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PRINCIPAL INVESTIGATOR: Frank McCormick, Ph.D.
Vivianne Ding, Ph.D.
David Toczyski, Ph.D.
Suchitra Ananthnarayan

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, CA 94143-0962

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Frank McCormick, Ph.D., Vivianne Ding, Ph.D., David Toczyski, Ph.D. and Suchitra Ananthnarayan

Email – mccormick@cc.ucsf.edu

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ABSTRACT

We proposed to study new functions of neurofibromin in the model organism, the budding yeast, *Saccharomyces cerevisiae*. The design of our study is mainly to generate dominant negative mutants of neurofibromin and its yeast homologs (Ira1 and Ira2), induce their expression in wild type yeast and elicit an interfering phenotype. We will then screen a yeast library to identify novel interaction partners of neurofibromin. We have constructed various mutants and fragments of Ira1 and neurofibromin. All of them have been tested in either transient or stably integrated expression. So far we have not observed any obvious interfering phenotype in the yeast strains that we have used. We have also experienced difficulties in detecting the expression of some of the mutants. We are continuing to test more yeast strains as difference strains have variable sensitivities to these mutants. A good rabbit anti-neurofibromin antibody was generated. Our second methodology to search for new functions of neurofibromin is gene expression profiling. We have obtained some preliminary data comparing Ira1/Ira2 deletion and Ras super-activation. We are working towards verifying these data and hopefully reaching some definitive answers in the near future.

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INTRODUCTION:

Neurofibromin is a GTPase activating protein (GAP) that down-regulates Ras [1-3]. Loss of neurofibromin and consequent sustained Ras activity is believed to account for many of the phenotypes associated with neurofibromatosis type 1 (NF1). Neurofibromin is a large protein containing 2818 amino acids. The function of the 330 amino acid GAP domain is well known, but little is know about the functions of the rest of the protein. These highly conserved regions may be responsible for allosteric regulation of the GAP domain in response to unknown signals, or to novel functions unrelated to Ras GAP regulation. By analogy with p120GAP, it is likely that the neurofibromin senses cellular signals, and, in response to these cues, down-regulates Ras. We hypothesize that the regions of neurofibromin flanking the GAP domain are involved in sensing these signals and that these signals are likely to interact with the conserved Ira proteins in a similar manner. Yeast *S. cerevisiae* has two neurofibromin homologs, Ira1 and Ira2, whose functions can be complemented by the human protein, and whose homology with neurofibromin extends across most of the protein, including sequences flanking the GAP domain [3-6] . We will study the functions of these flanking sequences and identify proteins or metabolites that they interact with. We will also search for non-GAP related functions of these highly conserved regions. Our specific aims are: (1) To establish a system for blocking signals detected by neurofibromin and Ira proteins using yeast as a model organism, (2) To identify the nature of the signals using genetic, biochemical and genomic approaches. Very little is known about functions of neurofibromin other than its GAP activity. To develop rational therapies for NF1, we need to fully understand how neurofibromin is regulated, and to understand all of its cellular functions.

BODY:

Identification of functions of neurofibromin distinct from the RasGAP domain.

Task 1. Establish a system in which Ira or neurofibromin proteins acts as dominant interfering mutants blocking signals normally detected by these highly conserved regions.

a. Construct a series of plasmids for inducible expression of fragments and mutants of Ira and neurofibromin

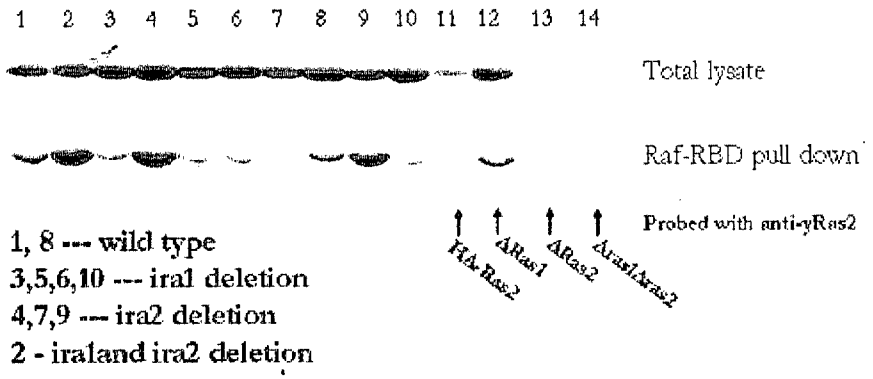
We have generated plasmids for inducibly expressing the following fragments and mutants of Ira and neurofibromin: Ira1N(aa. 1-1508), Ira1C(aa.1776-2939), Ira1ΔGRD (deleted for aa.1508-1776), Ira1-K1737E (modeled after the K1423E mutant in the GRD of neurofibromin that fail to bind Ras) [7]; Nf1-1 (aa. 1-375), Nf1-2 (aa. 372-718), Nf1-3 (aa. 719-1175), Nf1-GRD (aa. 1176-1552), Nf1-Sec14p (aa. 1552-1710), Nf1-4 (aa. 1707-2317), Nf1-5 (aa. 2314-2525), Nf1-6 (aa. 2522-2818). All of these fragments and mutants are HA eptiope-tagged and under the galactose inducible GAL1 promoter.

b. Perform assays to test these fragments and mutants do not interact with Ras

We were not able to detect expression of the different Ira1 fragments and mutants in total yeast cell lysates using antibodies against the HA epitope. All the Nf1 fragments are expressed in yeast and were successfully detected. We are currently working on immunoprecipitation experiments to examine whether these fragments can interact with Ras in yeast. So far, none of the fragments and mutants, except Nf1-GRD, can complement the heat shock

sensitivity phenotype of *ira*-deletion strains, supporting the prediction that these fragments do not interact with Ras. Ras-GTP level also did not change significantly in several yeast strains expressing different Ira1 and Nf1 constructs.

We established a simpler and more environment friendly assay to measure Ras-GTP level in yeast. Traditionally, in order to measure Ras-GTP level in yeast, one needs to metabolically label yeast with ^{32}P, then immunoprecipitate Ras, separate the nucleotides bound on Ras using thin-layer-chromotography [8]. We adapted the Raf-RBD pull down assay that is commonly used with mammalian system to measure yeast Ras-GTP level [9, 10]. In this protocol, yeast cultures were simply lysed with Cellytic-Y (Sigma) supplemented with protease inhibitors, phosphatase inhibitors, and 10mM $MgCl_2$. Ras-GTP is pulled down by GST-Raf-RBD immobilized on glutathione beads and visualized using Western blot. Using this protocol, we are able to detect increased yRas2-GTP in an *ira1* and *ira2* deletion strain, also *ira2* deletion strains (fig. 1). We did not detect obvious changes of yRas2-GTP in *ira1* deletion strains, possibly due to detection limits (fig. 1). Very recently, an Italian group has published a paper using a similar method to examine yRas2-GTP in yeast [11]. In their report, they also found that Ras-GTP level elevated about 2-3 fold in *ira1* strain while in *ira2* strain the elevation is at least 6 fold. We hope to further optimize this assay.



We also invested some time and effort to develop antibodies that can be used in immunoprecipitation experiments for neurofibromin/Ira. Several different fragments of neurofibromin was cloned into bacterial expression vectors and rabbit polyclonal antibodies were generated against the recombinant proteins. One of the antibodies against a portion of the N-terminus was able to immunoprecipitate endogenous neurofibromin protein from mammalian cell lysates.

c. Make yeast strains that have the Ira and neurofibromion fragments and mutants integrated into the genome and confirm the expression

All Ira1 fragment and mutants have been stably integrated into wild type yeast strain W303a, S288c and Σ1278b. Integrations were verified by PCR. However, we experienced the same problem of not being able to detect the HA tagged mutant protein.
We have also made various yeast strains that contain one IRA and one RAS. They will be subjected to the same tests.

d. Analyze phenotypes of interfering yeast strains

We have not observed any obvious phenotypes such as heat shock sensitivity or growth defects upon galactose induction.

e. Test phenotype of *ira1ira2* double deletion and Ira/neurofibromin overexpression in *ras*-deletion strains, also examine gene expression profiles

We have performed expression arrays comparing wild type strains, wild type strains expressing constitutively active yRas2(V19) and an *ira1ira2* double deletion strain. Our preliminary results suggest that not only there are common sets of genes regulated in similar fashion in both active yRas2(V19) cells and *ira1ira2* double deletion cells, there are also differentially expressed genes between them. We are currently verifying the gene expression profiles in more isogenic strain backgrounds using yeast strains that we constructed and more uniform culture conditions. *ira1ira2* double deletion strain has been made and markers used for integration have been removed using cre/lox system. Array analysis is underway.

Task 2. Identify the nature of the incoming signals that neurofibromin/Ira receive.

Pending further results from Task 1, we will then embark on the following tasks.

a. Map the minimal region of Ira/neurofibromin required to achieve intereference
b. Rescue the interference phenotype by introducing DNA library and recover candidates
c. Set up affinity purification and two-hybrid screens using the minimal region
d. Analyze candidates from various approaches to identify if any of them is the signal that we are pursuing
e. Test the influence of cAMP or other potential metabolites on Ira signaling
f. Move to mammalian systems to isolate mammalian homologs of the signals and analyze neurofibromin regulation

KEY RESEARCH ACCOMPLISHMENTS:

- Constructed various plasmids for inducible expression of fragments and mutants of Ira1 and Nf1
- Made various yeast strains containing integrated fragments and mutants of Ira1
- Established assay to detect Ras-GTP level in yeast
- Obtained preliminary gene expression patterns suggesting functions of Ira/neurofibromin distinct from Ras pathway.
- Generated a rabbit polyclonal antibody for immunoprecipitation of neurofibromin

REPORTABLE OUTCOMES:

Jaime Lopez, a student from San Francisco State University, participated in this project, has completed his Master program. He is currently a student in the Biomedical Sciences PhD program in University of California, San Francisco (from Fall 2004).

CONCLUSIONS:

We have devoted most of our effort in trying to establish an interference phenotype in yeast using rationally designed dominant negative mutants of Ira/neurofibromin. A series of constructs and yeast strains were made. So far we have not observed any obvious phenotype, also we had problem detecting the expression of certain mutants under induced conditions. In order to achieve a robust phenotype by dominant negative mutants, these mutants need to be expressed at levels that are higher than or at least close to that of the endogenous wild type protein, so to compete and titrate out signals that the endogenous proteins receive normally. We attempted to compare the expression level of mutants and the endogenous protein by using several tagged Ira strain either from the Yale HA-tagged yeast collection or those constructed by our own. So far tagged endogenous Ira proteins are as difficult to detect as the mutants in Western blots using whole cell lysates. We are working on using immunoprecipitation methods to detect these proteins. We would also like to expand the choice of yeast strains to strains that only contain one Ira protein, i.e. only having Ira1 or Ira2. The rationale is that in these strains the mutant proteins will only have to compete with half does of the wild type protein.

We successfully established a non-radioactive assay to measure endogenous Ras-GTP in yeast cells. With this method, we can measure Ras-GTP in a much more high-throughput and environmentally friendly manner. We also generated a rabbit polyclonal antibody that is capable of immunoprecipitating neurofibromin from mammalian cell lysates.

Expression array analysis has generated a large amount of data for us. Once we process all the yeast strains and verify the data, we should be able to draw some definitive conclusions in terms of whether Ira proteins regulate any non-Ras pathway molecules. These future results will give us new insights into the functions of Ira/neurofibromin.

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