Ponder, 1990). It was predicted, furthermore, that the *NF1* gene product might have an important role in modulating cell growth and differentiation in the nervous system.

Identification of the *NF1* Gene Product

Examination of the predicted protein sequence of the *NF1* gene product, termed neurofibromin, revealed sequence similarity between neurofibromin and various members of the GTPase-activating protein (GAP) superfamily (Figure 3) (Xu et al., 1990a, 1990b; Martin et al., 1990; Ballester et al., 1990). This family includes mammalian GAP, budding yeast IRA1 and IRA2, fission yeast sar1, and Drosophila Gap1. The first GAP

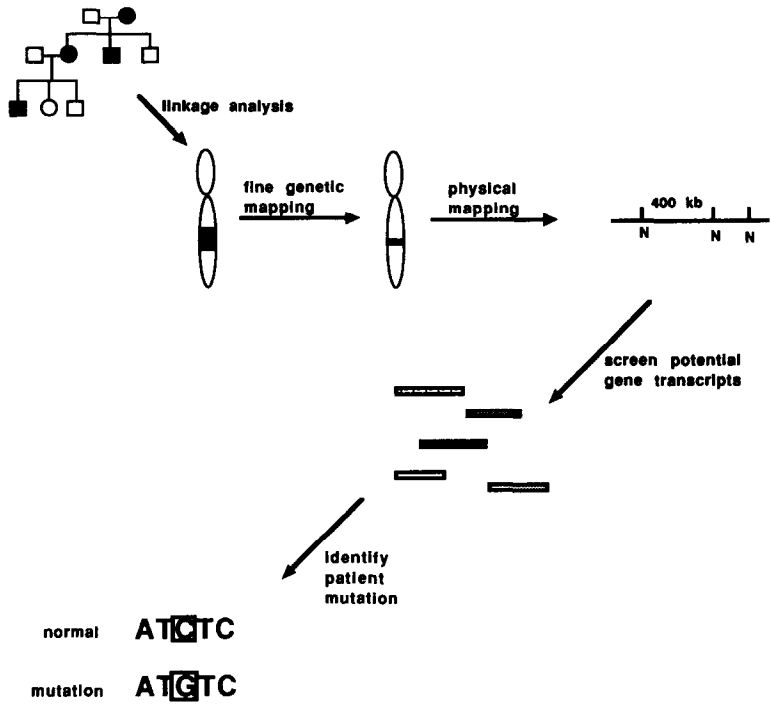

Figure 1. General Scheme Employed in Positional Genetic Cloning

Initially, many informative families are studied until a marker is found that shows linkage with the disease. The linked DNA markers are localized to a specific region of a chromosome. Additional DNA markers clustered within the originally defined broad chromosomal region are used to analyze families with the disease in order to narrow the interval to 1–2 Mb (1 cM ~ 1,000,000 nucleotides or 1 Mb), provided sufficient families are available. At this point, efforts are directed at cloning the entire region using the DNA markers most tightly associated with the disease, as determined by linkage analysis. This can be accomplished by various technologies, including yeast artificial chromosomes, chromosomal jumping, and cosmid cloning. The DNA segments covering the interval are then used to screen cDNA libraries in an effort to identify potential transcripts for the disease gene. Proof that the gene identified represents the desired disease gene requires the demonstration of mutations in that gene in patients with the disease (Taken from Collins, 1992.)

to be well characterized was mammalian p120–GAP (Trahey and McCormick, 1987; Vogel et al., 1988). This protein is highly expressed in brain and placenta and is involved in growth factor–mediated signal transduction pathways. The two yeast genes, *IRA1* and *IRA2*, code for proteins that regulate RAS in Saccharomyces cerevisiae and participate in the regulation of adenylate cyclase (Tanaka et al., 1989, 1990a, 1990b, 1991). In contrast, the *sar1* gene product from Schizosaccharomyces pombe regulates Ras1 but does not appear to participate in the modulation of adenylate cyclase (Wang et al., 1991). Recently the Drosophila homolog of GAP was identified and shown to be a negative regulator of Ras1 (Gaul et al., 1992). Activation of Ras1 is critical for the orderly development of the fruitfly compound eye and represents part of a signal transduction pathway involving receptor-mediated tyrosine phosphorylation (see below) (Pawson and Bernstein, 1990). The shared sequence similarity between these diverse proteins lies within a 250–400 amino acid domain, termed the catalytic domain, which interacts with a cellular proto-oncogene, $p21^{ras}$, accelerating its conversion from an active GTP-bound state to an inactive GDP-bound state (Figure 4) (Wigler, 1990). Activation of *ras* has previously been shown to promote cell proliferation in some mammalian cells and differentiation in others (see below) (Bourne et al., 1990, 1991).

The *NF1* gene product has no transmembrane domains, definite nuclear localization signals, or other common protein motifs. It contains several potential sites for phosphorylation, raising the possibility that neurofibromin could be regulated by kinases (Marchuk et al., 1991).

To understand the function of neurofibromin better, antibodies were generated by several groups against both fusion proteins and synthetic peptides (DeClue et al., 1991; Gutmann et al., 1991; Daston et al., 1992; Basu et al., 1992). These antibodies all recognize a protein with an M_r of ~250,000, expressed in all tissues but at highest levels in brain, kidney, and spleen. The broad expression of neurofibromin, including high levels in brain, was an unexpected finding given the disease phenotype. Although neurofibromin can be detected in crude homogenates from all tissues, careful quantification reveals that the expression of neurofibromin in brain is greater than it is in spleen, adrenal gland, or kidney (Gutmann and Collins, unpublished data). The cells within the kidney that express neurofibromin remain to be elucidated. On the other hand, the expression of neurofibromin in spleen is the result of expression in both T and B lymphocytes (Gutmann and Collins, unpublished data). Neurofibromin expression in other tissues (lung, muscle, skin, or gonads) is barely detectable by immunohistochemical analysis. With sensitive polymerase chain reaction–based methods, however, *NF1* mRNA appears to be ubiquitously expressed (Wallace et al., 1990).

Neurofibromin is expressed in many neuronal cell types, irrespective of neurotransmitter expression, neuronal pathway, or brain region. This protein in neurons appears to be most abundant in dendritic processes, where input messages would likely be transduced (Daston et al., 1992). This is in keeping with a protein that may be involved in signal transduction pathways, but other roles are possible. Other cells

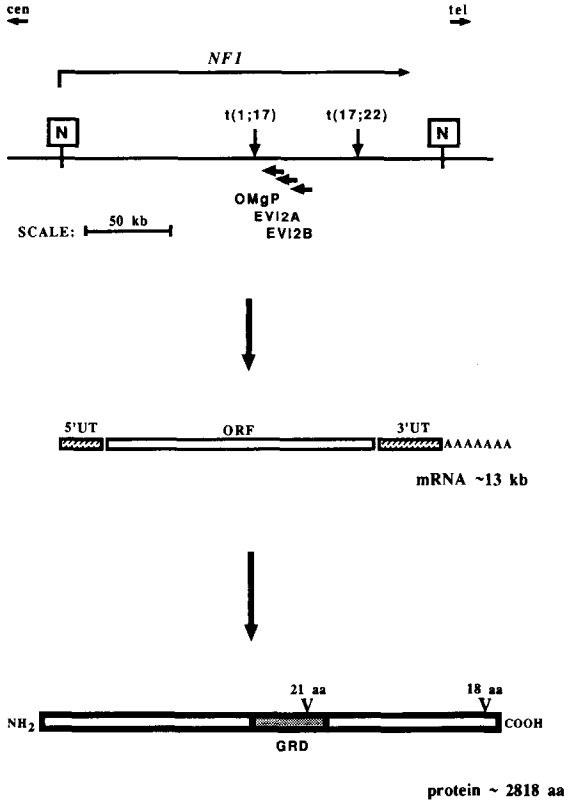

Figure 2. Genetic Organization of the *NF1* Locus

The genomic architecture of the *NF1* locus demonstrates the presence of two NotI restriction sites (N) separated by ~300 kb. The initiation codon is upstream of the centromeric NotI site and is found within a CpG-rich area, as has been described for many genes. The position of the two translocation breakpoints, t(1;17) and t(17;22), found in two patients with NF1 is depicted, and their interruption of the *NF1* gene is evident. Three embedded genes (*OMgP*, *EVI2A*, and *EVI2B*) are located within one intron on the opposite strand and are transcribed in the opposite orientation from the *NF1* gene. The mRNA for *NF1* is 13 kb and contains an open reading frame of 8454 nucleotides with at least 2 kb of 3′ untranslated sequence. Translation of the open reading frame predicts a protein of 2818 amino acids and an estimated molecular mass of 327 kd. Sequence similarity between a central 300–400 amino acid domain of the *NF1* gene product, neurofibromin, and a family of GAPs is denoted by the GRD. The presence of two alternatively spliced isoforms is denoted by the 21 amino acid and 18 amino acid insertions into the GRD and the carboxyl terminus of neurofibromin, respectively.

within the central nervous system that express neurofibromin include oligodendrocytes and cortical neurons. In the peripheral nervous system, this protein is expressed in nonmyelinating Schwann cells, dorsal root ganglia, and peripheral nerves (Daston et al., 1992).

The observed molecular size of neurofibromin (~250 kd) is smaller than would have been predicted based on translation of the *NF1* open reading frame (327 kd). This discrepancy is most likely the result of protein folding during migration through denaturing polyacrylamide gels. There is no evidence for glycosylation or processing of the full-length protein (Gutmann and Collins, unpublished data). There is one well-characterized alternatively spliced isoform of neurofibromin that has been termed type 2 neurofibromin (Nishi et al., 1991; Andersen et al., 1993a). This isoform contains an additional 21 amino acids inserted within the GAP-related domain (GRD) and is expressed at the RNA level in all tissues examined. Type 2 *NF1* mRNA is detectable in many species, including mouse, rat, and chicken. Expression of the type 2 NF1 GRD in yeast complementation studies demonstrates that it has weaker GAP properties than the type 1 NF1 GRD form. The relative abundance of the type 1 versus the type 2 form is modulated differentially during brain development and can be affected by stimulation with retinoic acid (Nishi et al., 1991). The predominant mRNA at 20 weeks of human fetal development is type 1 *NF1* mRNA. A rapid switch begins before week 22, culminating in mainly type 2 *NF1* mRNA in the adult brain. The expression of type 1 versus type 2 *NF1* mRNA has also been studied using neuroblastoma cell lines (Nishi et al., 1991). Stimulation of the neuroblastoma cell line, SH-SY5Y with retinoic acid (a treatment that promotes differentiation of SH-SY5Y cells to a more neuronal cell phenotype) results in the rapid switch from type 1 *NF1* mRNA to type 2 *NF1* mRNA expression. This mRNA switch is not observed with an astrocytoma cell line similarly treated with retinoic acid. The significance of both of these findings at the protein level awaits further elucidation.

Another isoform, generated by the insertion of an additional 18 amino acids near the extreme carboxyl terminus of the protein, has been reported at the mRNA level, but further work will be required to characterize this isoform (Cawthon et al., 1990b).

The availability of antibody reagents has allowed an examination of the subcellular localization of neurofibromin. It appears that neurofibromin is expressed predominantly in the cytoplasm and is in some way associated with the intracytoplasmic cytoskeleton. Recent experiments demonstrate the unexpected colocalization of neurofibromin with cytoplasmic microtubules (Gregory et al., 1993; Gutmann et al., 1992, J. Cell Biochem., abstract). Further support for this subcellular localization is provided by biochemical copurification experiments during which brain neurofibromin is enriched in polymerized microtubule fractions. It is not clear at this time whether the type 2 neurofibromin isoform also associates with microtubules. This location suggests that neurofibromin may be involved in signal transduction pathways associated with microtubules and in that fashion, play some role in mediating cytoarchitectural alterations coincident with neoplastic transformation.

Mutational Basis of Disease

Whereas the inherited predisposition to developing the clinical manifestations of NF1 is transmitted in a Mendelian autosomal dominant fashion, it is hypothe-

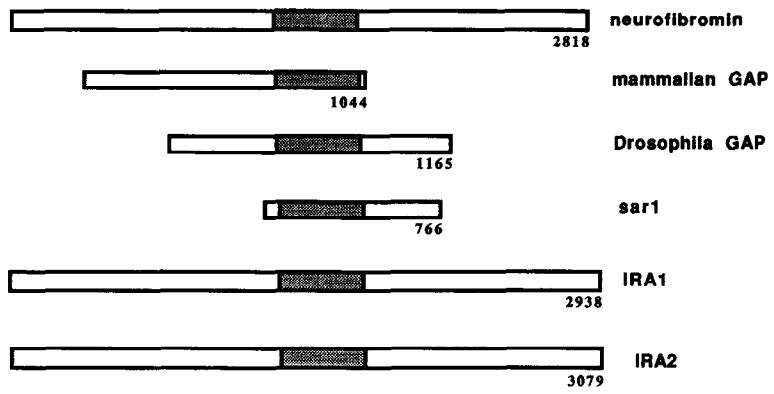

Figure 3. Sequence Similarity between Neurofibromin and Members of the GAP Family

The shaded areas represent the regions of sequence similarity between neurofibromin and the catalytic GRDs of these other proteins. The size of each protein is denoted by the number of amino acid residues. For details, refer to text.

sized that pathology arises only when both copies of the *NF1* gene are disrupted (Knudson, 1971). The inherited predisposition represents the *germline* mutation, which disrupts one of the two normal copies of the *NF1* gene and is found in all cells of NF1 patients. To produce a neurofibroma or any other clinical feature of NF1, a second mutational event must presumably occur to eliminate the remaining functional gene. This second "hit" represents the *somatic* mutation. Thus, although NF1 is an autosomal dominant disorder in families, it is recessive at the cellular level. This is a unifying feature of diseases attributable to tumor suppressor gene abnormalities.

Tumor formation arises as a result of alterations in genes that regulate cell proliferation. In normal cells, there are many such genes whose products control cell growth and whose absence, due to mutation, results in a state of uncontrolled cell proliferation or neoplasia (Marshall, 1991). These genes are collectively referred to as tumor suppressor genes. The fact that there are many such genes provides support for the notion that tumorigenesis represents a multistep process: Loss of one tumor suppressor gene product may enlarge a population of cells with mildly altered cell growth potential and create a substrate for subsequent tumor suppressor gene mutations, culminating in neoplasia.

The best example of an inherited predisposition to cancer has come from the study of retinoblastoma, the most common intraocular malignancy in children. Retinoblastoma can occur sporadically or as a heritable condition. The notion that there exists a primary inherited genetic alteration which predisposes to the development of retinoblastoma is supported by cytogenetic analysis demonstrating a visible deletion in one area of chromosome 13 (band q14), representing the *germline* mutation (Cavanee et al., 1983). Analysis of matched pairs of constitutional and tumor genotypes in cases of retinoblastoma demonstrated an additional alteration in the tumor chromosome 13, representing the acquired *somatic* mutation and disrupting both retinoblastoma tumor suppressor genes (Cavanee et al., 1985). Osteosarcoma and premenopausal breast cancer are also associated with retinoblastoma, and similar alterations on chromosome 13q14 were also found when these tumors from retinoblastoma patients were analyzed (reviewed in Hansen and Cavenee, 1988).

To date, only a small number of germline mutations have been characterized in NF1 patients. The large size of the gene has represented a considerable barrier to such investigations, and only a minority of patients have had the sort of gross rearrangements that are easily detectable. No hot spots for mutation have been identified, and there is no readily discernable genotype–phenotype correlation. The latter is not really surprising, since the very large degree of phenotypic variability commonly observed among affected members of the same family would preclude the possibility that severity is tightly defined by the specific

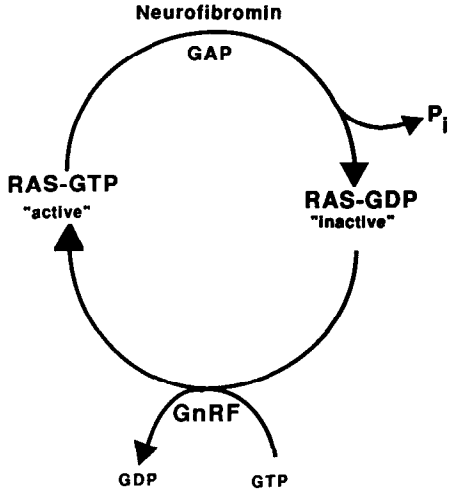

Figure 4. Regulation of Ras by GAP-Related Proteins

Ras is active in the GTP-bound state. Association of GAP-related proteins with ras accelerates the conversion of $p21^{ras}$·GTP to $p21^{ras}$·GDP by increasing the intrinsic GTPase activity of ras. Hydrolysis of $p21^{ras}$·GTP converts ras to the inactive GDP-bound form. Ras is converted to the active state by guanosine nucleotide replacing proteins, which substitute GTP for GDP and complete the cycle. In resting cells, the majority of ras is in the inactive, GDP-bound form.

Table 1. Characterized Germline *NF1* Mutations in Patients with NF1

Mutation	Result	Reference
Deletions		
40 kb deletion	–	Viskochil et al., 1990
190 kb deletion	–	Viskochil et al., 1990
11 kb deletion	–	Viskochil et al., 1990
90 kb deletion	–	Upadhyaya, 1992
10 Mb deletion	–	Kayes, 1992
Insertions		
320 bp Alu insertion	Splice disruption, frameshift	Wallace et al., 1991
Translocations		
1;17 translocation	–	Fountain et al., 1989 O'Connell, 1989
17;22 translocation	–	Fountain et al., 1989 O'Connell 1989
Point Mutations		
Lys-1423 to Glu Lys-1423 to Gln	Nonconservative amino acid changes; reduced GAP activity	Li et al., 1992
Arg-1610 to stop	Creation of stop codon	Cawthon et al., 1990b Estivill et al., 1991
Leu-1595 to Pro	Conservative amino acid change; ? polymorphism	Cawthon et al., 1990b

NF1 mutation. The majority of mutations described thus far have been alterations (nonsense mutations, deletions, frameshift mutations, and insertions) that would be predicted to result in no neurofibromin production from that allele (Table 1). One convincing missense mutation within the GRD at residue 1423 has been identified in an NF1 family (Li et al., 1992). The NF1 GRD harboring this mutation, when expressed in insect Sf9 or bacterial cells, had 200- to 400-fold reduced GAP activity, suggesting that this mutation affects the normal function of neurofibromin.

Examination of tumors from NF1 patients is beginning to uncover somatic alterations of *NF1* on the DNA and protein levels. Benign neurofibromas are difficult to examine for altered neurofibromin expression, since they harbor a mixed population of cells. Although they presumably contain some cellular element with a second hit, the expression of neurofibromin is presumably normal in the other unaffected cells. On the other hand, neurofibrosarcomas, malignant schwannomas derived from neurofibromas, have been shown to be clonal and to express nearly undetectable levels of neurofibromin (Basu et al., 1992; DeClue et al., 1992). In one NF1 neurofibrosarcoma, a specific somatic 200 kb deletion eliminating the amino-terminal half of one *NF1* gene was detected (Legius et al., 1993).

In general, tumor suppressor genes that predispose to familial cancer syndromes when inherited through the germline are also capable of playing a role in sporadic malignancy by somatic mutation. The same appears to be true of *NF1*. Examination of sporadic metastatic malignant melanoma cell lines revealed the absence of protein and/or mRNA in 2 of 9 cell lines and a large homozygous deletion involving the amino-terminal half of the *NF1* gene in one tumor (Andersen et al., 1993b). The codon 1423 mutation described above has also been found in a sporadic colon adenocarcinoma, a patient with myelodysplastic syndrome, and a sporadic anaplastic astrocytoma (Li et al., 1992). These findings are consistent with the notion that neurofibromin, like p53, is a tumor suppressor gene product that is disrupted on the more general pathway to malignant transformation and neoplasia. Further work will be required to determine what role neurofibromin plays in the progression to the malignant state.

Function(s) of Neurofibromin

Three specific properties of neurofibromin have been suggested by recent studies: regulation of cellular proto-oncogenes (*ras*) important in growth and differentiative pathways; association with cytoplasmic microtubules; and involvement in phosphorylation-mediated signal transduction pathways. Neurofibromin, like its homologs in yeast and mammals, is a GAP and serves, at least in part, to accelerate the conversion of the active $p21^{ras} \cdot GTP$ to the inactive $p21^{ras} \cdot GDP$ (Figure 4). In yeast, ras is involved in regulation of cAMP levels through adenylate cyclase. However, in mammals its role is much less clear.

Two models have been proposed for the interaction between ras and GAP (McCormick, 1989): One model (the upstream model) envisions GAP acting as a regulator of ras, accelerating the conversion of ras from the active to the inactive form through GTP hydrolysis. The second model (the downstream model) predicts that GAP functions as an effector for ras and that conversion of ras to the GDP-bound form transmits a sig-

nal through GAP to other proteins within the cytoplasm. Neither of these models is mutually exclusive, and it is equally possible that GAP and ras transmit a signal only when complexed together.

Ras has been shown to mediate differentiation in some tissue types (Schwann cells and PC12 pheochromocytoma cells) and proliferation in others (fibroblasts) (Bar-Sagi and Feramisco, 1985; Feramisco et al., 1984; Noda et al., 1985). Regulation of this important cellular modulator is therefore critical for the maintenance of the differentiated or proliferative state. To this end, neurofibromin in its role as a tumor suppressor protein may keep the brake on ras, and its absence through mutation may result in disturbed growth or differentiative properties. Support for this notion derives from the study of malignant neurofibrosarcoma cells from patients with NF1 (Basu et al., 1992; DeClue et al., 1992). In these cells, a large proportion of $p21^{ras}$ is in the active, GTP-bound form and little or no neurofibromin can be detected. Given no neurofibromin to down-regulate ras in these cells, $p21^{ras}$ remains active and presumably contributes to the abnormal growth characteristics of these cell lines. In addition, these neurofibrosarcoma cells harbor other chromosomal aberrations that also may contribute to the phenotype of these malignant cells.

The fact that ras is activated in these neurofibrosarcoma cell lines which do not express neurofibromin yet have functional GAP molecules raises the question, what does GAP actually have to do with ras regulation? The inability of GAP to down-regulate ras in these cells may be explained in a number of ways: First, it is possible that GAP is actually a target or effector of ras function and does not down-regulate ras in vivo in Schwann cells. Second, in Schwann cells, it may be that neurofibromin, and not GAP, is the predominant regulator of ras, and although both are present and functional, only neurofibromin performs this function. Support for this notion comes from experiments demonstrating the selective inhibition of GAP versus neurofibromin catalytic activity by lipids. In Schwann cells, the predominant GAP activity is attributable to neurofibromin and not GAP (Bollag and McCormick, 1991). Lastly, although both are present and functional, the ability to regulate ras depends on the presence of other intracytoplasmic proteins, and the use of neurofibromin versus GAP as the primary ras regulator may depend on the availability of these proteins. To this end, in Schwann cells, the proteins that interact with GAP for adequate GAP function may not be available, and neurofibromin is used preferentially. There are currently no experimental data available to support this model.

The association of neurofibromin with microtubules suggests additional roles for the *NF1* gene product. Neurofibromin could function as a regulator of *ras* when released from microtubules, but may be kept distant from $p21^{ras}$ by virtue of its association with the cytoskeleton when it is necessary that ras remain active. It is also possible that neurofibromin plays some specific role in the regulation of microtubule-mediated functions through its interaction with tubulin.

Many cellular proto-oncogenes are regulated through phosphorylation events initiating at the cell surface and culminating in transcriptional regulation within the nucleus (Aaronson, 1991). Mammalian GAP is tyrosine phosphorylated in response to growth factor stimulation and in this fashion may be regulated either through its association with a distinct subset of proteins (such as c-mos, c-raf, or ERK proteins) or by down-regulation of its GTPase activating properties (Ellis et al., 1990; Downward et al., 1990). Neurofibromin is also phosphorylated in response to growth factors, but not on tyrosine residues (Gutmann et al., 1992, Neurology, abstract). Instead, neurofibromin is heavily phosphorylated on serine and threonine residues in response to epidermal and platelet-derived growth factors. This phosphorylation does not result in alteration of its GAP activity, but it is conceivable that phosphorylation could change the intracellular localization of neurofibromin in relation to microtubules or its association with other proteins.

The Role of Neurofibromin in the Nervous System

Role of Neurofibromin in Nervous System Development

There is a growing body of evidence to substantiate the claim that ras is important in the proper, orderly development of the nervous system. In the best studied system to date, the regulation of ras is critical to the development of the Drosophila compound eye (Fortini et al., 1992; Pawson, 1990). In this photoreceptor system, several gene products have been identified that form a cascade involving receptors with tyrosine kinase activity (receptor tyrosine kinases with the ability to phosphorylate proteins on tyrosine residues), GTPase-activating proteins, and ras. The proper differentiation of one of these photoreceptor cells (R7 cell) requires an inductive signal from a neighboring photoreceptor cell (R8 cell) (Simon et al., 1991). The R7 cell expresses a receptor tyrosine kinase that is activated by a ligand expressed on the surface of the R8 cell. Stimulation of the R7 receptor tyrosine kinase results in activation of Ras1 and proper differentiation of the R7 cell. A recently identified Drosophila GAP-like protein, Gap1, appears to down-regulate Ras1 and negatively regulates R7 determination (Gaul et al., 1992). The orderly expression of the growth factor ligand and its interaction with the R7 receptor tyrosine kinase constitute the upstream signaling components that regulate GAP and ras in a receptor-mediated signal transduction pathway. Similar processes, perhaps involving neurofibromin, may be operative in the development of the mammalian nervous system.

Role of Neurofibromin in Neural Crest-Derived Tissues

The expression of neurofibromin is presently being investigated in two neural crest-derived tissues: Schwann cells and the pheochromocytoma cell line, PC12. In cultured Schwann cells, elevation of cAMP results in differentiation at least in part by up-regulation of growth factor receptor expression (Weinmaster and Lemke, 1990) and perhaps also through cAMP-dependent phosphorylation events in the cytoplasm or transcriptional events in the nucleus (Sobue et al., 1986). If ras is involved in the regulation of cAMP in mammalian cells, as it is in yeast, it is likely that the regulation of ras by neurofibromin may constitute a pathway important in modulating cell differentiation. In the PC12 model system, elevation of cAMP levels results in differentiation to a neuronal phenotype with sprouting of neuritic processes, similar to the phenotype induced by nerve growth factor treatment (Richter-Landsberg and Jastorff, 1986). Likewise, activation of ras by nerve growth factor stimulation or introduction of activated ras protein leads to PC12 cell differentiation (Bar-Sagi and Feramisco, 1985). There is evidence to suggest the existence of at least two different pathways that culminate in PC12 neurite extension: one pathway involves ras whereas the other is independent of ras and is mediated by cAMP (Golubeva et al., 1989; Miyasaka et al., 1991; Hagag et al., 1986; Hama et al., 1986; Sugimoto et al., 1988). Further work will be required to determine whether these two seemingly independent pathways converge on a common protein modulator to culminate in differentiation.

Summary and Future Directions

There are many more questions raised than answered by the cloning of the *NF1* gene. Now that neurofibromin has been shown to be involved in the regulation of ras, to participate in protein kinase phosphorylation cascades, and to be potentially associated with cytoplasmic microtubules, many new unexpected areas for investigation are open: Studies aimed at better defining the role of neurofibromin in the regulation of ras will need to focus on determining the critical residues for neurofibromin-ras interaction, dissecting the mechanism for regulation of neurofibromin function, and deciding between the upstream and downstream models for neurofibromin-ras interactions. In this regard, further definition of the range of germline and somatic *NF1* mutations should assist in a better understanding of structure-function relationships, especially if more missense mutations can be found. Studies directed at understanding the role of neurofibromin in signal transduction cascades may shed some light on the potential sites for neurofibromin phosphorylation in response to growth factors, the consequences of neurofibromin phosphorylation, and determination of the intracellular protein targets for interaction with neurofibromin. An investigation of the association of neurofibromin with microtubules should elucidate the nature of this physical association, the elements required for regulation of this interaction, and the effects of up-regulating or down-regulating neurofibromin expression on microtubule-mediated functions. Lastly, the role of neurofibromin in the nervous system may be better understood through an examination of its role in Schwann cell-mediated functions, neuronal regeneration, and Schwann cell and oligodendrocyte myelination. Investigations along these lines should provide potentially exciting insights into the multiple roles of neurofibromin within the nervous system.

Acknowledgments

The authors thank the members of the Michigan NF1 group, past, present, and future, as well as our collaborators for their intellectual contributions to this project: Lone Andersen, Roymarie Ballester, Dafna Bar-Sagi, Tanya Basu, Mike Boyer, Anne Brereton, Shawn Clothier, Jeffery Cole, Scott Diehl, Steven Doran, Julian Downward, Jane Fountain, Tom Glover, Paula Gregory, Amit Hajra, Eric Legius, Doug Marchuk, Anna Mitchell, Jane Nicholson, Hana Odeh, Lynn Rutkowski, Ann Saulino, Manju Swaroop, Susan Tarlé, Roxanne Tavakkol, Gihan Tennekoon, Peggy Wallace, Michael Wigler, Susan Wilson-Gunn, and Deborah Wood. F. S. C. is an Investigator in the Howard Hughes Medical Institute. This work was supported by National Institutes of Health grant NS23410 (F. S. C.) and Clinical Investigator Award NS01590 from the NINDS (D. H. G.).

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