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Award Number: DAMD17-01-1-0715

TITLE: "High Sensitivity SELDI Analysis of NFI Interactors in Mammals, Drosophila, and Yeast"

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REPORT DATE: August 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Annual (15 Jul 01 - 14 Jul 02)

4. TITLE AND SUBTITLE
"High Sensitivity SELDI Analysis of NFI Interactors in Mammals, Drosophila, and Yeast"

5. FUNDING NUMBERS
DAMD17-01-1-0715

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The role of neurofibromin in cellular growth control is complex with available data suggesting that neurofibromin is a bifunctional modulator that may regulate proliferation by coordinating the activities of numerous molecular pathways. One important step in clarifying the function of neurofibromin is the identification of critical interactors whose detection and characterization may define downstream targets of neurofibromin and regulators of its activity. The purpose of our research is identification of neurofibromin-associated proteins in multiple experimental systems using high sensitivity surface enhanced laser desorption/ionization (SELDI) mass spectrometry. 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 using tetracycline-inducible HSV amplicon systems to re-introduce wild type neurofibromin into neurofibromin-deficient primary mouse embryo fibroblasts and human neuroblastoma cells. Our progress has been severely limited by technical difficulties that have prevented controlled exogenous neurofibromin expression in the target cells. We are now utilizing a recently available inducible amplicon system that affords much tighter regulation of transgene expression. This feature will overcome the major obstacle that has hampered our progress to date. This research will open new avenues of experimentation to clarify neurofibromin's role in cellular growth control.

14. SUBJECT TERMS
neurofribromin, interacting proteins, mass spectrometry, amplicons

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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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 *NF1* gene encodes the neurofibromin tumor suppressor protein 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 although considerable evidence suggests that neurofibromin has important regulatory functions distinct from its RasGAP activity and its role in cellular signal transduction and growth control is complex (4-8). One critical step in clarifying the function of neurofibromin is the identification of crucial interacting proteins whose detection and characterization may help to place neurofibromin in alternative signal transduction pathways and may define both regulators of neurofibromin activity and downstream targets of neurofibromin. Current information obtained from studies using conventional methods to detect functionally relevant neurofibromin-interacting proteins is limited, suggesting that alternative approaches to this long-standing question may prove valuable in the definition of critical interacting proteins. The purpose of our research is the identification of functionally relevant neurofibromin-associated proteins using high sensitivity surface enhanced laser desorption/ionization (SELDI) mass spectrometry in multiple experimental systems. Through these powerful analyses, we will identify neurofibromin-associated proteins in mammals, Drosophila, and yeast and assess the functional relevance of these interactions using mutant forms of neurofibromin. We expect that these studies will lay the groundwork for further research on neurofibromin function and will open new avenues of experimentation to clarify the complex role of neurofibromin in cellular growth control.

Body

Task 1. Detect neurofibromin interactors in human and mouse cells by high sensitivity SELDI

Our initial approach to the identification of neurofibromin-associated proteins in mammalian cells is simple co-immunoprecipitation analysis of neurofibromin-containing complexes isolated from neurofibromin-deficient primary mouse embryo fibroblasts (MEFs) and the human neuroblastoma cell line 90-4 following re-introduction of epitope-tagged wild type neurofibromin. The proposed experiments using SELDI to detect neurofibromin-associated proteins through co-immunoprecipitation require the use of carefully matched samples to insure that any protein detected is in fact a true associated protein and not a non-specific co-precipitating protein that is detected because of background differences used in the initial immunoprecipitation. 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. These vectors have proven useful in the introduction and stable expression of genes in cell lines like 90-4 and the MEFs that are not readily transfected through conventional methods. More recently, the Breakefield laboratory has developed a series of vectors that allow regulatable transgene expression and afford more control of exogenous gene expression. This is an important feature for our experiments since even low level (2-3

fold) overexpression of neurofibromin inhibits cellular proliferation (9). The Breakefield laboratory provided us with the amplicon shown in Figure 1.

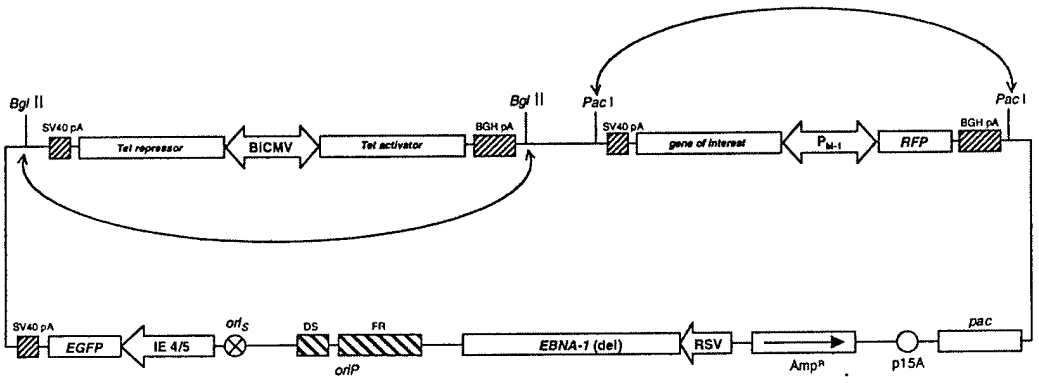

Figure 1 Tetracycline-regulated amplicon HET2

The HSV/EBV amplicon backbone was derived from previously described constructs (10) 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 (11). 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 with broad host range that infect target cells with high efficiency (10, 12). The HET2 vector shown in Figure 1 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 1 also contains a tetracycline-independent reporter (enhanced green fluorescent protein) to monitor infection efficiency. We tested the utility of this tetracycline-inducible amplicon in our studies by first analyzing the potential leakiness of the tetracycline-controlled promoter. We infected NIH3T3 cells with the amplicon carrying no transgene since this cell line is easily infected at high efficiency (S. Camp, pers. comm.). We then examined both GFP and RFP expression in the absence of the tetracycline analog doxycycline and the results are shown in Figure 2.

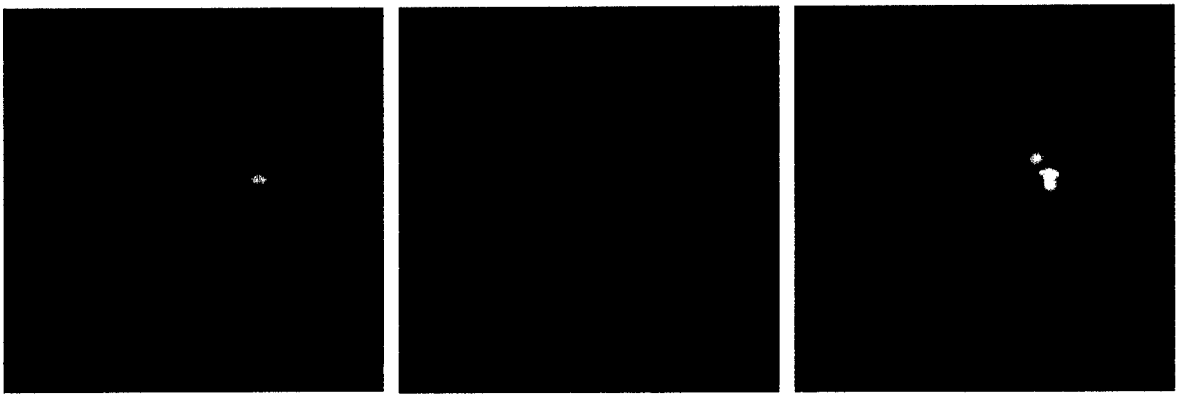

Figure 2 Reporter gene expression in NIH3T3 cells infected with the tetracycline-inducible amplicon. Expression of green fluorescent protein (GFP, left panel) and red fluorescent protein (RFP, center panel) in HET2-infected NIH3T3 cells was assessed by fluorescence microscopy 48 hours post-infection. 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. In parallel analyses of the 90-4 neuroblastoma cell line, we also observed variable constitutive expression of RFP that correlated with the level of GFP expression in the infected cells although the overall infection efficiency was low.

The high constitutive level of RFP expression following infection with this amplicon precluded its use in our experiments since our studies require tight regulation of exogenous neurofibromin expression to generate well-controlled samples for our co-immunoprecipitation analyses. Rather than proceed with this particular system, we chose to wait for a modified tetracycline-inducible amplicon system that was being developed in the Breakefield laboratory. The vector is shown in Figure 3.

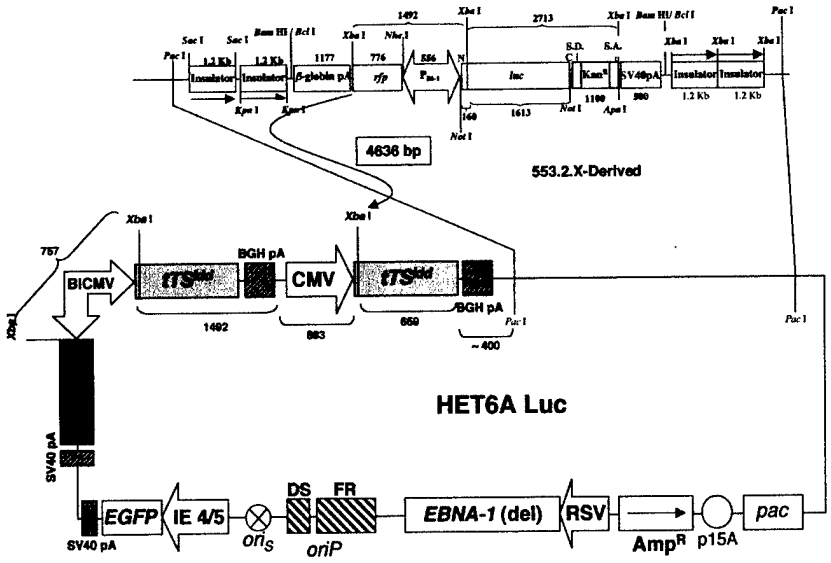

Figure 3 The modified tetracycline-inducible amplicon HET6A Luc

Amplicon HET6A Luc is derived from the HET2 vector shown in Figure 1 and contains many of the same features of the amplicon backbone. The expanded region shows the elements surrounding the cloning site in which the transgene of interest replaces the luciferase coding sequence. The bidirectional promoter driving RFP and transgene expression is tetracycline-responsive.

The HET6A Luc amplicon is an improved version of the original HET2 vector and contains elements that are designed to reduce constitutive transcription from the bi-directional tetracycline-responsive promoter that drives transgene expression. The major modification is the presence of two copies of the *tTS*^{kid} gene which encodes a chimeric silencer protein that binds to the inducible promoter in the absence of tetracycline and blocks expression. Preliminary analysis of this amplicon indicates that the level of constitutive expression from the tetracycline-inducible promoter is significantly reduced as compared to the levels observed with the original HET2 vector (M. Sena-Esteves, pers. comm.). We have obtained the HET6A Luc amplicon and are initiating experiments to test the level of constitutive transgene expression in our cell lines. We are simultaneously generating a version of the HET6A Luc vector carrying FLAG-tagged wild type neurofibromin for use in our experiments in MEFs and the 90-4 human neuroblastoma cell line. We expect that the improved control of tetracycline-regulated expression afforded by the HET6A amplicon system will allow us to carry out well-controlled co-immunoprecipitation experiments to identify neurofibromin interactors. The generation of the neurofibromin-expressing HET6A-derived amplicon and its use in the MEF and 90-4 cell lines is the major goal of the coming year.

Clearly, our difficulty in developing a reliable method for controlled expression of exogenous neurofibromin in the mouse and human cell lines has limited our progress in the identification of relevant neurofibromin interactors. This obstacle has also led us to delay the optimization of conditions for SELDI analysis of immunoprecipitated neurofibromin-containing complexes. The development of

Task 2. Analyze association of mutant neurofibromin proteins with identified interactors

The goal of Task 2 is to generate mutant forms of neurofibromin in the tetracycline-inducible expression vector and test the ability of each to associate with the candidate neurofibromin interactors identified in Task 1. Such analyses are one means to assessing the relevance of the identified interactors to the normal function of neurofibromin. The planned neurofibromin constructs outlined in the proposal included a series of point mutants with disease-causing amino acid substitutions in both the well-characterized GAP-related domain and a proposed second functional domain comprising exons 11-17 (8, 13). Our progress in the construction of these mutants, which was to be initiated in month 6 of the funding period, has been hampered by the difficulties in developing a reliable inducible expression system in the neurofibromin-deficient mouse and human cells as described in Task 1. We will generate these neurofibromin mutants once we have determined the utility of the HET6A amplicon system in the MEF and 90-4 cell lines.

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 establishing reliable exogenous neurofibromin expression using the HET2 tetracycline-inducible amplicon system (task 1) in primary mouse embryo fibroblasts and human neuroblastoma cells has proven difficult and has limited our ability to identify cellular proteins which interact with neurofibromin. The limitations of the HET2 amplicon system have also prevented us from initiating studies of the association between candidate interacting proteins and neurofibromin mutants harboring disease-causing alterations (task 2). We feel that these obstacles can be overcome through use of the recently developed HET6A amplicon vector that has been engineered to prevent unregulated exogenous expression of neurofibromin. Our efforts will now focus on using this system to identify neurofibromin-associated proteins in human and mouse cells through the co-immunoprecipitation and SELDI analyses described in the original proposal. We expect that this research will define new routes of neurofibromin function and open up unexplored areas in NF1 research. 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

1. Riccardi, V.M. 1981. Von Recklinghausen neurofibromatosis. N Engl J Med **305:** 1617-1627.

2. Marchuk, D.A., Saulino, A.M., Tavakkol, R., Swaroop, M., Wallace, M.R., Andersen, L.B., Mitchell, A.L., Gutmann, D.H., Boguski, M., and Collins, F.S. 1991. cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the NF1 gene product. Genomics **11:** 931-940.
3. Bernards, A., Haase, V.H., Murthy, A.E., Menon, A., Hannigan, G.E., and Gusella, J.F. 1992. Complete human NF1 cDNA sequence: two alternatively spliced mRNAs and absence of expression in a neuroblastoma line. DNA Cell Biol **11:** 727-734.
4. Johnson, M.R., DeClue, J.E., Felzmann, S., Vass, W.C., Xu, G., White, R., and Lowy, D.R. 1994. Neurofibromin can inhibit Ras-dependent growth by a mechanism independent of its GTPase-accelerating function. Mol Cell Biol **14:** 641-645.
5. Li, Y. and White, R. 1996. Suppression of a human colon cancer cell line by introduction of an exogenous NF1 gene. Cancer Res **56:** 2872-2876.
6. Guo, H.F., The, I., Hannan, F., Bernards, A., and Zhong, Y. 1997. Requirement of Drosophila NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. Science **276:** 795-798.
7. The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Y., Gusella, J.F., Hariharan, I.K., and Bernards, A. 1997. Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. Science **276:** 791-794.
8. Fahsold, R., Hoffmeyer, S., Mischung, C., Gille, C., Ehlers, C., Kucukceylan, N., Abdel-Nour, M., Gewies, A., Peters, H., Kaufmann, D., Buske, A., Tinschert, S., and Nurnberg, P. 2000. Minor lesion mutational spectrum of the entire NF1 gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet **66:** 790-818.
9. Johnson, M.R., DeClue, J.E., Felzmann, S., Vass, W.C., Xu, G., White, R., and Lowy, D.R. 1994. Neurofibromin can inhibit Ras-dependent growth by a mechanism independent of its GTPase-accelerating function. Mol Cell Biol **14:** 641-645.
10. Sena-Esteves, M., Saeki, Y., Camp, S.M., Chiocca, E.A., and Breakefield, X.O. 1999. Single-step conversion of cells to retrovirus vector producers with herpes simplex virus-Epstein-Barr virus hybrid amplicons. J Virol **73:** 10426-10439.
11. Freundlieb, S., Schirra-Muller, C., and Bujard, H. 1999. A tetracycline controlled activation/repression system with increased potential for gene transfer into mammalian cells. J Gene Med **1:** 4-12.
12. Sena-Esteves, M., Saeki, Y., Fraefel, C., and Breakefield, X.O. 2000. HSV-1 amplicon vectors-simplicity and versatility. Mol Ther **2:** 9-15.
13. Upadhyaya, M. and Cooper, D.N., The mutational spectrum in neurofibromatosis type 1 and its underlying mechanisms. In Neurofibromatosis type 1: from genotype to phenotype, M. Upadhyaya and D.N. Cooper, Editors. 1998, BIOS Scientific Publishers: Oxford. p. 65-88.