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TITLE: Characterization of sur-2, a Novel Ras-Mediated Signal Transduction Component in C. elegans

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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. One avenue of cancer research studies the function of normal growth-control genes (proto-oncogenes), as well as their transformation into cancer genes (oncogenes). A subset of proto-oncogenes comprise the RAS signal transduction pathway.

Vulval development in the nematode worm Caenorhabditis elegans is controlled by a RAS signal transduction pathway. This research will identify novel components in this RAS pathway, as well as gain insight into how the pathway is regulated. The sur-2 gene, which encodes a large, novel protein, was identified by a screen for suppressors of activated RAS and determined to act downstream of RAS in the signaling pathway. This project studies the function, regulation and targets of SUR-2 using both genetic and biochemical approaches. This information will improve our understanding of mitogenic signaling and may eventually lead to the design of novel cancer therapeutic agents which target control points in the RAS pathway.
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Abstract

Alterations in the cellular genome which affect the expression or function of genes controlling cell growth or differentiation underlie the genesis of all cancers. One avenue of current research in cancer biology endeavors to identify and understand the function of normal growth-control genes (proto-oncogenes), as well as understand their transformation into cancer genes (oncogenes). Ultimately, it is hoped that this basic research will yield novel cancer therapies that target these oncogenes. A subset of proto-oncogenes comprise the RAS signal transduction pathway, a highly conserved collection of signaling molecules used by all eucaryotes. This pathway is of special significance in that it is used by many mitogenic signaling cascades, and mutations in RAS and other components of this pathway are found in many human tumors. This proposed research uses Caenorhabditis elegans as a model system to identify novel components in the RAS pathway, as well as gain insight into how the pathway is regulated.

Vulval development in C. elegans is controlled in part by a RAS signal transduction pathway. Previous studies have identified components within this pathway using a screen for suppressors of the activated let-60/RAS multivulval phenotype. One of the genes identified in this screen is sur-2 (37). Worms homozygous for sur-2 loss-of-function alleles show the vulvaless phenotype. Epistasis analysis verified that sur-2 acts downstream of the let-60/RAS pathway and possibly downstream or in parallel to sur-1/MAP kinase, lin-1, lin-25 and lin-31 in the vulval signaling pathway. The sur-2 gene product bears no significant homology to known signaling proteins; hence, its biochemical activity remains unknown.

Work is in progress to characterize the structure, function and regulation of the sur-2 gene and protein. These studies utilize both biochemical and genetic approaches. These include: 1) Expression studies using a sur-2::GFP reporter construct. 2) Production of anti-SUR-2 antibodies. 3) A yeast two-hybrid screen to identify SUR-2 interacting proteins. 4) Analysis of the temporal requirements for SUR-2 expression using a heat shock inducible expression vector. 5) A genetic screen to identify pathway components which lie downstream of sur-2 in the regulation of vulval induction.
Background/Significance

RAS-signaling is a highly conserved pathway

RAS-mediated signal transduction is a pathway utilized by all eucaryotes to regulate basic cellular functions. This pathway is used to transduce diverse physiological signals in multiple tissues and at all stages of development. Although a variety of physiological signals are received by distinct transmembrane receptors, many of the subsequent cellular responses appear to be mediated by a cascade of common intermediates, within which RAS is a key component (reviewed in 5). The signaling activity of the RAS protein is modulated by its bound guanine nucleotide, as well as the ancillary proteins SOS (an exchange factor, aka guanine nucleotide releasing factor), GAP (a RAS-GTPase activating protein), and adapter proteins (eg. GRB2). Following receptor and RAS activation, RAS recruits and activates the serine/threonine kinase RAF at the cell membrane through a poorly understood mechanism. Activated RAF then triggers a kinase cascade which includes MEK (a dual-specificity kinase, aka MAP kinase kinase) and ultimately MAP kinase, another serine/threonine kinase. Following its phosphorylation and activation, MAP kinase is translocated to the nucleus, where it is thought to control programs of gene expression by phosphorylating target transcription factors (reviewed in 6, 7, 8). Despite our reasonably complete knowledge of this pathway, an underlying question still remains. Since a variety of extracellular signals utilize this same intracellular cascade, there must be other aspects to regulation of the pathway or targets of the MAP kinase which confer the specificity of a cellular response.

Ras pathway intermediates are frequently abrogated in breast cancer

Alterations in the cellular genome which affect the expression or function of genes controlling cell growth or differentiation are considered to be the underlying cause of all cancer progression and maintenance of the neoplastic phenotype. One avenue of current research in cancer biology endeavors to identify and understand the function of normal growth-control genes (proto-oncogenes), as well as understand their transformation into cancer genes (oncogenes). Ultimately, it is hoped that basic research into the nature of proto-oncogenes and oncogenes will yield cancer therapies that target these genes. A subset of proto-oncogenes comprise the RAS signal transduction pathway, a highly conserved collection of signaling molecules used by all eucaryotes.

The RAS-mediated signal transduction pathway includes growth factors, growth factor receptor tyrosine kinases, RAS proteins, other signaling protein kinases, nuclear proteins, and transcription factors. These signal transducing molecules play a vital role in regulating cell proliferation and differentiation. It is these two processes that are disrupted in cancers and give rise to the
destructive neoplastic phenotype. Breast cancer, specifically, shows deregulation of a number of signal transduction components. The following is a partial list of genes (and their respective proteins) that have been theorized to play a role in breast cancer progression or offer important indicators for prognosis and prediction of response to therapy: RAS (10, 11), EGF receptor/c-erbB1 (12, 11, 13), EGF receptor-related oncogenic receptors erbB-2 (aka HER2/neu), erbB-3, erbB-4 (12, 11, 14), CSF-1 and CSF-1 receptor (15), Grb2 (16), EGF and TGF-α (12), estrogen and progesterone receptors (11), and insulin-like growth factors (IGF-I and IGF-2), their receptors, and IGF binding proteins (IGFBPs) (12, 17).

C. elegans vulval differentiation entails multiple signaling events.

The vulva is the nematode mating and egg-laying organ in the adult hermaphrodite worm. The structure arises from relatively few precursor cells, is formed from simple cell lineages, and is readily observed under the dissecting microscope. The presence or absence of this structure provides a sensitive and easily assayed phenotype for the study of the vulval development program. Prior to vulval induction, six vulval precursor cells (VPCs), numbered P3.p thru P8.p, have equal developmental potential (see Figure 1). Each has the ability to assume a vulval developmental fate or become part of the surrounding hypodermis. During the third larval stage, three of the six VPCs (P5.p-P7.p) differentiate into one of two vulval cell types (1° or 2° fates). The remaining three VPCs become part of the surrounding hypodermis (3° fate). The 1° and 2° cells are distinct in both the lineages they produce and their morphologies.

The fate assumed by each of the VPCs is determined by three different intercellular signaling events (see Figure 1). These signals are: (1) an inhibitory signal emanating from the hypodermis to repress the vulval 1° and 2° cell fates; (2) an inductive signal from a specialized cell residing in the adjacent gonad called the anchor cell (this inductive signal induces three of the six VPCs to assume 1° or 2° vulval cell fates); (3) a lateral signal acting between VPCs to specify 2° cell fate, called lateral inhibition (reviewed in 18).
Multipotent Pn.p cells (VPCs)

Surrounding Hypodermis

Figure 1. Schematic of vulval precursor cell (VPC) signaling. VPCs P3.p thru P8.p are shown, as well as the 1°, 2°, or 3° fates they will eventually assume. The following signaling events are indicated: (1) an inhibitory signal from the surrounding hypodermis; (2) an inductive signal emanating from the anchor cell within the gonad; (3) a lateral signal between the VPCs.

Mutations that result in mis-specification of VPC fates have defined many genes necessary for normal VPC differentiation. In these mutants, one of two phenotypes may be manifested (see Figure 2). There may be more than three VPCs that assume a vulval development pathway (1° or 2° cell fates), resulting in extra vulval tissue in the mature worm (the multivulva or "Muv" phenotype). Alternatively, greater than three VPCs may assume a hypodermal cell lineage (3° fate), resulting in a vulvaless (or "Vul") phenotype (19, 20, 21, 22, reviewed in 18).

Ras-mediated signal transduction components are utilized in the C. elegans vulval induction signaling cascade.

The invertebrates Drosophila melanogaster and Saccharomyces cerevisiae have been fruitful model systems in the study of RAS-mediated signal transduction (reviewed in 23, 24). The nematode worm C. elegans has also been a rich source for study. A large number of mutations (and genes) that
disrupt nematode vulval cell lineages have been described. A subset of these genes comprise a RAS signal transduction pathway controlling vulval development (19, 20, 21, 22, reviewed in 25, 26, 27). Most of the signal transduction intermediates identified in mammals have counterparts in this C. elegans signaling cascade. Specifically, the inductive signal emanating from the anchor cell in the C. elegans gonad to induce 1° and 2° vulval lineages in the VPCs requires the same genes found in mammalian mitogenic signaling systems (see Figure 3).

In an attempt to identify additional components which regulate this RAS-mediated signal transduction pathway (including signaling components downstream of MAP kinase), a genetic suppressor screen was used to identify genes which, when mutated, would suppress the Muv phenotype demonstrated by a let-60/ras gain-of-function mutant allele. The mutations identified in the screen would be anticipated to disrupt genes which regulate activity of the RAS signaling pathway, or regulate another contributing parallel pathway. This screen obtained mutations in the lin-45 raf, mek-2 and sur-1/MAP kinase genes, indicating that the screen was successful in identifying known RAS-mediated signal transduction intermediates (34, 36). One of the novel genes identified in this screen is called sur-2, for suppressor of activated ras (37).

Epistasis analysis verified that SUR-2 acts downstream of the let-60/RAS pathway and is a positively acting gene. Worms homozygous for sur-2 loss-of-function alleles are vulvaless, and show the bag-of-worms phenotype. sur-2 mutant alleles demonstrate pleiotropic effects, implicating sur-2 in a number of developmental events in the maturing worm. These effects include an incompletely penetrant larval lethality, males are unable to mate, possibly due to male tail developmental defects, and a variably penetrant sterility and gonadal defects. Genetic experiments also suggest that sur-2 may act downstream of sur-1/MAP kinase. However, the genetic and molecular relationship of sur-2 to other downstream components in the vulval signaling pathway, including lin-1, lin-25 and lin-31, remain somewhat ambiguous.

The SUR-2 protein open reading frame encodes a 1,587 amino acid, 190-kD product bearing no significant homology to any known protein; hence, its biochemical activity remains unknown. The only recognizable protein motif within SUR-2 lies within the C-terminus, and contains repeating glutamine
and histidine residues resembling opa repeats. Opa repeats are found in a wide variety of proteins, and may be sites of protein/protein interactions (38). SUR-2 also contains a highly acidic domain reminiscent of transcription factor activation domains. The protein also contains a single consensus MAP kinase phosphorylation site as well as eight proline-directed serine/threonine phosphorylation sites.

The experiments described in this research investigate the structure, function and regulation of the sur-2 gene and gene product in the vulval development program. An understanding of sur-2 will also address a larger question in the study of ras-mediated signal transduction. That is, how can the same signal transduction intermediates (tyrosine kinase receptors, adapter proteins, RAS, RAF, MEK, MAP kinase) be used in the same organism to manifest a variety of developmental fates? The answers to this question are likely to lie in the downstream targets of the kinase cascade.

Unlike mammalian systems, the easily manipulated genetics of C. elegans will allow us to take genetic as well as biochemical approaches to identify control points in the RAS signal transduction pathway. This information will improve our understanding of mammalian mitogenic signaling and may deepen our understanding of the molecular basis of cancer. An exciting observation has recently been made by Arnold Berk at UCLA (personal communication). He has identified a human homologue of the nematode SUR-2 protein, and has evidence that the protein may be involved in regulating transcription and cell proliferation in human cells. Our groups are currently collaborating to further characterize the function of the SUR-2 protein. Our work may ultimately permit the development of novel "molecular therapies" for the treatment of breast cancer which target control points in the RAS pathway (2, 3), including sur-2. Our lab has also demonstrated the utility of C. elegans as a tool in cancer research. Our lab has shown that C. elegans vulval development is a sensitive multicellular assay system to directly test the effectiveness of potential anticancer drugs that control activity of the RAS pathway (4).

My efforts to characterize the structure, function and regulation of the sur-2 gene and protein utilize both molecular and genetic approaches, and are outlined below. These include: 1) Production of anti-SUR-2 antibodies, which will enable the visualization of SUR-2 expression, and possibly permit the visualization of subcellular localization. 2) A yeast two-hybrid screen is currently in progress to identify SUR-2 interacting proteins. 3) A reporter construct containing the sur-2 promoter region and the GFP coding sequence will be analyzed in wildtype worms as well as worms containing mutations in upstream signaling components. 4) An expression vector containing the sur-2 coding sequence driven by an inducible heat shock promoter is being made. This construct will allow us to address the temporal requirements of SUR-2 expression. 5) A genetic screen is currently in progress to identify pathway components which lie downstream of sur-2 in the regulation of vulval induction.
Analysis of sur-2 gene expression

Worms containing the sur-2(ku9) mutant allele show pleiotropic defects, indicating that sur-2 may be expressed and play a role in multiple development programs in addition to vulval induction. In light of this observation, we wished to visualize the pattern of sur-2 transcription in the developing worm. This will also allow us to confirm that the sur-2 gene is expressed in the vulval lineages during the time of vulval precursor cell induction, as well as presumably observe its expression in other tissues.

Construction of a sur-2 gene reporter construct will also allow us to ask a second critical question. That is: how is sur-2 regulated by the RAS signaling pathway. Two possibilities exist. First, sur-2 activity may be regulated at the level of transcription, whereby the RAS pathway controls the activity of transcription factors which act on the sur-2 promoter region. Second, sur-2 transcription may be constitutive, and SUR-2 protein activity may be regulated by post-translational events (such as phosphorylation), triggered by the RAS signaling pathway. By analyzing the activity of a sur-2 reporter construct in the context of mutations in upstream RAS signaling intermediates, we may be able to distinguish between these two possibilities.

Methods/Results

Analysis of sur-2 transcriptional regulation was initiated by construction of a fusion gene containing the E. coli lacZ gene placed downstream of the sur-2 gene promoter and transcriptional regulatory region. This reporter contains approximately 5 kb of genomic sequence upstream of the initiator methionine as well as 2.8 kb of 5' exon/intron sequence. This chimeric gene was stably integrated into a wildtype host worm genome. Activity of the sur-2 promoter was observed by fixation of whole worms and incubation with X-gal, the colorimetric substrate for β-galactosidase. This technique is well established in C. elegans. As might be predicted, Singh and Han (37) have shown that the sur-2::lacZ chimeric gene is expressed in the vulval precursor cells whose daughters eventually give rise to the 1° and 2° vulval lineages. The chimeric gene is also expressed in embryos and early larval stages, and in the distal tip cells of the gonad, consistent with sur-2 possibly being an integral part of multiple development programs in the worm.

We wished to expand on this initial analysis by making a homologous sur-2 reporter construct using the jellyfish green fluorescent protein (GFP) as a marker in place of β-galactosidase. This would be of great utility, since samples do not have to be fixed or treated prior to visualization, allowing rapid visualization in living animals. This construct contains the identical sur-2 genomic regulatory sequences as that contained in the sur-2::lacZ reporter construct. Analysis of this sur-2::GFP reporter construct in stably
integrated transgenic worms confirmed observations made using the sur-2::lacZ reporter. Expression is observed in the VPC lineages, and is strongest in the P5.p, P6.p and P7.p primary and secondary lineages, and weaker in the P3.p, P4.p and P8.p tertiary lineages. However, unlike the results using the lacZ reporter, sur-2::GFP expression persists in the adult vulva. In addition, GFP expression was observed in the distal tip cells of the hermaphrodite gonad, embryos, the male tail, seam cells, and pharynx.

To examine whether sur-2 is regulated by the RAS pathway transcriptionally or post-transcriptionally, I am currently putting the sur-2::GFP reporter construct into worms carrying mutations in upstream RAS signaling components. I am initially testing the effect of mutations in the lin-45(raf) gene on sur-2::GFP activity. I am also testing the effect of mutations in lin-1 on sur-2::GFP activity. LIN-1 is an ETS family transcription factor known to act in the RAS signaling cascade downstream of MAP kinase and possibly in parallel to SUR-2. These experiments are currently in progress.

Discussion/Alternative Approaches

Previous work from Singh and Han (37) demonstrated that sur-2 is expressed in vulval precursor cells in addition to other developmental programs in the maturing worm. Their experiments used a sur-2::lacZ reporter construct stably integrated into wildtype worms. I have verified these observations using an analogous sur-2::GFP reporter construct. I am in the process of examining the effects of mutations in RAS signaling components on the activity of this reporter construct. These results will indicate if sur-2 is regulated by the RAS pathway at the level of transcription or regulated post-transcriptionally.

Production of anti-SUR-2 antibodies

The production of anti-SUR-2 antibodies will provide a useful tool for studying a number of aspects of SUR-2 function. Using such antibodies, worms can be fixed and subject to standard C. elegans immunohistochemical techniques using the SUR-2 specific antibodies. This experiment will reveal tissue specific SUR-2 expression patterns. The SUR-2 expression pattern observed using an anti-SUR-2 antibody will augment our analysis of the sur-2 transcriptional expression pattern using the sur-2::GFP reporter construct discussed above.

Also with an anti-SUR-2 antibody, we may be able to visualize subcellular localization when viewed under higher magnification. By knowing the subcellular localization of the SUR-2 protein, hypotheses may be made as to its function. For example, a nuclear localization may indicate a role in transcriptional regulation.
Methods/Results

Numerous attempts have been made to raise anti-SUR-2 antibodies. The antigens used in these immunizations are summarized as follows:

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Amino Acids</th>
<th>Animal Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide I w/ His Tag</td>
<td>1-462</td>
<td>rat</td>
</tr>
<tr>
<td>Peptide II w/ His Tag</td>
<td>456-927</td>
<td>rat</td>
</tr>
<tr>
<td>Peptide III w/ His Tag</td>
<td>947-1387</td>
<td>rat</td>
</tr>
<tr>
<td>Synthetic Peptide I</td>
<td>950-963</td>
<td>rabbit</td>
</tr>
<tr>
<td>Synthetic Peptide I</td>
<td>950-963</td>
<td>rat</td>
</tr>
<tr>
<td>Synthetic Peptide II</td>
<td>1377-1390</td>
<td>rabbit</td>
</tr>
<tr>
<td>Synthetic Peptide II</td>
<td>1377-1390</td>
<td>rat</td>
</tr>
</tbody>
</table>

Following immunization protocols, antisera were tested for specific immune-affinity for SUR-2 protein on western blot. Antisera reacted specifically to recombinant SUR-2 protein produced in bacteria, but do not show any reactivity to SUR-2 protein produced in yeast or from *C. elegans* lysates.

Discussion/Alternative Approaches

All of the antisera raised against the SUR-2 peptide fragments have been negative on western blot when tested against worm protein lysates and recombinant SUR-2 produced in yeast. Why this might be remains unknown. One possible explanation is that SUR-2 protein levels in the worm lysates may be too low to be detected on the western blot. However, this explanation does not account for its inability to detect recombinant SUR-2 produced in yeast.

As an alternative to raising anti-SUR-2 antibodies, a chimeric gene can be constructed by fusing full length *sur-2* coding sequence with a previously characterized epitope tag such as HA (hemagglutinin), FLAG or MYC tags. A transgenic worm stably expressing this fusion gene (under the control of its endogenous promoter) will then be made. Analysis of tissue specific expression and subcellular localization can then be done as above using a commercially available primary antibody specific for the epitope tag. This epitope tag may also permit *in vitro* SUR-2 protein interaction studies in the future.

I may also try to use green fluorescent protein (GFP) as a marker for tissue expression and subcellular protein localization (47). As with the epitope tags described above, I will construct a fusion gene consisting of the complete *sur-2* coding and regulatory sequences and GFP. A transgenic animal expressing this protein will then be made. The most obvious advantage to using a GFP marker is that visualization of the reporter does not
require any sample preparation and allows rapid visualization in a living animal. GFP has been used successfully to identify the subcellular localization of a protein involved in *Drosophila* oocyte development (48).

### Identify SUR-2 interacting proteins

A primary goal in our experiments is to ascribe a biochemical function to the SUR-2 protein. Assigning a function to a novel protein is a difficult task, and often only comes after results from a variety of experiments have contributed to the proposal of a model for protein function. We hope to make significant progress towards this goal by identifying the proteins which interact with SUR-2. By identifying SUR-2 interacting proteins, hints as to the biochemical function of SUR-2 may become apparent. For example, if the screen identifies a protein with known transcription factor motifs, we might speculate that SUR-2 may regulate that activity of that transcription factor or act as a coactivator. If SUR-2 interacts with components of the basal transcription machinery, we might also propose that SUR-2 is involved in transcriptional regulation.

By identifying SUR-2 interacting proteins, we might also obtain clues as to how SUR-2 protein is regulated. For example, if SUR-2 is associated with a known kinase activity, such as MAP kinase, the resulting phosphorylation events may regulate SUR-2 activity. By further mapping the domain of kinase interaction, we may be able to also identify the key phosphorylation sites on SUR-2.

I have employed the yeast two-hybrid interaction trap to test known signaling proteins for interaction with SUR-2 as well as screen *C. elegans* libraries for SUR-2 interacting proteins (39, 40, 41). As with any result obtained in the yeast two-hybrid assay, observations I make will be further confirmed by *in vitro* and *in vivo* protein interaction experiments.

### Methods/Results

I have used the yeast two-hybrid assay to test candidate proteins which may physically interact with SUR-2. The SUR-2 bait construct consists of the first 1412 of the 1587 amino acids in the SUR-2 protein cloned into the yeast two-hybrid bait vector (pAS2, Clontech). All attempts to subclone the full length *sur-2* cDNA into the bait vector have been unsuccessful, despite multiple subcloning strategies. I am continuing to try to subclone the entire full length cDNA into the bait vector.

I will first test candidate proteins for interaction with SUR-2. These candidate proteins are listed below:

*sur-1* /MAP kinase -MAP kinase activities have been shown to directly regulate the activity of transcription factors in multiple pathways and organisms. *C. elegans sur-1* /MAP kinase had been shown to likely lie upstream or in
parallel to sur-2 by indirect epistasis evidence (35, 36, 37). We are most eager to test whether SUR-2 is a direct target of C. elegans MAP kinase activity. The C. elegans MAP kinase cDNA is currently being subcloned into the two-hybrid prey vector (pACT2, Clontech).

**lin-1** - The *lin-1* gene has been shown to be a negative regulator of the vulval induction pathway and acts downstream of MAP kinase in the pathway. The *lin-1* cDNA encodes an ETS family transcription factor and contains MAP kinase consensus phosphorylation sites. The genetic relationship between *sur-2* and *lin-1* is complex, with *sur-2* mutations partially suppressing the Muv phenotype of *lin-1* mutations. A LIN-1 prey vector is currently being tested in the SUR-2 interaction assay.

**lin-25** - The *lin-25* gene is a positive regulator of the vulval induction pathway, and was shown to lie late in the induction pathway. The *lin-25* cDNA encodes a novel protein of unknown function. Interestingly, it is observed that LIN-25 protein is downregulated in *sur-2* mutant worms (Simon Tuck, personal communication). A LIN-25 prey vector is currently being made.

**lin-31** - The *lin-31* gene also lies downstream in the vulval induction pathway, and may lie downstream of MAP kinase. However, its phenotype is complex, and a null allele results in a randomization of VPC fates. The genetic relationship between *lin-31* and *sur-2* is unclear, with *sur-2* mutations able to suppress some but not all of the Muv phenotype arising from *lin-31* mutations. The *lin-31* cDNA encodes an HNF-3/fork head family transcription factor and contains consensus MAP kinase phosphorylation sites. In addition, LIN-31 has been shown to physically interact with both LIN-1 and MAP kinase in *in vitro* assays (Stuart Kim lab, Stanford University). A LIN-31 prey vector will be made and tested in the SUR-2 interaction assay.

**lin-39** - The *lin-39* gene encodes a homeodomain transcription factor of the *Drosophila* Antennapedia/sex combs reduced class which controls a variety of cell fates in the central body region of the developing worm, including the fates of the vulval precursor cells. Mutations in the *lin-39* gene result in vulvaless worms. Epistasis analysis demonstrates a dual role for *lin-39* both before and after the RAS/MAP kinase cascade. The LIN-39 signal may be required for VPC's to respond to the RAS/MAP kinase inductive signal. A LIN-39 prey vector is currently being tested in the SUR-2 interaction assay.

In addition to testing these candidate proteins for interaction with SUR-2, I have started a library screen for protein interactions. The library used in this screen is the Robert Barstead C. elegans mixed stage two-hybrid library. I will be testing two forms of this library, one oligo-dT primed, the other random
primed. Both libraries have an estimated $10^7$ unique clones. I have screened approximately $2 \times 10^6$ clones from the oligo-dT primed library, and have not yet identified any positive clones.

**Discussion/Alternative Approaches**

I have initiated a yeast two-hybrid screen to identify proteins which interact with SUR-2. Such proteins may give clues as to the biochemical activity of SUR-2, SUR-2 substrates, and how SUR-2 is regulated. High levels of false positive background using the two-hybrid screen is frequently reported, especially when using bait proteins that are components of the transcriptional machinery. Fortunately, this has not been a problem in my case. However, my screening has yet to yield any SUR-2 interacting proteins. This may be due to a toxic effect from the SUR-2 protein in the yeast host cells. If my screening continues to be unsuccessful, I will try using smaller domains of the SUR-2 protein in the screening. Also, I will try screening the random primed library in addition to the oligo-dT primed library.

If our two hybrid screen is unsuccessful, I will use an alternative approach to identifying SUR-2 interacting proteins. I will use a technique called "interaction cloning" or "functional cloning." This will entail screening a λphage C. elegans cDNA expression library with a radiotagged SUR-2 protein. This technique has proven very effective, especially in the study of transcription factor interactions (42, 43, 44).

**Heat shock promoter driving sur-2 expression**

It has been previously observed that a sur-2::lacZ reporter construct is expressed in the VPC's in the L1 through L3 stages. This expression of the reporter construct presumably recapitulates a requirement for sur-2 activity for induction of the vulval lineage. We wished to expand this analysis by experimentally demonstrating the temporal requirement for sur-2 activity.

**Method/Results**

To experimentally demonstrate the temporal requirement for sur-2 expression during vulval induction, I have constructed an inducible expression vector containing the complete sur-2 genomic sequence under the transcriptional control of a C. elegans heat shock promoter. Genomic sequence was used to ensure proper processing of the message, as well as the fact that we have not yet been able to subclone the last 190 amino acids in the 1,587 amino acid protein. This heat shock inducible construct was injected into sur-2 mutant worms and maintained as an extrachromosomal array. When these worms were exposed to a heat shock of 30°C for 30 minutes, a very small number of worms were later able to lay eggs, presumably
following vulval induction. In an attempt to improve the penetrance of the heat shock rescue, the extrachromosomal array was stably integrated into the host genome using standard irradiation integration techniques. These worms carrying the stably integrated array are currently being outcrossed and tested again for rescue of the vulval induction phenotype following heat shock.

Discussion/Alternative Approaches

I have made a heat shock inducible sur-2 expression vector. This tool will enable me to determine at what stage(s) sur-2 expression is required for proper vulval induction, and also the duration of expression that is required. It will also enable us to ask if sur-2 expression is sufficient to induce ectopic vulval induction, which is a question that remains unanswered. This construct will also be valuable in examining the temporal requirement for sur-2 expression in other C. elegans development programs, including male tail development.

The initial analysis of the heat shock vector in worms carrying the vector as an extrachromosomal array showed a very low degree of rescue following heat shock. It is not clear why this would be the case. This same sur-2 genomic fragment showed very good rescue when injected into mutant worms when under the control of its endogenous promoter. Similarly, the heat shock promoter in the construct is used successfully by many groups in similar heat shock experiments. It is possible that I have not optimized the conditions for the actual heat shock. A wider range of heat shock temperatures and times will be tried in hopes of improving the penetrance of rescue. DNA maintained in the worm as an extrachromosomal array is lost in subsequent generations due to inaccurate segregation to daughter cells following cell divisions. Therefore, it is possible that sur-2 expression may not be faithfully reproduced in the vulval precursor cell lineages. Hopefully, stably integrating the array will ensure fidelity of inheritance of the array to all cells in the worm and improve the frequency of rescue following heat shock.

*sur-2 suppressor screens*

Little is known of how events downstream of MAP kinase or sur-2 regulate vulval induction. As described above, the two-hybrid screen will allow us to identify factors acting as targets of the SUR-2 protein. As a complementary approach, here I propose a genetic screen to identify other genes which lie further downstream of sur-2 in the vulval signaling cascade. If SUR-2 is a transcription factor, genes identified in this screen may represent genes activated or repressed by SUR-2. The genes identified in such a screen will lie downstream of sur-2 and may not be direct physical targets of the SUR-2 protein.
Methods/Results

In an attempt identify other genes which lie further downstream of sur-2 in the vulval induction signaling cascade, a genetic suppressor screen will be employed. Worms homozygous for the severe loss-of-function allele sur2(ku9) are unable to lay eggs (Egl, bag-of-worms) with 100% penetrance. In this screen, sur-2(ku9) worms were mutagenized, and the resulting F1 and F2 progeny were screened for their ability to lay eggs (Egl').

\[ 
\begin{align*}
\text{sur-2(ku9);+} & \xrightarrow{\text{EMS}} \text{sur-2(ku9);suppressor} \\
\text{Vulvaless, Egl} & \xrightarrow{\text{mutagenesis}} \text{WT or Multivulval} \\
\text{bag-of-worms} & \xrightarrow{\text{EgT}} \text{Egl'}
\end{align*}
\]

Approximately 20,000 haploid chromosome sets have been mutagenized in this screen. No suppressors have yet been identified in the screen.

Discussion/Alternative Approaches

A genetic suppressor screen was used in hopes of identifying genes which lie downstream of sur-2 in the vulval induction pathway. Unfortunately, no such genes were identified in the screen. This may be due to a few different reasons. First, any such suppressor may be impossible to produce, as the induction pathway may be branched following sur-2, therefore, a mutation in any single gene may never recover the egg-laying phenotype. Second, the allele used in the suppressor screen, ku9, is a very strong allele, showing 100% penetrance of the Egl phenotype. Better luck may be had by using a weaker allele of sur-2. The sur-2(ku60) allele by itself shows 50% penetrance (50% Egl, bag-of-worms), but is able to completely suppress the multivulval phenotype of a gain-of-function RAS allele, let-60(n1046).

\[ 
\begin{align*}
\text{let-60(n1046);sur-2(ku60);+} & \xrightarrow{\text{EMS}} \text{let-60(n1046);sur-2(ku9);suppressor} \\
\text{non-Muv} & \xrightarrow{\text{mutagenesis}} \text{Multivulval}
\end{align*}
\]

This allele may yield a better chance of generating a suppressor mutation that will rescue the Egl phenotype. let-60(n1046);sur-2(ku60) worms (non-Muv) will be mutagenized, and their progeny screened for reversion to the Muv phenotype.
Bibliography


