Tumor suppressor genes as negative growth regulators in development and differentiation

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Introduction

Tumor suppressor genes are typically thought of as genes whose expression is reduced or lost in cancer cells (Knudson, 1993). This lack of expression results from mutations in the genes encoding their proteins. Since these proteins are believed to suppress cell growth and thereby act as negative growth regulators, loss of their expression in tumor cells leads to the increased cell proliferation observed and contributes to malignant transformation. However, this loss of expression in tumors represents only one side of the coin. As negative growth regulators, tumor suppressor gene products are likely to have normal functions critical to the development of differentiated tissues. In this respect, tumor suppressor genes may have important roles in the growth arrest necessary for the onset of cellular differentiation, as growth regulation is a normal feature of development and differentiation. In this review, the possible functions of three representative tumor suppressor genes in normal cellular differentiation and embryonic development will be examined.

Nuclear tumor suppressor genes

Many of the events that culminate in growth suppression occur in the nucleus where different proteins directly interact with DNA to promote or inhibit gene transcription and the production of messenger RNA (Weinberg, 1991, 1993). These DNA binding proteins (transcriptional regulators) are intimately involved in the control of gene expression and regulate cell growth. Nuclear tumor suppressor genes may function here by modulating the expression of genes necessary for cell proliferation or differentiation. Alternatively, nuclear tumor suppressor genes may interact with proteins involved in regulating progression through the cell cycle. Since dividing cells must move through the cell cycle (undergoing DNA synthesis and mitosis) as opposed to differentiated cells which remain in G0 or G1, nuclear tumor suppressor genes that alter the expression or function of cell cycle regulatory proteins disrupt the balance between cell proliferation and differentiation.

One class of nuclear tumor suppressor gene product is comprised of proteins intimately involved in cell cycle regulation. These include proteins that phosphorylate cell-cycle regulatory proteins, such as p15 or p16 (Serrano et al., 1993; Kamb et al., 1994). p15 and p16 belong to a newly described family of cyclin-independent kinase inhibitory proteins that function to negatively regulate cell growth through direct interaction with other proteins involved in cell cycle progression. By inhibiting the function of growth-promoting proteins and preventing progression through the cell cycle, p15 and p16 cause growth arrest. Other cell cycle regulatory proteins (e.g., p21-WAF1/CIP1) are involved in the formation of regulatory protein complexes that inhibit DNA polymerase and proliferating cell nuclear antigen (PCNA) (Li et al., 1994; Wang et al., 1994). Inhibition of DNA replication results in cell cycle growth arrest. In addition, p21-WAF1/CIP1 is an inhibitor of cell cycle-dependent kinases in much the same fashion as p15 and p16. Loss of the expression of genes encoding these proteins would result in an increase in cell proliferation by allowing the cells to enter the cell cycle. p53, another nuclear tumor suppressor gene product, acts as a regulator of p21-WAF1/CIP1 such that its loss in tumors allows cells to progress through the cell cycle and proliferate. A final group of nuclear tumor suppressor genes function as transcriptional activators and repressors, like the retinoblastoma and Wilm's tumor (WT-1) gene products. These DNA binding proteins are critical transcriptional regulators and their loss leads to unchecked cell proliferation. In this regard, the retinoblastoma protein, p110-Rb, has been demonstrated to play an important role in the production and maintenance of the terminally differentiated muscle cell phenotype (Gu et al., 1989). Activation of p110-Rb inhibits myogenesis through direct interactions with the muscle-specific DNA binding protein, MyoD. Inhibition of MyoD activity leads to a failure in myoblast differentiation presumably due to inhibition of MyoD transcriptional activation of the muscle differentiation program.

Non-nuclear tumor suppressor genes

Another class of tumor suppressor genes encodes proteins whose locus of action resides outside the nucleus. The way these proteins function is not nearly as clear as that described for their nuclear counterparts. Non-nuclear tumor suppressor proteins probably exert their effects through a variety of mechanisms involving signal transduction pathways, cell membrane receptors and changes in the cytoskeleton. Although the precise mechanisms by which this novel class of tumor suppressor genes regulates cell growth are not understood, it is likely that an elucidation of their modes of action will provide significant insights into the many diverse pathways that serve to maintain the balance between cell proliferation and differentiation.

To date, 7 non-nuclear tumor suppressor genes have been identified through positional cloning of disease genes associated with specific familial cancer syndromes. Three of these, neurofibromatosis 1 (NF1), neurofibromatosis 2 (NF2), and tuberous sclerosis 2 (TSC2) will be discussed in greater detail below. Von Hippel Lindau (VHL) is an inherited cancer syndrome manifested clinically by tumors composed of blood vessels (hemangioblastomas), renal cell carcinomas, and adrenal gland tumors (pheochromocytomas) (Malher et al., 1980). The VHL gene was identified and found to code for a small 16-21 kDa protein with no apparent sequence similarity to known proteins (Lafl et al., 1993). It appears to be localized in the cytoplasm, however studies directed at determining its precise subcellular distribution have not been performed (Gao et al., 1995).

Three colon cancer tumor suppressor genes have been identified by positional cloning. These genes, adenomatous polyp-
sis coli (APC), deleted in colorectal carcinoma (DCC) and mutated in colorectal carcinoma (MCC), all code for cytoplasmic or membrane-associated proteins. Of the three colon cancer gene products, APC has been best studied (Groden et al., 1991). The APC protein has been shown to associate with two proteins, α and β catenin, which are critical determinants of the adherens junction (Rubinfeld et al., 1993; Su et al., 1993). The adherens junction (also called the zona adherens) is important for the establishment and maintenance of epithelial layers. The proteins involved in forming adherens junctions also mediate adhesion between cells, provide signals that neighboring cells are present, and anchor the actin cytoskeleton. In addition, several lines of evidence suggest that these proteins may become phosphorylated on tyrosine residues during cell growth and differentiation and might provide a signal for "contact inhibition". Catenins represent one component of this complex and act as calcium-regulated cell adhesion transmembrane molecules. The association between APC and catenins raises the possibility that APC regulates the transmission of a contact inhibition signal to the cell which would instruct a cell to stop proliferating when it finds its nearest neighbor. This process is critical to the proper formation of tissues during differentiation and development.

The other two colon cancer cytoplasmic tumor suppressor genes, DCC and MCC, are less well understood. MCC codes for a predicted 829 amino acid protein with significant sequence similarity to the G protein-coupled muscarinic acetylcholine receptor (Joslyn et al., 1991). Little is known about its expression during embryogenesis. The DCC protein, on the other hand, is expressed at highest levels in brain and shares sequence similarity with domains common to a family of cellular adhesion molecules (CAMs) (Fearon et al., 1990). Using antisense oligonucleotides, decreased expression of DCC was shown to be associated with inhibition of cell adhesion in fibroblasts (Narayan et al., 1992). In addition, abrogating DCC expression in PC12 cells prevents neurite outgrowth in response to nerve growth factor stimulation, suggesting a role for this protein in neuronal differentiation (Lawlor and Narayan, 1992).

Neurofibromatosis 1

Neurofibromatosis 1 is a common autosomal dominant disorder in which affected individuals develop both benign and malignant tumors at increased frequency (Riccardi, 1991). The clinical features of NF1 are variable but include glial cell tumors, Schwann cell tumors and tumors of the adrenal medulla (pheochromocytomas). Although typically regarded as a "tumor syndrome", individuals with NF1 also develop abnormalities of the central nervous system (epilepsy and learning disabilities), skeleton (sphenoid wing dysplasia and scoliosis) and the circulatory system (renal artery dysplasia and abnormalities of cerebral blood vessels) (Riccardi and Eichner, 1992).

The NF1 gene was identified by positional cloning in 1990 and found to encode a large cytoplasmic protein (neurofibromin) (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990). Neurofibromin is a 220-250 kDa phosphoprotein expressed predominantly in neurons, Schwann cells, adrenal medullary cells, oligodendrocytes, white blood cells and vascular endothelial cells in adult tissues (DeClue et al., 1991; Gutmann et al., 1991; Daston et al., 1992; Golubic et al., 1992).

Fig. 1. Structure and proposed functions of the NF1 product, neurofibromin. Neurofibromin is a 250 kDa phosphoprotein that contains a central 300-400 amino acid domain capable of functioning as a GTPase activating protein as well as mediating interactions with cytoplasmic microtubules. Active GTP-bound ras is converted to its inactive GDP-bound conformation by binding to neurofibromin. The same region of neurofibromin important for p21-ras regulation is essential for microtubule association in some cell types.

Comparison of the predicted protein sequence of neurofibromin demonstrated striking similarity with a family of proteins involved in the regulation of the p21-ras proto-oncogene, termed GTPase activating proteins or GAPs (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990a,b) (Fig. 1). p21-ras is a critical protein in normal cells which has been shown to trigger proliferation or differentiation in a tissue-specific fashion (Wigler, 1990). As a protein involved in growth suppression, the ability of neurofibromin to inactivate another protein which stimulates cell proliferation offered an exciting explanation for how loss of neurofibromin expression in tumors might result in increased cell proliferation. p21-ras is found in two forms within the cell, an active GTP-bound and an inactive GDP-bound form (Bourne et al., 1990 and 1991). Interaction of p21-ras with neurofibromin or other members of the GTPase-activating protein (GAP) family converts p21-ras from its active GTP-bound conformation to an inactive GDP-bound state. In selected tumors, loss of neurofibromin has been shown to be associated with elevated levels of GTP-bound, activated p21-ras (Basu et al., 1992; DeClue et al., 1992). However, this GAP homology domain only accounts for 10% of the entire neurofibromin protein. Recent studies have demonstrated that neurofibromin can suppress cell growth and perhaps activate cell differentiation through mechanisms unrelated to p21-ras regulation (Johnson et al., 1994).

When antibodies to neurofibromin became available, a number of groups demonstrated that neurofibromin associated with cytoplasmic microtubules in some, but not all cell types (Gregory et al., 1993). This association represented a 1:1 stoichiometric binding of neurofibromin with alpha-tubulin (one of
the building blocks of microtubules) (Bollag et al., 1993). Further definition of the region of neurofibromin responsible for this microtubule association demonstrated that it was the same region responsible for p21-ras regulation (Gregory et al., 1993). Moreover, the binding of free tubulin to neurofibromin inhibits the ability of neurofibromin to function as a GAP molecule towards p21-ras, suggesting that the association of neurofibromin with microtubules might act to modulate neurofibromin's GAP activity (Bollag et al., 1993). Studies on lymphocytes have also confirmed a link between neurofibromin and microtubules (Boyer et al., 1994). In B cells stimulated by surface immunoglobulin (slg) crosslinking, p21-ras and neurofibromin translocate to one pole of the cell to interact with these activated slg receptors in a microtubule-dependent fashion. In other cell types, some studies have failed to demonstrate an association between neurofibromin and microtubules in vivo (Golubic et al., 1992; Nordlund et al., 1993).

Another feature of the anatomy of NF1 is the presence of at least two alternatively spliced exons (exons 23a and 48a) which produce at least four neurofibromin protein isoforms (Marchuk et al., 1991), termed type 1 neurofibromin (lacking either exon 23a and 48a), type 2 neurofibromin (containing only exon 23a), type 3 neurofibromin (containing only exon 48a), and type 4 neurofibromin (containing both exons 23a and 48a) (see Fig. 4). Type 1 neurofibromin is the predominant isoform in neurons of the central nervous system and dorsal root ganglia whereas type 2 neurofibromin predominates in neural crest-derived tissues (Schwann cells and adrenal medulla) (Gutmann et al., 1995b). Neurofibromin proteins containing exon 48a are restricted to skeletal and cardiac muscle tissues (Gutmann et al., 1993a, 1995a). Previous studies have shown that type 2 neurofibromin is 10-fold less able to downregulate p21-ras (Andersen et al., 1993). It is not clear at this point whether these isoforms have different capacities to associate with the cytoskeleton. Recent studies have demonstrated the expression of an alternatively spliced exon (exon 9a) which predominates in cerebral cortex, but not cerebellum or spinal cord (Danglot et al., 1995). This exon encodes 10 amino acids which are conserved between human and rodent species.

**Expression during embryonic development**

Examination of the expression of neurofibromin during embryonic development has centered around three types of studies: (1) analysis of total neurofibromin expression, (2) analysis of neurofibromin isoform expression, and (3) analysis of development in mice with homozygous targeted disruptions of NF1 ("NF1 knock-out mice"). Studies examining total neurofibromin expression in rat, mouse and chick development have demonstrated ubiquitous expression during early to mid-embryonic development with later enrichment in central nervous system tissues (Daston and Ratner, 1993; Stocker et al., 1995). Sometime during the late first week after birth in rodent species, the neurofibromin expression pattern resembles that seen in adult tissues. This widespread embryonic expression suggests that neurofibromin might play a significant role in the development of tissues where it has no function in adult tissue counterparts. In the rat, roughly equal levels of neurofibromin are detected in brain, spinal cord, lung, skeletal muscle and skin at embryonic day 16 whereas by postnatal day 6, expression predominates in the brain with 10-fold less expression in spinal cord and undetectable levels in lung, muscle and skin (tissues where adult tissue expression has been observed) (Daston and Ratner, 1993). In central nervous system neurons, increased neurofibromin expression was detected in differentiated neurons. In embryonic day 12 cerebral cortex, intense neurofibromin expression was found in neurons in the cortical plate, but not in the proliferating periventricular zone of the developing cortex. This finding is consistent with the notion that neurofibromin acts as a negative growth regulator whose expression correlates with cell differentiation.

In similar studies on chick development, widespread NF1 expression was detected in the central nervous system, spinal ganglia, mesonephros and muscle masses in the stage 30 (e7) embryo (Stocker et al., 1995; Kavka et al., submitted). NF1 expression was also found in e7 and e13 brain, heart, liver, gut, muscle and kidney. Neurofibromin expression was expressed in migrating trunk and cranial neural crest cells of early chick embryos (e1.5-e2) as well as in vascular endothelial cells and cells of the dermatomyotome. At later stages (e3-e7), neurofibromin expression was also seen in heart, skeletal muscle and kidney. In addition, the subcellular localization of neurofibromin was shown to differ in non-neural crest-derived fibroblasts and neural crest-derived Schwann cells, suggesting that neurofibromin associates with different proteins in different cell types and may perform cell-type specific functions.

Since neurofibromin has been shown to represent the composite of four isoforms with different patterns of expression, some studies have focused on the expression of neurofibromin isoforms during rodent embryonic development. In these studies, type 1 NF1 was shown to predominate in neurons of the central nervous system, but not the peripheral nervous system, where type 2 NF1 is found in spinal cord motor neurons (Huynh et al., 1992, 1994; Gutmann et al., 1995b). In the developing brain, type 2 NF1 expression is more abundant early in development (E12-E14) after which time, type 1 NF1 expression increases. This increase in type 1 NF1 expression correlates with the differentiation of central nervous system neurons. Similar patterns of isoform predominance have been observed in the developing cerebellum. In the spinal cord, there is a gradual increase in type 2 NF1 expression during embryonic and postnatal development concomitant with maturation and differentiation of spinal motor neurons. These results suggest that increases in total neurofibromin expression are associated with cellular differentiation, but also that the particular neurofibromin isoform expressed may impact on the differentiation of these tissues. Type 3 and 4 neurofibromin species were detected throughout development in heart and skeletal muscle tissues with highest levels of expression during mid to late embryonic development and the first week of postnatal life (Gutmann et al., 1995a). These results are consistent with the tissue-specific expression of neurofibromin proteins containing exon 48a.

Another way to determine the effect of neurofibromin expression on embryonic development and differentiation is to generate animals lacking NF1 expression through targeted disruption of the gene. Two groups of investigators have created NF1 knockout mice and have shown that absent neurofibromin expression is lethal during mid-embryogenesis (Brannan et al., 1994; Jacks et al., 1994). Homozygous NF1 knockout mice die
between embryonic days 12.5 and 13.5 of generalized edema and cardiac failure secondary to a defect in the development of the cardiac great vessels. These mice exhibit a double outlet right ventricle in which the aorta and pulmonary artery are joined. Previous studies in the chick have demonstrated that this abnormality is observed when migrating neural crest cells are ablated (Kirby et al., 1983), suggesting that neurofibromin has a critical function in neural crest-derived cells that contribute to the development of the cardiac vessels. Since these animals die so early in embryogenesis, it has not been possible to determine what effect neurofibromin absence has on the developing nervous system. In addition to the double outlet right ventricle abnormality, these mutant mice also demonstrate hypoplastic endocardial cushions, suggesting that myocyte infiltration failed to occur. In other organs, such as kidney, liver and skeletal muscle, there is an 18 to 24 h delay in development. Hypoplastic muscles in the abdomen and shoulder girdle were also observed in these mutant mice. These results are consistent with the notion that neurofibromin might play a role in muscle development (see below). Lastly, hyperplasia of sympathetic ganglia was seen in these NF1 knockout mice, suggesting that lack of neurofibromin expression in these structures is associated with lack of growth control. The increased size of the neural crest-derived ganglia resulted from an increase in cell number rather than cell size. Dissociation and culturing of these cells demonstrated greater than 2-fold more proliferation than normal ganglionic neurons with evidence of increased mitosis and cell division, in keeping with the proposed function of neurofibromin as a negative growth regulator during embryonic development and cell differentiation (Brannan et al., 1994).

**Expression during cell differentiation**

In an effort to determine what role(s) neurofibromin might have during cell differentiation, a number of groups have employed *in vitro* differentiation systems. The major tumor type in individuals with NF1 is the neurofibroma composed of predominantly Schwann cells and fibroblasts. Initial studies demonstrated that nonmyelinating, but not myelin-producing, Schwann cells express neurofibromin (Daston et al., 1992). These results suggest two possibilities. One possibility is that neurofibromin expression discriminates between myelinating and non-myelinating Schwann cells (two distinct cell types). Alternatively, neurofibromin expression differentiates between immature Schwann cells and myelinating Schwann cells that have undergone differentiation (different developmental stages of same cell type). To determine whether neurofibromin expression correlates with Schwann cell differentiation, a rat Schwann cell line capable of differentiating *in vitro* in response to treatments that elevate intracellular cAMP levels was employed. This cell line, MT,H1, expresses myelin Po and galactocerebroside (markers of Schwann cell differentiation) in response to forskolin or dibutyryl cAMP treatments (Tennekoon et al., 1987). Concomitant with an increase in galactocerebroside and myelin Po expression in these stimulated Schwann cells, there is an increase in neurofibromin expression (Gutmann et al., 1993b). Not only does NF1 expression increase, but the NF1 isosform predominance changes from type 1 to type 2 NF1. This finding is intriguing given the reduced ability of type 2 neurofibromin to function as a GAP molecule. In Schwann cells, the introduction of an activat-
neuronal differentiation (Dichter et al., 1977). Reducing neurofibromin expression with antisense oligonucleotides in PC12 cells resulted in a dose-dependent inhibition of neurite outgrowth in response to NGF treatment (Huynh and Pulst, 1995). Previous experiments have demonstrated that the introduction of activated p21-ras into PC12 cells results in the elaboration of neuritic processes and "differentiation." If neurofibromin functions to inactivate p21-ras in PC12 cells, one would predict that reduced NF1 expression would result in "spontaneous" or potentiated differentiation. The observation that antisense NF1 oligonucleotide treatment is associated with differentiation blockade suggests that neurofibromin might function to suppress cell growth through mechanisms unrelated to p21-ras inactivation.

Given the existence of muscle-specific isoforms of neurofibromin and the abnormal phenotype of the NF1 knockout mice, neurofibromin might function as a negative growth regulator during myoblast differentiation. Studies by Eric Olson's group in the 1980s demonstrated that the introduction of activated p21-ras results in a failure of C2C12 mouse myoblasts to undergo differentiation in response to serum deprivation (Olson et al., 1987). This effect of p21-ras results in inhibition of MyoD1 gene expression (Payne et al., 1987; Konieczny et al., 1989). As mentioned earlier, MyoD1 expression is essential for the onset of the myoblast differentiation program. In C2C12 myoblasts differentiating in response to serum starvation, there is an increase in the expression of neurofibromin concomitant with a decrease in the levels of activated p21-ras (Gutmann et al., 1994). It is not clear at this time whether this decrease in p21-ras activity is a direct consequence of neurofibromin downregulation or represents a separate effect. Future studies aimed at determining the role of neurofibromin in muscle cell differentiation may shed some light on this interaction.

Lastly, melanocyte abnormalities have been proposed to underlie some of the clinical features of NF1. In the melanoma cell line MeWo, the introduction of NF1 leads to an increase in the levels of tyrosinase and is associated with extension of processes similar to those seen in differentiated melanocytes (Johnson et al., 1994). In experiments studying transcriptional regulation of tyrosinase gene expression, neurofibromin overexpression results in an increase in tyrosinase promoter activity as measured using a reporter gene assay (Suzuki et al., 1995). These results suggest that neurofibromin may play an essential role in melanocyte differentiation through direct effects on DNA transcription.

**Neurofibromatosis 2**

Neurofibromatosis 2 is a distinct autosomal dominant disorder characterized by vestibular Schwann cell tumors, meningiomas, ependymomas and astrocytomas (glial cell tumors) (Evans et al., 1992). In addition to these tumors, individuals with NF2 manifest cataracts, retinal abnormalities and peripheral nerve deficits. The NF2 gene was identified by positional cloning in 1993 and found to encode a protein termed merlin or schwannomin (Rouleau et al., 1993). Expression of NF2 mRNA has been detected in a wide variety of adult human tissues including brain, heart, liver, lung, skeletal muscle, kidney and pancreas (Rouleau et al., 1993; Trofatter et al., 1993; Gutmann et al., 1995c). Homologs have now been identified in mouse and rat which bear striking sequence conservation (Claudio et al., 1994; Haase et al., 1994; Hara et al., 1994; Gutmann et al., 1995c). Expression of rat NF2 was detected in nervous system tissues (cerebral cortex, brainstem, cerebellum, and spinal cord), dorsal root ganglia (DRG), testis, ovary and adrenal gland with significantly lower expression in other tissues. In situ hybridization analysis of NF2 expression in the rat central nervous system using an NF2 riboprobe demonstrated a restricted pattern of mRNA expression with predominant expression in the hippocampus, cerebellum, and brainstem nuclei (Gutmann et al., 1995c). In the spinal cord, expression of NF2 mRNA was apparent in all neurons in the grey matter and primary sensory neurons in the DRG.

A number of studies have demonstrated the presence of alternative splicing on the RNA level that could potentially lead to the elaboration of multiple merlin protein species (Bianchi et al., 1994; Pykett et al., 1994; Gutmann et al., 1995c). Three regions of alternative splicing have been reported which correspond to the three recognized domains of merlin (see Fig. 4). In the amino terminal region thought to mediate interactions with cell surface proteins, variable deletions of exons 2 and 12 as well as insertion of exon 2 (117 nucleotides) have been reported (Bianchi et al., 1994; Pykett et al., 1994). In the central portion of merlin, deletions of exons 8 and 10 have also been described while in the carboxyl terminus of the protein, deletions of exons 15 and 17 as well as insertion of exon 16 (45 nucleotides) have been observed. No consistent pattern of isoform expression has been elucidated for most of these alternatively spliced forms with the notable exception of exon 16. Exon 16 contains 45 nucleotides and a premature stop codon which is predicted to result in a mature merlin protein 5 amino acid residues shorter than the merlin protein lacking this exon. In
cerebral cortex and cerebellum, relatively more type 2 NF2 isoform expression (containing exon 16) was observed whereas spinal cord and brainstem demonstrated relatively more type 1 NF2 isoform expression (lacking exon 16) (Gutmann et al., 1995c). Roughly equivalent amounts of type 1 and type 2 NF2 isoforms were seen in tests and DRG, as opposed to adrenal gland and cultured human Schwann cells which demonstrated relatively more type 1 NF2 isoform expression. In situ hybridization using a type 2 NF2 oligonucleotide probe demonstrated expression of type 2 NF2 in the cortex, hippocampus and cerebellum with significantly less expression in the spinal cord.

The generation of antibodies against the NF2 product by a number of different groups has demonstrated a 55 kDa or 65-70 kDa protein identified as merlin (or schwannomin) (Sainz et al., 1994; den Bakker et al., 1995). In one study, merlin was expressed in greatest abundance in adult human vascular smooth muscle and Schwann cells, migrating as a 55 kDa protein (den Bakker et al., 1995). The authors suggested that this 55 kDa protein may represent an isoform of merlin lacking exons 2 and 3. NF2 exogenously expressed in COS cells resulted in punctate and membranous staining which may relate to an association with the cytoskeleton. In other studies, a 65-70 kDa merlin protein was observed in cortex, cerebellum and Schwann cells with less expression in other tissues (Sainz et al., 1994; Gutmann, unpublished observations).

Expression during embryonic development

Expression of NF2 in the developing rat has been studied by in situ hybridization (Gutmann et al., 1995c). During embryonic development, expression of NF2 RNA was detected in a variety of tissues which in the adult counterparts lacked NF2 expression. Expression of NF2 RNA was observed in developing rat kidney, skeletal muscle, and lung where no expression was detected in the adult. In the cerebral cortex, there is relatively more type 1 NF2 expression at embryonic day E14 with increasingly more type 2 NF2 expression observed in later embryonic development and in the adult. This is consistent with the NF2 isoform expression pattern observed during in vitro neuronal maturation of murine neocortical cultures in which increasing expression of type 2 NF2 was observed as these neurons matured in vitro. Higher levels of NF2 expression in cerebral cortex were observed during embryonic development than in the adult. NF2 isoform expression in the developing spinal cord and brainstem demonstrated a change from relatively (more) higher type 2 expression at day E16 and postnatal day 1 to equal NF2 isoform expression or relatively more NF2 type 1 isoform expression in the adult. In the DRG, NF2 expression increased during the first two weeks of postnatal life whereas expression in the superior cervical ganglia (SCG) decreased to nearly undetectable levels in the adult. By in situ hybridization using an oligonucleotide riboprobe corresponding to exon 16, high levels of type 2 NF2 were detected in the hippocampus, most brainstem nuclei and cerebellar Purkinje cells of the rat CNS at both postnatal day 1 and in the adult. Levels of NF2 expression were reduced in the adult brain relative to corresponding areas in the postnatal day 1 rat brain. This is consistent with previous experiments using an NF2 riboprobe capable of detecting all isoforms of NF2.

Fig. 4. Alternative splicing of the NF1, NF2 and TSC2 genes generate multiple neurofibromin, merlin and tuberin isoforms. (A) Three alternatively spliced exons of neurofibromin include exon 9a (10 amino acids), exon 23a (21 amino acids) and exon 48a (18 amino acids). Neurofibromin containing exon 9a is highly expressed in brain tissues while those containing exon 23a and 48a are found in neural crest derived and muscle tissues, respectively. Neurofibromin lacking exons 23a and 48a predominate in central nervous system tissues. (B) Two major isoforms of merlin are formed by the alternative use of exon 16. The tissue specific patterns of expression are less obvious than that described for neurofibromin. (C) One alternatively spliced exon of tuberin (exon 25) has been reported. Some tissues express TSC2 lacking exon 25 whereas others express TSC2 containing exon 25.

Proposed functions of merlin

Analysis of the predicted protein sequence of the NF2 product demonstrated significant sequence similarity between merlin and a family of proteins that link the cell membrane to the actin cytoskeleton (Rouleau et al., 1993; Trofatter et al., 1993) (Fig. 2). Members of this family include moesin, ezrin, radixin and the erythrocyte 4.1 protein. The structure of merlin resembles the structure of the other members of this family in that there is a large amino terminal domain followed by an alpha helical domain and a small hydrophobic carboxyl terminus. These proteins are most similar in the amino termini (62% amino acid identity for moesin, ezrin, and radixin; 46% for erythrocyte 4.1 protein). Members of this novel family of proteins have been proposed to function as links between the cell membrane and the actin cytoskeleton. Highly related genes have also been detected in the nematode C. elegans. Protein 4.1 is the best studied of this family of proteins. It plays a critical role in maintaining membrane stability and cell shape in the erythrocyte by connecting integral membrane proteins, glycophorin and protein 3, to the spectrin-actin lattice of the cytoskeleton. Binding of protein 4.1 to glycophorin has been mapped to the amino terminus of the protein.
whereas spectrin binding is mediated through the alpha helical region. Moesin is a 77 kDa protein expressed in muscle, spleen and heart where it is hypothesized to function as a membrane organizing extension spike protein (Lankes and Furthmayr, 1991). Antibodies against moesin have biological effects in that they interfere with membrane protrusion budding.

Ezrin is an 80 kDa phosphoprotein expressed in parietal cells and associated with the cytoskeleton (Bretscher, 1989; Gould et al., 1989; Krieg and Hunter, 1992). It may play a key role in the assembly of secretory apical microvilli important for the regulation of acid secretion in the gastrointestinal tract (Hanzel et al., 1991). Highest levels of ezrin RNA expression are found in kidney, intestine, skin and lung with lower levels in ovary, thymus, heart, brain and spleen. No ezrin expression has been detected in testis, skeletal muscle and liver. Multiple isoforms of ezrin have been described that associate with microfilaments and microtubules in differentiating neuronal cells (Birgbauer et al., 1991).

Ezrin is also phosphorylated on tyrosine residues (Y145 and Y353) in response to epidermal growth factor stimulation, suggesting a link between growth regulation and changes in the cytoskeleton (Bretscher, 1989).

Radixin is a similar 82 kDa protein localized to the cellular adherens junction (Tsukita et al., 1989). It is expressed in liver, small intestine and at lower levels in cardiac muscle. Less is known about the function of radixin.

Recently, it was shown that merlin (or schwannomin) can function in vitro as a negative growth regulator by suppressing the growth of NIH-3T3 fibroblasts (Lutchman and Rouleau, 1995). In this study, the amino terminal 100 amino acids were required for growth suppression, suggesting that the mechanism by which this tumor suppressor protein regulates cell growth is through interactions with the cell membrane. No evidence for EGF effects on merlin expression were demonstrated.

**Tuberous sclerosis 2**

Tuberous sclerosis (TSC) is another autosomal dominant disorder characterized by the development of benign growths (hamartomas) in many different tissues and organs (Gomez, 1988). Individuals affected with tuberous sclerosis develop astrocytomomas, hamartomas of the eyes, skin and central nervous system and cardiac primitive muscle cell tumors (rhabdomyosarcomas). Using physical mapping techniques and positional cloning, the TSC2 gene on chromosome 16 was identified in 1993 (European Chromosome 16 Tuberous Sclerosis Consortium, 1993). TSC2 produces a 5.5 kb transcript widely expressed in human and rat tissues by Northern blot analysis (European Chromosome 16 Tuberous Sclerosis Consortium, 1993; Yeung et al., 1994). In human rat and mouse tissues, the highest levels of expression were observed in gonadal tissues (ovary and testis), cerebellum and spinal cord with lower levels of expression in other tissues (Geist and Gutmann, 1995). Within the central nervous system 5-10 fold more expression was detected in cerebellum and spinal cord compared to brainstem and cerebral cortex. Intense labeling of neurons in the hippocampus cingulate gyrus, piriform cortex, olfactory tract and cerebellum (both Purkinje and granule cells) were seen by in situ hybridization. Expression of TSC2 in the spinal cord was restricted to dorsal root ganglion cells, motor neurons in the ventral horn and neurons in the dorsal horn. Recently, alternative splicing of exon 25 has been reported in brain and muscle tissues (Xiao et al., 1995; Xu et al., 1995; see Fig. 4). Studies aimed at determining the significance of this alternatively spliced tuberin isoform are presently in progress.

**Expression during embryonic development**

Rat embryonic tissues analyzed by RT-PCR and in situ hybridization during late embryogenesis and during the first week of postnatal life demonstrated high levels of TSC2 expression in the developing forebrain and spinal cord at embryonic day 12 (E12) and E15 with increasing levels of expression after E15 in the hindbrain region that gives rise to the cerebellum (Geist and Gutmann, 1995). In non-CNS tissues, there is expression in the developing adrenal gland and heart at stages E12 and E15 with diminishing expression by E17. No appreciable differences in expression were detectable during the first two weeks of postnatal life. In this analysis, there is abundant expression of TSC2 in cerebellum and spinal cord at relatively constant levels from E16 through postnatal day 14 (PN14). Detectable levels of TSC2 were observed in all other tissues but in amounts 5-fold less than those observed in cerebellum and spinal cord.

**Proposed functions**

Analysis of the predicted protein product, tuberin, demonstrates a region of sequence similarity with another GTPase-activating protein, rap1-GAP (European Chromosome 16 Tuberous Sclerosis Consortium, 1993) (Fig. 3). This region of homology with rap1-GAP extends over 58 amino acids predicted to interact with the rap1 p21-ras-related protein. The rap1 (Krev-1) gene encodes a ras-related protein that suppresses transformation by p21-ras (Rubinfeld et al., 1991, 1992). Its regulatory GAP molecule, rap1-GAP, is an 88 kDa protein expressed predominantly in brain (Rubinfeld et al., 1992). During development, rap1-GAP is expressed at high levels in fetal brain with less so in lung and liver. The adult lung and liver do not express rap1-GAP. Increased expression of rap1-GAP was also found in several tumor cell lines, suggesting that immature, undifferentiated cells express higher levels of rap1-GAP than their mature, differentiated counterparts. To this end, the expression of rap1-GAP is dramatically reduced in HL60 tumor cells stimulated to differentiate in vitro. Subcellular localization studies of rap1-GAP demonstrated that it is associated with the membrane fraction in brain. Interestingly, brain expresses an isoform of rap1-GAP containing a duplicated 26 amino acid sequence. Since both forms of rap1-GAP (with and without this duplicated 26 amino acid sequence) are efficient p21-rap1 regulators, it is possible that this duplicated sequence relates to the ability of rap1-GAP to associate with the cell membrane.

Recent studies have conclusively demonstrated that tuberin functions in vitro as a GAP molecule for rap1 as was predicted by sequence analysis (Weinecke et al., 1995). Antibodies directed against tuberin demonstrate expression of tuberin in brain with lower levels of expression in heart and kidney. In preliminary studies, tuberin migrates as a 180 kDa protein which partitions into an insoluble fraction. Further analysis of tuberin expression in adult nervous system tissues and during development are in progress.

Several studies have shown an interaction between rap1 and the cytoskeleton, suggesting a possible link between tumor suppressor genes that regulate small GTPase proteins (p21-ras,
Negative growth regulators (tumor suppressor gene products) can be envisioned to result in growth arrest and differentiation through a variety of mechanisms. Interactions between growth factors and their receptors at the cell surface trigger intracellular events involving signal transduction pathways (p21-ras phosphorylation cascades), cell adhesion molecules, microtubule-mediated processes and transcriptional regulation in the nucleus. The site of function of the tumor suppressor gene products discussed in the text are depicted.

p21-rap1) and cytoskeletal changes associated with cell differentiation, development and malignant transformation. Examination of p21-rap1 function in yeast cells demonstrated that rap1 interacts with some of the components of the yeast budding pathway, a process involving changes in the cytoskeleton (McCabe et al., 1992). In this system, expression of p21-rap1 interfered with yeast budding which could be reversed by the introduction of rap1-GAP. Similarly, rap1 associates with the cell membrane in platelets (White et al., 1993). However, upon platelet activation with thrombin, rap1 translocates to a cytoplasmic compartment containing the cell cytoskeleton. This redistribution occurs in two phases: 20% of rap1 protein is initially translocated within seconds followed by a slower phase during which the majority of rap1 moves into the cytoplasm over the course of ten minutes. As is true of most cytoskeletal interactions, this redistribution was inhibited by alterations in calcium homeostasis.

**Negative growth regulators as critical determinants during differentiation and development**

The focus on tumor suppressor genes as negative growth regulators has partially ignored their normal roles in promoting cell differentiation and development (see Fig. 5). If tumorigenesis reflects a de-differentiated stage where the normal constraints on cell growth are lost, it should not be surprising that tumor suppressor genes function during differentiation and development. In the case of nuclear tumor suppressor genes, it is likely that their ability to regulate cell growth and promote cell differentiation relates to their functions as transcriptional regulators. These genes, like retinoblastoma and p53, are predicted to operate by switching on and off the transcription of selected genes important in cell growth and differentiation. Cytosplasmic tumor suppressor genes, like the ones described herein, act through pathways linking cues from the extracellular environment with transcriptional events in the nucleus. Elucidation of these pathways are critical to our understanding of cell growth and differentiation as well as the development of cancer, as has been demonstrated by a number of elegant studies on Drosophila tumor suppressor genes (recently reviewed by Gateff, 1994).

The regulation of cell growth can be envisioned to involve a number of distinct, but overlapping, pathways. These include interactions at the cell surface, signal transduction cascades, and events involving the cytoskeleton. This panoply of events is
schematically represented in Figure 5. Interactions at the cell surface provide a porthole for the cell to the external world. In this fashion, the individual developing (or differentiating) cell obtains information about its position relative to other cells, receives signals for growth and differentiation, and forms mutually beneficial relationships with other cells. Interruption of these normal processes would lead to the dissemination of incorrect information to the interior of the cell responsible for responding to these external signals. At the cell surface, cues are provided to the cell about its neighbors. Normally cells cease growing when they encounter another cell (contact inhibition). This information is processed through specific cell surface proteins. Failure to relay contact inhibition signals to the interior of the cell would result in continued cell proliferation (and tumor development). As mentioned earlier, the APC gene product associates with two adherens junction proteins that may relate a contact inhibition signal to the cell. Loss of APC expression in tumors might lead to continued cell division owing to improper contact inhibition.

Soluble diffusible substances, such as growth and differentiation factors, have their effect on the cell through events occurring at the cell membrane. Each of these factors has a specific membrane-bound receptor which has one end in the extracellular space where it contacts the factor and another end within the cell where it can interact with cytoplasmic proteins to propagate the external signal. Many of the factors thus far identified operate through cascading pathways involving protein phosphorylation. Growth factors bind their respective receptors and initiate activation of these receptors by tyrosine phosphorylation which in turn allows the activated receptors to interact with other proteins found near the cell membrane (Snider, 1994). Mutations in receptors providing cues for cell proliferation, such as the epidermal growth factor receptor and the RET proto-oncogene (another receptor tyrosine kinase protein), have been identified in cancer cells and are hypothesized to lead to increased cell proliferation due to continued (constitutive) signaling from an aberrantly activated receptor.

In order for cells to form mutually beneficial relationships with other cells, they must interact on their external surfaces. These docking events are mediated through cellular adhesion molecules (CAMs). Inefficient docking would fail to provide the necessary cues to the cell and might result in an inability to differentiate. The DCC protein is a cytoplasmic tumor suppressor gene with significant homology to members of the CAM family. As mentioned earlier, reduced expression of DCC in PC12 cells prevents neurite outgrowth and differentiation, suggesting that tumor suppressor genes may function as negative growth regulators by promoting cell adhesion.

Signal transduction cascades provide other avenues for relating events outside the cell to the nucleus. These signaling pathways have been well studied in non-vertebrate development where they have been shown to function as critical determinants of cell fate determination. One heavily investigated cascade involves the p21-ras proto-oncogene in which p21-ras is activated by phosphorylation of receptor tyrosine kinases (such as the epidermal growth factor or the nerve growth factor trkA receptor). Binding of epidermal growth factor (EGF) to its receptor results in receptor autophosphorylation and the binding of a number of proteins. These proteins in turn activate p21-ras which initiates signaling to the nucleus through a series of phosphorylation events. As mentioned above, one of the functions of the NFI product, neurofibromin, is to inactivate p21-ras and terminate its ability to propagate any growth or differentiation-promoting signals to the nucleus. Increased expression of neurofibromin during development might serve to inactivate p21-ras and release the cell from growth signaling. Termination of this p21-ras signal might allow the cell to begin differentiation. Loss of neurofibromin expression, as observed in tumors, would result in uninterrupted growth promotion mediated through p21-ras and increased cell proliferation.

The importance of p21-ras signaling in normal development and differentiation has been extensively characterized. In the developing Drosophila compound eye, p21-ras acts to transmit a differentiative signal provided by a surface receptor (Bonfini et al., 1992). Mutants in Drosophila p21-ras interfere with normal eye development. A similar pathway involving p21-ras exists in the developing C. elegans vulva and is critical for normal worm genital development (Bethel et al., 1990; Greenwald and Broach, 1990). Proteins involved in the inactivation of p21-ras and other related growth promoters would be excellent candidates for tumor suppressor proteins due to their abilities to function as negative growth regulators for normal cell differentiation and development.

One additional mechanism for effecting changes in cell growth and differentiation involves the cytoskeleton. The cytoskeleton represents the scaffolding of the cell, but in addition to providing cellular infrastructure, it is critical for transport of proteins, movement of cells, and segregation of genetic material during cell division. As cell differentiation is often associated with changes in cell shape, motility, and the transport of specific proteins along cytoskeletal guidewires, tumor suppressor proteins with ties to the cytoskeleton might be subserving novel functions essential for cell growth and differentiation. The three tumor suppressor proteins, neurofibromin, Merlin and tuberin have all been hypothesized to interact with cytoskeletal elements. Neurofibromin associates with microtubules and seems to stoichiometrically form complexes with tubulin molecules. The region of neurofibromin responsible for mediating this microtubule interaction is the same region involved in p21-ras regulation. In the case of neurofibromin, its association with microtubules might provide a convenient place for sequestration. When neurofibromin is required at the cell surface, it can be rapidly translocated along microtubule guidewires. Alternatively, neurofibromin might be housed on the microtubules in an inactive state (since it cannot interact with p21-ras while microtubule-bound) and in that fashion be unable to transmit or terminate p21-ras-mediated growth or differentiation-promoting signals. Lastly, it is conceivable that neurofibromin might have novel microtubule-related functions. The formation of microtubules is a GTP-dependent process regulated by several GTPase molecules. Perhaps, neurofibromin acts as an efficient GAP in vivo for one of these microtubule-GTPase molecules.

Merlin has a structure similar to members of the ERM family which serve to link the cell membrane to the cytoskeleton. Merlin might function in developing and differentiating cells as do ezrin and radixin. These proteins are critical determinants involved in remodeling events seen in both gastrointestinal and neuronal cell differentiation. Likewise, tuberin by virtue of its proposed
similarity to rap1-GAP, might regulate a rap1-like protein and allow for differentiation through changes in cytoskeletal-related events. Little is known at this point about the expression, distribution or functions of the Merlin and tuberin tumor suppressor proteins.

As more tumor suppressor genes are identified by scientists interested in cancer genetics, more negative growth regulators will present themselves as potential players in normal growth and differentiation. It is clear that there is an intimate relationship between development, differentiation and the neoplastic state, so it should not be surprising that genes involved in cancer progression have important roles in normal cell differentiation and embryonic development. In addition, it is more than likely that the next important advances in our understanding of the function of these tumor suppressor genes will come from developmental biologists and researchers studying cell growth and differentiation. The convergence of developmental biology and cancer genetics offers the promise of greater insights into both fields than could be appreciated by either discipline separately.

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Summary

Tumor suppressor genes have received much attention for their roles in the development of human malignancies. However, their gene products (proteins) function as negative growth regulators and are highly expressed in many tissues during embryonic development, suggesting that they might function as critical proteins in differentiation and development. The evidence implicating tumor suppressor proteins in cellular differentiation and embryonic development will be presented with special attention to the neurofibromatosis 1, neurofibromatosis 2, and tuberous sclerosis 2 gene products.

KEY WORDS: neurofibromatosis, tuberous sclerosis, ras, GTPase activating protein, tumor suppressor gene

References


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