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Functional Analysis of Neurofibromin: Clues from Drosophila Applied to Mammalian Systems

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The purpose of our research is the generation of relevant biological assay systems in which Ras-independent effects of neurofibromin on cellular proliferation can be readily assessed. Our primary efforts have been aimed at overcoming the longstanding difficulties of manipulating normal and mutant forms of neurofibromin in mammalian cells. We have focused on the development of tightly controlled expression systems using both a vector-based ec dysone system and a tetracycline-regulated HIV amplicon system in established NIH3T3 murine fibroblasts and primary neurofibromin-deficient mouse embryo fibroblasts. Our progress has been severely limited by technical difficulties with both systems that have prevented the establishment of reliably inducible exogenous neurofibromin expression in either cell type. We are now utilizing a recently described retroviral based ec dysone expression system since stable trans-activator-expressing NIH3T3 lines in this system are available. Additionally, expanded transgene delivery into poorly transfected cell lines is afforded by retrovirus infection. These important features will overcome the two major obstacles that have hampered our progress to date. The revised experiments outlined in this report will lay the foundation for further investigation of the complex role of neurofibromin in cellular growth control mediated through its Ras-dependent and Ras-independent functions.

Neurofibromin, proliferated, ec dysone-iducible expression system

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Introduction

Neurofibromatosis type 1 (NF1) is a common human autosomal dominant disorder characterized by a complex disease phenotype that includes the development of both benign and malignant tumors of the nervous system (1). The \textit{NFI} gene encodes a 250 kD tumor suppressor protein, designated neurofibromin, with clear homology to Ras GTPase activating proteins (GAPs) (2, 3). The ability of neurofibromin to negatively regulate Ras through stimulation of GTP hydrolysis is consistent with its tumor suppressor function. Considerable evidence suggests that neurofibromin has important regulatory functions distinct from its RasGAP activity suggesting its role in cellular signal transduction and growth control is complex. Studies of the Drosophila \textit{Nfl} gene have determined that the primary function of neurofibromin in flies is the regulation of cAMP-mediated signaling (4). The activity of neurofibromin in this process can be dissociated from its established function in Ras regulation (5). A RasGAP-independent function of neurofibromin is further suggested by the observation that 2-3 fold overexpression of neurofibromin in NIH3T3 cells blocked their proliferation (6). This growth-inhibitory effect was not dependent on the ability of neurofibromin to negatively regulate Ras GTPase activity.

One additional indication of Ras-independent functions of neurofibromin is derived from comprehensive mutational analyses of the \textit{NFI} gene in affected patients which suggest that defects in the GAP-related domain (GRD) are not solely responsible for the disease phenotype and the region immediately amino-terminal to the GRD may represent a distinct functional domain of neurofibromin (7, 8). The purpose of this research is to test our hypothesis that neurofibromin has important functional domains distinct from the GRD using a cell proliferation assay in which we can test the effects of various mutant forms of neurofibromin on cell growth in both established and primary mouse fibroblasts. Through these analyses, we expect to define critical domains of neurofibromin and clarify its role in the control of cellular proliferation. Further understanding of neurofibromin function will aid in our understanding of the complex NF1 disease phenotype.

Body

Task 1. Generation of stable NIH3T3/EcR cell lines

The primary goal of task 1 is the development of stable NIH3T3 lines that allow controlled expression of exogenous neurofibromin. The anti-proliferative effect of neurofibromin overexpression in NIH3T3 fibroblasts (6) provides a basis for the development of a cell culture system to study this property. The additional observation that this function of neurofibromin is not dependent on its RasGAP activity makes this assay system ideal for identifying additional functional domains of neurofibromin. We initially utilized a tightly regulatable vector-based ecdysone-inducible expression system (Invitrogen) to generate cell lines in which the anti-proliferative effects of neurofibromin overexpression could be first confirmed and then further studied using mutant forms of the protein. An NIH3T3 cell line that stably expresses the ecdysone-responsive transcriptional activator (EcR) is no longer available from the manufacturer. Consequently, the major focus of our effort in the past year has been the generation of such stable lines for use in our analysis of potential anti-proliferation effects of controlled
exogenous expression of neurofibromin. We generated multiple NIH3T3 cell lines that carried the EcR plasmid and tested the ability of each to reproducibly induce expression of an ecdysone-responsive lacZ reporter gene following transient transfection. In each experiment, we also assayed lacZ reporter gene expression in a U2OS/EcR cell line (RxR2a, provided by Rene Bernards) that reproducibly induces lacZ expression in a dose-dependent manner in response to the ecdysone analog GSE (data not shown). A representative analysis of candidate NIH3T3/EcR cell lines and RxR2a is shown in Figure 1.

![Graph showing reporter gene induction in candidate NIH3T3 cell lines.](image)

**Figure 1** Reporter gene induction in candidate NIH3T3 cell lines.

Twelve candidate NIH3T3/EcR cell lines and the RxR2a positive control cell line were transiently transfected with the lacZ reporter plasmid (pIND LACZ) and a control plasmid (pIND). Induced beta-galactosidase activity was quantitated in an ONPG assay following a 20 hour incubation with the ecdysone analog GSE (5 μM). The absorbance at 420 nm indicates the relative beta-galactosidase activity of each transfected line. Each sample was assayed in duplicate.

In this experiment, we assessed lacZ reporter gene expression in 12 individual candidate NIH3T3/EcR cell lines using a quantitative beta-galactosidase assay. Although the RxR2a cell line is capable of high level induction of the lacZ reporter gene over background beta-galactosidase activity, no similar induction of lacZ expression was detected in any of the NIH3T3/EcR cell lines. The low-level induction seen in clone 10 was detectable only after initial establishment of the line and was not seen after passaging of the cell line, a result that was obtained with other candidate NIH3T3/EcR cell lines. We screened over 140 NIH3T3/EcR candidate cell lines and found none that could reproducibly induce ecdysone-dependent transcription of the lacZ reporter gene. We also established stable Cos, Swiss 3T3, and human neuroblastoma EcR cell lines using the ecdysone expression system in an effort to generate an alternative assay system in which potential anti-proliferative effects of tightly controlled exogenous neurofibromin overexpression could be assessed. Similar analysis of reporter gene expression in these lines also indicated that no candidate line significantly induced expression of the lacZ reporter gene following treatment with the ecdysone analog. We further examined the utility of the Invitrogen system by testing the effect of exogenous neurofibromin expression on the proliferation of the RxR2a cell line.
There was no detectable effect on overall cell number following transfection of the RXR2a cell line with an ecdysone-inducible full-length neurofibromin construct using GSE concentrations that induced high levels of beta-galactosidase activity (data not shown). The disappointing results of our work with the Invitrogen system in the NIH3T3 and U2OS cell lines led us to consider alternative methods for establishing cell culture based assays in which we could tightly control exogenous neurofibromin expression.

Xandra Breakefield and colleagues in the Molecular Neurogenetics Unit have considerable expertise in the development and use of amplicon vectors that permit reproducible transgene expression in a wide variety of cell lines (9). These vectors have proven useful in the introduction and stable expression of genes in cells that are not readily transfected through conventional methods which would be useful in our studies of exogenous neurofibromin expression in primary mouse fibroblasts (see Task 3). More recently, the Breakefield laboratory has developed a series of vectors that allow regulatable transgene expression and afford more control of exogenous gene expression and they have provided us with the amplicon shown in Figure 2.

Figure 2    Tetracycline-regulated amplicon
The HSV/EBV amplicon backbone was derived from previously described constructs (9) and includes the reporter gene enhanced green fluorescent protein (EGFP, Clontech) under an immediate early viral promoter (HSV IE4/5), and the amplicon elements oriP, a latent origin of DNA replication, and a mutant version of the EBNA-1 gene. The amplicon also contains a cassette carrying the tet-silencer protein and the tet-activator protein under control of a bidirectional CMV promoter (10). Another cassette contains a multicloning site (MCS) for insertion of the gene of interest and the reporter gene red fluorescent protein (RFP, Clontech) both of which are under the control of a bi-directional tet-responsive promoter (Clontech).

The amplicon vector system is designed to produce high titer helper-free virus stocks that infect target cells with high efficiency (11). The vector shown in Figure 2 has multiple cloning sites where the gene of interest can be expressed from a tetracycline regulatable promoter that also directs expression of
a reporter (red fluorescent protein). The tetracycline-regulated amplicon is capable of inducing expression 350-fold and expression in response to the application of the tetracycline analog doxycycline is dose-dependent (M. Sena-Esteves and X. Breakefield, pers. comm.). The amplicon shown in Figure 2 also contains a tetracycline-independent reporter (enhanced green fluorescent protein) to monitor infection efficiency. We tested the potential utility of this tetracycline-inducible amplicon in our studies by infecting NIH3T3 cells with the amplicon carrying no transgene and examined both GFP and RFP expression in the absence of doxycycline. The results are shown in Figure 3.

![Figure 3](image)

**Figure 3** Reporter gene expression in NIH3T3 cells infected with the tetracycline-inducible amplicon.
Expression of green fluorescent protein (GFP, left panel) and red fluorescent protein (RFP, center panel) in amplicon-infected NIH3T3 cells were assessed by fluorescence microscopy 48 hours post-infection. The right panel is an overlay of the GFP and RFP panels and shows dual expression of the reporter proteins in a subset of the infected cells.

We could readily detect GFP+ NIH3T3 cells (left panel) indicating efficient amplicon infection although the level of GFP expression varied among individual cells. The expression of RFP in the infected cells was also variable (center panel) and largely correlated with GFP expression levels in the dual positive cells (right panel). Although we could detect GFP+/RFP- cells, the predominant expression of the RFP reporter protein in the absence of doxycycline indicates a high degree of constitutive transcription from the tetracycline-dependent promoter. Because low (2-3 fold) levels of neurofibromin overexpression in NIH3T3 cells inhibit proliferation (6), our studies require tight regulation of exogenous neurofibromin expression. This requirement precludes the use of the amplicon system in our studies and led us to again consider alternative expression systems.

Recent reports in the literature describe a retroviral version of the ecdysone-inducible mammalian expression system developed by Stratagene (12) that may overcome the obstacles we have faced in developing our cell proliferation assay. The major advantage of this retroviral system is the availability of well-characterized stable NIH3T3 cell lines which express the ecdysone-responsive trans-activator component proteins at optimal levels for efficient and reproducible induction of introduced transgenes (13). This retroviral system is the best approach to developing the cell proliferation assay systems in NIH3T3 cells and primary MEFs since it would both bypass the need to generate stable 3T3
inducer cell lines and overcome the difficulty of delivering exogenous transgenes to the poorly
transfected MEF cell lines (see Task 3). Our major goals of the coming year are the introduction of wild
type and mutant neurofibromin proteins into the established NIH3T3 cell line available from Stratagene
and subsequent analyses of the proliferation effects of each following controlled exogenous
neurofibromin expression.

Task 2. Construction and expression of mutant forms of neurofibromin

The goal of Task 2 is to generate mutant forms of neurofibromin in the ecdysone-inducible
expression vector and test the ability of each to block NIH3T3 cell proliferation as a means to defining
critical neurofibromin functional domains that are distinct from the GRD. The initial collection of
mutants outlined in the grant proposal included single amino acid substitutions in the GRD that were
predicted to eliminate interaction of neurofibromin with the Ras pathway and we will pursue analysis of
those mutant neurofibromin proteins. More recently, comprehensive mutational analysis of the entire
NFI gene in a large cohort of affected patients has suggested that the region immediately aminoterminal to the GRD has a relatively high concentration of point mutations and may represent a distinct
functional domain of neurofibromin. This region of homology is conserved in the Drosophila protein
(4) and the yeast Ira1 and Ira2 proteins (14). We will focus our efforts on constructing mutations in this
domain and will specifically analyze reported amino acid substitution mutants in exons 12a (1581T,
K583R), 15 (W777S, T780K, H781P), and 16 (L847P) in the proliferation assay since those exons are
suggested to be critical regions within the proposed functional domain (8). We will also generate small
in-frame deletion mutants in this domain by use of convenient restriction sites or PCR-based
mutagenesis. Our progress in the construction and analysis of these mutants has been hampered by the
difficulties in developing a reliable inducible expression system in NIH3T3 cells. The generation and
characterization of these mutants in the cell proliferation assay developed with the Stratagene system as
outlined in Task 1 is a high priority for the coming year.

Task 3. Generation of stable primary mouse embryo fibroblast/FcR cell lines

Task 3 is directed toward establishing stable isogenic neurofibromin-deficient and wild type
primary mouse embryo fibroblasts (MEFs) that express the ecdysone-responsive transcriptional activator
and can be utilized in subsequent analyses of cellular proliferation following exogenous neurofibromin
expression in the primary fibroblasts. We are interested in these analyses since the MEFs, unlike the
pre-neoplastic NIH3T3 line, are not likely to have suffered any additional genetic changes and may have
da different response to neurofibromin overexpression. Our progress in generating these lines using the
Invitrogen system has been severely limited by the low transfection efficiency of these cells and low cell
viability following drug selection. These difficulties may have been overcome by the use of the
amplicon system but the poor control of tetracycline-dependent expression in that system precluded its
use in the MEF cell lines. The retroviral ecdysone-induced expression system described in Task 1 offers
an alternative method to efficiently introduce exogenous wild type neurofibromin into the MEF lines
and tightly control its regulation in subsequent proliferation assays. These experiments are an important
goal of the coming year.
Key Research Accomplishments N/A

Reportable Outcomes N/A

Conclusions

Our efforts of the past year have been directed at surmounting one major obstacle to progress in NF1 research, namely the inability to manipulate normal and mutant neurofibromin expression in a relevant cell system. Our work in developing reliable inducible expression in NIH3T3 cells using both the vector-based ecdysone controlled system and the tetracycline-inducible amplicon system (task 1) has proven difficult and has limited our ability to define critical functional domains of neurofibromin through analysis of mutant proteins (task 2). We feel that these limitations can be overcome by the use of a recently commercially available retroviral ecdysone expression system (Stratagene), particularly since stable NIH3T3 lines expressing optimized levels of the ecdysone-responsive trans-activator are now available. Our efforts will now focus on using this system to efficiently assess the effects of wild type and mutant neurofibromin on cellular proliferation. Additionally, the retroviral system will allow us to analyze potential anti-proliferation effects of exogenous neurofibromin expression in the isogenic neurofibromin-deficient and wild type MEF cell lines (task 3). Our work over the coming year will lay the foundation for further investigation of the complex role of neurofibromin in cellular growth control mediated through its Ras-dependent and Ras-independent functions and may suggest additional cellular pathways in which the consequences of disease causing mutations in the NF1 gene can be investigated. Moreover, these studies will provide insight into the molecular pathology of NF1 and identify potential targets for novel therapeutic approaches and management of this disorder.

References


