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Alterations in the cellular genome affecting the expression or function of genes controlling cell growth or differentiation are the mechanism underlying the genesis of all cancers. Human cancers often require mutations in genes called proto-oncogenes which mutate to oncogenes (or cancer genes). A subset of proto-oncogenes comprise the RAS signal transduction pathway.

Vulval development in the nematode worm C. elegans is controlled by a RAS signal transduction pathway. C. elegans has proven to be a pwerful model system for understanding regulation of the RAS pathway. This research studies two novel components of the RAS pathway in C. elegans, sur-2 and sur-9. A novel gene has been identified that may physically interact with sur-2. In addition, a second novel gene, sur-9, is in the process of being cloned to facilitate further analysis of its function in the RAS signal transduction pathway. These studies will improve our understanding of mitogenic signaling and may eventually lead to the design of novel cancer therapeutic agents which target control proteins in the RAS pathway.

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Andrew G. Spencer, Ph.D. 4/21/00

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INTRODUCTION

Our knowledge of animal development has increased exponentially during the 1990's. Studies in a variety of organisms make clear that the events responsible for executing developmental programs are conserved not only at the level of individual proteins, but throughout their functional pathways. For example, the Wnt pathway members (Wnt, Frizzled, GSK, β-catenin, TCF/Lef, and APC) are all found in flies, frogs, worms, mice, humans, and other metazoans (Cadigan, 1998). The Ras signal transduction pathway (Fig. 1) is found in all metazoans and used in the regulation of disparate cellular events (e.g., cell proliferation, differentiation, and migration) (Sternberg and Han, 1998). It seems likely that complex signaling networks upstream and downstream of pathways like Ras. This report contains the results from work by Dr. Edward DesJardins and the author on the characterization of sur-2, a novel Ras-mediated signal transduction component in C. elegans. Ras pathway and its role in *C. elegans* vulval development to further our knowledge of downstream control of a specific cell signaling and differentiation event.

In *C. elegans*, activation of the Ras pathway is required to trigger the events of vulval development. The vulva is the mating and egg laying organ of *C. elegans* hermaphrodites. Derived from ectodermal precursor cells (Pn.p cells), the vulval cell lineages are quite simple and therefore relatively easy to score at the level of individual cells (Sulston and Horvitz, 1977). A wild-type vulva is developed during the third and fourth larval stages. The organ is formed by 22 cells (nuclei) which are derived from the posterior daughters of three ectodermal P cells. Vulval differentiation can be divided into three sequential steps: generation of the vulval precursor cells (Pn.p cells or VPCs), determination of the differential fates of these precursors, and execution of vulva-specific functions. The Han laboratory focuses on the latter two steps.

Vulval cell fates are specified by multiple intercellular signals (Sternberg and Han, 1998). Prior to vulval differentiation, six Pn.p cells (P3.p – P8.p) have equal developmental potential (Fig. 2). During the third larval stage, three of the six VPCs (P5.p – P7.p) differentiate into two vulval cells types (1° or 2° fates) and the remaining three become part of the epidermis (3° fates)(Fig. 2). In mutants, the cell fates for these precursor cells can be altered in opposite directions to cause either the Multivulva (Muv) phenotype due to extra VPCs differentiating into vulval cells, or the Vulvaless (Vul) phenotype due to less than three VPCs differentiating into vulval cells. For example, a gain-of-function mutation in the let-60 Ras gene causes an inappropriate activation of the pathway resulting in all Pn.p cells adopting 1° or 2° fates (Han, 1990). In turn, multiple vulval inductions occur to give the characteristic Muv phenotype. Screens identifying suppressors of the Muv phenotype have led to the identification of many genes involved

in Ras signaling, including *sur-2* and *sur-9* (reviewed by Sundaram and Han, 1996; Sternberg and Han, 1998; M. Han, unpublished.).

Previous studies suggest that the precise pattern of vulval cell differentiation for the six VPCs is specified by three different intercellular signals. These include an inhibitory signal mediated by the Rb-E2F pathway to repress the vulval cell fates (Lu and Horvitz, 1998), a gonad-derived RTK/Ras/MAP kinase-mediated inductive signal to induce three of the six VPCs to become the vulval cells, and a lin-12/Notch-mediated lateral signal acting between VPCs to specify 2° vulval cells (Kornfeld, 1997; Sundaram and Han, 1996).

C. elegans in particular has proven to be a extremely powerful system with which to explore the Ras signaling pathway. As alluded to above, screens aimed at understanding vulval induction have identified an EGF-like growth factor, a receptor tyrosine kinase, RAS, RAF, MEK and MAP kinase. Downstream modulators of the Ras pathway identified by genetic suppressor screens in the Han laboratory have identified a number of new genes. Many of these, including *sur-2, sur-4, sur-5, sur-6, sur-7, sur-8,* and *ksr-1* are now known to have mammalian homologues, proving *C. elegans* to be an excellent model system for understanding mammalian signaling pathways (Sternberg and Han, 1998).

Other genes have been identified that act downstream of MAP kinase (Sternberg and Han, 1998). LIN-1 and LIN-31 are two downstream factors that are likely regulated by MPK-1 MAP kinase phosphorylation. LIN-1 acts negatively on vulval induction and phophorylation of LIN-1 in its C-terminal domain is likely to repress its function as an inhibitor of vulval cell fate (Jacob et al., 1998). Recent results also suggest that LIN-1 and LIN-31 interact directly to form a LIN-1/LIN-31 complex that inhibits vulval formation (Tan, et al., 1997). Phosphorylation of LIN-31 by MPK-1 disrupts the complex and thus disrupts the inhibitory function of LIN-1. Two other genes, LIN-25 and SUR-2, are likely to act as positive factors downstream of MPK-1 in vulval differentiation. They could act in a branch downstream of *mpk-1* and have been proposed to act as transcription cofactors based on their position in the genetic pathway.

Work supported by this fellowship resulted in progress towards cloning a new suppressor of Ras, sur-9, and the identification of a potential sur-2 interacting protein, B0379.4.

The sur-2 gene.

Sur-2 was identified a few years ago in the Han lab in a screen for suppressors of a gain-of-function Ras mutation (Singh and Han, 1995). Alone, mutations in *sur-2* result in a severe Vulvaless phenotype suggesting a role for its gene product in positive modulation of the Ras pathway. Genetic epistasis analyses indicate that *sur-2* is likely to

act downstream of Ras/MAP kinase. The *sur-2* mutation can partially suppress mutant *lin-1* and *lin-31*, indicating that *sur-2* may act in parallel to these genes and may modulate transcription. *Sur-2* gene expression is found in vulval precursor cells at the time of vulval induction (Singh and Han, 1995). Studies in the Han laboratory also indicate that *sur-2* expression patterns change in vulval precursor cells and in response to a lin-1 mutation (M. Han, unpublished). Human *sur-2* (h*sur-2*) was identified and cloned from a biochemical assay for proteins that interact with the adenovirus E1A zinc finger domain (Boyer, et al., 1999). These studies showed that h*sur-2* mediates a stable interaction between viral E1A and a human homologue of the yeast Srb/Mediator protein complex. The role of h*sur-2* in transcription is consistent with the genetic analysis performed by the Han laboratory on *C. elegans sur-2*. It is therefore quite possible that further studies on the role of *sur-2* in *C. elegans* will reveal mechanisms used in human systems. By focusing on the *sur-2* interacting protein encoded by the gene B0379.4, we hope to characterize more fully the *sur-2* complex and its role downstream of Ras signal transduction.

The sur-9 gene.

The *sur-9* locus was identified recently in the Han laboratory in a temperature-sensitive screen for suppressors of a gain of function mutation in the *C. elegans* Ras gene, *let-60*. Thus, the wild-type *sur-9* gene product is a positive regulator of the Ras pathway in vivo. *Sur-9* mutants by themselves result in abnormal vulval morphogenesis and other developmental defects. Preliminary genetic epistasis experiments performed by a former postdoctoral fellow in the Han laboratory indicate that *sur-9* acts downstream of the Ras signal transduction pathway. Double mutant analyses indicate that *sur-9* mutations suppress the Muv phenotypes caused by mutations in *lin-1, let-60,* and *lin-15*[1]. Preliminary observations that *sur-9* suppresses *lin-1* mutations are of particular interest since few known Ras suppressors have been reported to act downstream of *lin-1*. We expect that identification of *sur-9* and its subsequent analysis will lead to interesting advances in our understanding of the downstream events controlled by the Ras pathway.

[1] *Lin-15* encodes a novel protein that negatively regulates the activity of let-23, a receptor tyrosine kinase (Clark, et al., 1994).

METHODS

Identification of SUR-2 interacting proteins: the candiate approach

The yeast two-hybrid assay to test candidate proteins which may physically interact with SUR-2. The SUR-2 bait construct consisted of the first 1412 of the 1587 amino acids in the SUR-2 proteins cloned into the yeast two-hybrid GAL4 DNA binding domain vector pAS2 (Clontech). All attempts to subclone the full length sur-2 CDNA into the bait vector were unsuccessful despite multiple subcloning strategies. The cDNAs for candidate SUR-2 interactors (lin-31, lin-25, sur-1, lin-1, and lin-39) were subcloned into the two-hybrid GAL4 activation domain prey vector, pACT2 (Clontech). The yeast strains used in the screen contain two reporter genes, HIS and lacZ, which are activated upon reconstitution of the GAL4 DNA binding and transcriptional activation domains.

Identification of SUR-2 interacting proteins by library screening

The cDNA libraries used in this screen are courtesy of Robert Barstead and are C. elegans mixed stage two-hybrid libraries, each cloned into the pACT2 GAL4 activation domain prey vector. One library, RB-1 is an oligo-dT primed library, while the second, RB-2 is a random primed library. Each of these libraries contains an estimated 1×10^7 unique clones. The yeast strains used in the screen contain two reporter genes, encoding HIS and lacZ. 2.4×10^6 and 3.64×10^7 clones from the RB-1 and RB-2 libraries, respectively, were screened using SUR-2 as bait.

Generation and genetic characterization of sur-9

As described in previous annual reports, a genetic suppressor screen was conducted to identify signaling components that lie downstream of Ras. Widely used genetic and C. elegans methods were used to attempt to map the sur-9 mutation to a discrete position on a chromosome (three-point mapping, epistasis, etc).

Characterization of a candidate SUR-2 interactor, BO79.4

Since we have no mutants of B0379.4 in hand, we attempted to probe its loss-of-function phenotype by using RNAi[2]. The vulva can be a rather difficult organ in which to employ RNAi, so we plan to use both injection and soaking methods (perhaps in combination) to increase our chances of success. Young adult worms were injected with approximately 0.5×10^6 molecules of double stranded RNA corresponding to the

[2] RNA-mediated gene interference (RNAi) results in the inactivation of the gene to which injected double stranded RNA sequences correspond. The mechanism by which this phenomenon occurs is unknown, but RNAi has proven to be an effective way to phenocopy the null phenotype of many *C. elegans* genes (Fire, et al., 1998).

B0379.4 coding sequence. Each injected worm was then placed on its own plate, and F1 progeny will be scored for phenotype.

RESULTS AND CONCLUSIONS

Identification of SUR-2 interacting proteins

None of the candidates (lin-31, lin-25, sur-1, lin-1, and lin-39) used in the initial yeast two-hybrid tests showed an interaction in the yeast two-hybrid system. However, after eliminating false positives, the library screening identified three candidates that may interact with SUR-2. Unfortunately, two of the three genes were vitellogenin, an egg protein found in many animals that is probably not related to Ras signaling in the C. elegans vulva. The third candidate is a novel gene identified as B0379.4 by the C. elegans genome project. B0379.4 lies on cosmid B0379 and covers approximately 20 kilobases. This project led to the realization that the genome project's predicted genes B0379.4 and B0379.5 are in fact a single gene.

We attempted to further explore the role of B0379.4 by "knocking out" the gene's function by RNAi. No phenotype was observed when B0379.4 sequences were used to perform RNAi. Of 23 worms injected with B0379.4 sequences, all 20 gave rise to progeny whose development appeared normal when examined using Nomarski light microscopy. Control worms injected with RNA sequences corresponding to a gene known to cause embryonic lethality did give rise to dead eggs. This result allowed for three possible interpretations. First, the B0379.4 gene product may not play a significant role in vulval development. Second, and probably more likely based on similar results observed for RNAi on vulval genes, conventional RNAi may not work for B0379.4 sequences. A third possibility was that the role of B0379.4 in vulval development may only be detectable in a Ras gain-of-function (let-60 n1046) background. This is observed with several suppressors of Ras identified in the Han laboratory. For example, mutations in sur-2 have no obvious phenotype alone but can strongly suppress the multivulva phenotype in a let-60 (gf) (n1046) gain of function worm. To examine the possibility that a B0379.4 knockout is silent in a wild type background, RNAi was performed by injecting B0379.4 double stranded RNA sequences into the let-60 (gf) (n1046) background. A similar result was observed; i.e., no changes in the n1046 phenotype were observed.

In short, we are unable to make any firm conclusions about the role of B0379.4 in vulval development. A request to construct a strain lacking a functional B0379.4 gene has been submitted to the C. elegans Gene Knockout Consortium. If the Consortium is successful in making this strain, we will have a valuable tool with which to explore the

role of B0379.4. However, it should be emphasized that no hard evidence exists that B0379.4 interacts with SUR-2 of the Ras pathway other than the two-hybrid experiments, which could be artifactual. It will be important to determine if the B0379.4 gene product is actually expressed in the same cells as SUR-2, which an in vivo interaction would obviously require. Currently, efforts are being made to get an RNAi phenotype using newer, tissue-specific methods. In addition, construction of B0379.4/Green Fluorescent Protein constructs may help us determine the expression pattern of this novel gene.

Characterization and cloning of sur-9

A single isolated allele of the *sur-9* gene results in the suppression of the Muv phenotype caused by a gain-of-function mutation in Ras *let-60*. Genetic analysis indicates that *sur-9* acts downstream of MAP kinase in the Ras signaling network. We attempted to characterize the *sur-9* gene using molecular and genetic methods to learn more about its role in cell signaling. It is highly likely that *sur-9*, like other genes isolated in the Han lab (*sur-2, sur-6, sur-8, ksr-1*, etc.), will encode an unknown *C. elegans* homologue of a mammalian signaling protein.

In short, sur-9 has proven to be a very difficult gene with which to work. Preliminary mapping an analysis of the *sur-9* mutation places the gene within a 0.42 map unit region of chromosome III between two cloned genes, *unc-32* and *emb-9*. We will further three-point map *sur-9* using the *unc-32* and *emb-9* markers to pin down the position of this gene to a region containing less than 10 cosmids. We originally planned to clone *sur-9* using microinjection transformation methods. However, a graduate student in the Han lab, Dennis Eastburn, noticed that the sur-9 mutation acts in a dominant fashion, which makes typical cosmid rescue cloning strategies difficult. This will likely lead us to mapping by single nucleotide polymorphisms in an outcrossed strain, which has been used to map mutations down to a single gene. The *C. elegans* genome project is essentially complete, and it is almost certain that the gene corresponding to the *sur-9* mutation has been sequenced. It may therefore be possible to identify sur-9 by a candidate gene approach. Here, after mapping the mutation to a small genomic region, we would manually scan the genes present and sequence candidates with the hope of finding a molecular lesion that could explain the sur-9 phenotype.

Summary

This project has not led to the publication of any articles to date. However, it is highly probable that manuscripts will be submitted once we come to a more complete understanding of the roles of B0379.4 and sur-9 in C. elegans development.

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