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The work done in this grant aims at elucidating the function of the Wnt signaling molecules in cancer. Wnt proteins are secreted and play important growth controlling roles, in particular in the mammary gland. They can act as oncogenes in mouse mammary tumors. The work is specifically aimed at identifying a receptor for Wnt proteins. The isolation of a receptor is critical to our understanding of normal growth control of the mammary gland. Using genetic and biochemical approaches we wish to identify and to clone the receptor for a Wnt gene product in Drosophila, called wingless. About a year ago, we succeeded in finding a receptor for wingless called frizzled. During the past year, we have further characterized this receptor, as well as other genes in Drosophila involved in wingless function.
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Introduction

The goal of this grant is to understand more about the function of the Wnt signaling molecules in cancer and in embryogenesis. The emphasis is on finding a receptor for Wnt proteins. Wnt genes encode secreted proteins involved in cell-to-cell signaling. The Wnt gene family includes a Drosophila gene wingless, which genetically has been well characterized. Wnt genes control growth, in particular in the mammary gland and, importantly, can act as oncogenes in mouse mammary tumors. Problems with working with Wnt proteins in vitro had precluded the isolation and characterization of Wnt receptors.

Our approach to identify a Wnt receptor was two-fold:

1. We use an assay for soluble wingless protein and an in vitro cell culture assay to identify wingless receptors.

2. In Drosophila, we perform genetic screens to genetic modifiers of a wingless phenotype. These genes will be cloned and their properties will be examined.

About a year ago, we found that the Dfrizzled-2 protein fulfills the criteria to act as a receptor for wingless. We thereby accomplished the major goal of this grant. During the past year, we have characterized the interaction between frizzled and wingless proteins further, and we have made progress in identifying a genetic enhancer of wingless in Drosophila.
Body

1. *wingless* signaling in vitro; identification of a receptor

Our assays for wingless function is based on a cell line (clone 8 or cl-8) derived from *Drosophila* imaginal discs. To measure a response to *wingless*, we found a large increase in *armadillo* levels in the cl-8 target cells (6, 8). We also found that, in contrast to clone-8 cells, *Drosophila* S2 cells do not respond to the wg protein, indicating that they lack one or more components of Wg signaling (11). This finding suggested a complementation strategy to identify such missing components and we therefore tested whether transfection of receptor candidates would make S2 cells responsive to the *wingless* protein. One interesting receptor candidate was identified during the characterization of a large family of putative cell surface receptors with extensive homology to the *Drosophila* tissue polarity gene *frizzled* (*fz*).

Frizzled proteins are part of the large family of seven membrane spanning domain receptors (sometimes referred to as serpentine receptors). In *Drosophila*, this gene family counts three members, as far as known (3). While *fz* is expressed in the *Drosophila* embryo, it appears not to be essential for early embryogenesis. In addition, the *smoothened* (*smo*) gene, which is implicated in *hedgehog* (*hh*) signal transduction is a frizzled family member. Finally, a *Drosophila* frizzled-related gene, *Dfz2*, is expressed in the embryo in a pattern reminiscent of some segment polarity genes, such as Wg (1).

We found *Dfz2* is expressed in a *Drosophila* clone-8 cell line that is *wg*-responsive, but not in a non-responding S2 cells (the assay for *wg* activity being the stabilization and subsequent accumulation of the Arm). To produce control conditioned medium or conditioned medium containing soluble Wg protein, untransfected S2 cells or S2 cells stably transfected with a construct in which the *wg* coding region is under the control of a heat shock promoter were used as described. S2 cells stably transfected with *Dfz2* under the control of the metallothionein promoter were generated by hygromycin selection following transfection with a plasmid carrying the *Dfz2* coding region inserted into pMK33. Clone 8, S2, and *Dfz2* transfected S2 cells were incubated with the concentrated media for 2 hrs. Following the incubation period, cells were lysed and the resulting protein extracts were analyzed using a monoclonal anti- armadillo
antibody 7A1. The bound antibody was visualized using the ECL system (Amersham).

After transfection with the Dfz2 gene, S2 cells are able to transduce the wg signal. In addition, the S2 cells can now bind wg protein on their cell surface (1). In these assays, untransfected S2 cells and S2 cells expressing Dfz2 were washed twice in PBS and incubated with 1.5 ml of 10x concentrated conditioned medium at 4 C for 3 hrs. After three 10 minute washes with cold PBS the cells were fixed in 2% paraformaldehyde (Polysciences, Inc) for 15 minutes at room temperature. Following three more 10 minute washes with PBS, affinity purified anti-Wg antibody diluted 1:25 in 5% donkey serum/PBS was added to the cells and incubated overnight at 4 C. After additional washes in PBS, the cells were incubated with fluorescent Cy3 secondary antibody (Jackson Immunoresearch) and mounted. For transient expression in 293T or 293 cells, the Dfz2 coding region was inserted into the pCIS expression vector under the control of the cytomegalovirus immediate early promoter/enhancer and with an optimized translation initiation context, and transfected into 293T or into 293 cells with a T-antigen expression plasmid using the calcium phosphate method. Confocal images were collected with Bio-Rad MRC 1000 confocal laser attached to a Zeiss Axio scope microscope. The same number of scans (20) were taken to visualize the fluorescence of each sample. Transfection of cells with Dfz2 constructs lacking either the extracellular or intracellular domain of the protein demonstrated that the extracellular domain was required for binding. Although a direct interaction between the Wg protein and Dfz2 is still lacking, the data suggest that Dfz2 can bind to and transduce the Wg signal (1, 7, 10). The Smo protein, when tested in the same assay, does not bind Wg, but of the identified mouse and human Fz proteins, several are also positive in the Wg binding assay described above (7, 9).

We have also tested for signal transduction, by transfection of Fz constructs into S2 cells and measuring the Arm protein concentration before and after adding soluble Wg protein. Without exception, the Fz proteins that were able to bind Wg did also transduce the Wg signal to Arm (7). The original fz gene can confer wg responsiveness to non-responding cultured cells, as well as wg binding. One possibility is that fz acts redundantly with Dfz2 or other as yet unidentified frizzled proteins to transduce the wg signal. However, it does appear likely that some Drosophila Wnt gene is the ligand for the polarity function of fz.

We have obtained evidence that Dfz2 can act as a receptor for Wg in vivo by expressing the extracellular domain of the protein as a GPI-linked cell surface
protein in *Drosophila* imaginal discs, using the GAL-4/UAS system. The wings of
the resulting flies have marked defects in the margin, known to be specified by
Wg, and in other structures such as the eyes and the legs. All the phenotypes
observed are similar to loss of Wg function, which suggests that the extracellular
domain binds Wg and inhibits its function (7).

Hence, we have shown that the *Dfz-2* gene fulfills two criteria to be a receptor
for the Wg protein: Wg binds to the *Dfz-2* and binding leads to a biological
response; an increase in intracellular Arm concentration. In most vertebrates,
more than 10 *Wnt* genes have been identified. As expected, there exists indeed a
large family of *fz*-like genes in vertebrates, likely candidates for receptors for the
other *Wnt* proteins. At this moment, there is no genetic evidence that *Dfz-2* is
required for Wg signaling, as no mutants at the gene are available. Possible
candidates for *Dfz2* mutant may have arisen from the genetic screen described in
6.2.

2. **A genetic screen for suppressors of a wingless phenotype in Drosophila**

A second route to the identification of components of *wingless* signal
transduction in *Drosophila* is to take advantage of the genetic tools developed in
this organism. By performing genetic screens for suppressors of a *wingless*-caused
phenotype in the fly, one can uncover mutations in genes that are essential to
generate this phenotype. Those genes could encode components of the *wingless*
signaling pathway, including the receptor.

We have made several P-element based constructs to obtain ectopic
expression of *wingless* in larval imaginal discs, the progenitors of adult tissues.
These include a construct in which *wingless* expression is driven by the *sevenless*
promoter, pSEW-*wingless*, which is known to be active only in the eye imaginal
disc. The transgenic flies that were obtained have a very specific phenotype in the
eye: an almost complete absence of interommatidial bristles (2). This phenotype is
100% penetrant and easy to score with a dissecting microscope. This phenotype is
also generated by a *wingless* temperature sensitive allele, but in a temperature
dependent manner.

This penetrant adult viable *wingless* phenotype has been used to perform a
screen for dominant suppressors or enhancers of *wingless*. The principle behind
this screen is to search for mutations that will give a phenotype when one allele
has undergone a loss-of-function mutation. Normally, complete absence of one
allele will not give a phenotype. But in a genetic background where the phenotype of one gene (in this case *wingless*) is dosage-sensitive, absence of one copy of an interacting gene may modify this phenotype. This screen can be done in the F1 generation. Especially since the phenotype is semi-quantitative (i.e. the number of bristles on the eye can be approximated) this screen is very sensitive to dosage of gene products interacting with *wingless* and can identify not only suppressors but also enhancers of the pSEW-wingless(ts) phenotype. Male flies were mutagenized with EMS and mated to females carrying the pSEW-wingless(ts) transgene. This transgene is present on a balancer chromosome, which makes it easier to identify individual F1 animals carrying both the transgene and a possible mutation in a modifying gene. These F1 animals were be crossed further to examine whether the modification persists.

Of several modifiers obtained, we performed a clonal analysis. In such an analysis, one makes individual cells (a clone) in an animal mutant for the gene by mitotic recombination, a standard technique in *Drosophila* research. If the mutation is cell autonomous, the clone will display the mutant phenotype. It is to be expected that genes that operate in the receiving end of *wingless* signaling will be cell autonomous. By examining the phenotype of clones in the eye of the pSEW-wingless(ts) strain, we can establish whether they act in cell-autonomous way and whether the mutation completely suppresses the pSEW-wingless(ts) phenotype, i.e. allow normal bristle formation. We have performed such clonal studies on Mutant alleles of *dsh*, *zwo* and *arm*. These were recombined unto a P[hs-neo; FRT]18A chromosome, porc onto P[hs-neo; FRT]19A, wg onto P[hs-neo; FRT]40A and a P[sev-wg; w+] mapping to 3L onto P[hs-neo; FRT]80A, all in a w background. w clones were induced in animals heterozygous with the appropriate P[mini-w+], P[FRT] chromosome: P[mini-w+; hs-pM]5A, 10D, P[hs-neo; FRT]18A; P[mini-w+]18A, P[hs-neo; FRT]19A; P[mini-w+ ;hs-pM]21C, 36F, P[hs-neo; FRT]. FLP recombinase was provided from the FLP-99 chromosome. Clones were induced by a one hour heat shock (37°C) 24-48 hours after egg laying and scored for the absence of pigmentation in the adult eye.

For production of N germ-line clones, the N null alleles were recombined onto a P[mini-w+; FRT]101 chromosome. N, P[mini-w+; FRT]101/FM7 females were crossed to a w ovoD1, P[mini-w+; FRT]101/Y; P[hs-FLP]38 stock and progeny were heat shocked late 3rd instar/early pupation for 2 hours at 37°C (earlier heat shocks resulted in lethal lethality due to somatic clones). Mosaic mothers were
crossed to \( P[ftz-lac\text{Z}]C \) males, or \( P[ftz-lac\text{Z}]C; P[hs-wg]/TM3 \) males. Embryos with no \( lac\text{Z} \) staining lacked both maternal and zygotic expression of \( N \).

We have now isolated approximately 20 suppressors and enhancers of the \textit{wingless} phenotype in the eye. These genes have been mapped and have been assembled into complementation groups. We have also performed clonal analysis of these genes, indicating that some of them also have a phenotype in the homozygous state. Five interesting complementation groups have been found, two of them consisting of known genes.

One is \textit{daughterless} (\textit{da}), a helix-loop-helix protein heterodimerizing with other such proteins and required for neurogenesis. We found that \textit{wg} expression in the eye reduces the level of \textit{da} expression, the first demonstration of regulation of \textit{da} expression by an extracellular signal.

A second known suppressor is a \textit{Drosophila} tumor suppressor gene, called \textit{warts}. This gene encodes a protein kinase but its biochemical function is not clear. We are currently addressing this by producing antibodies to \textit{warts}.

We have characterized one enhancer in much detail. This enhancer, called \textit{10A20}, maps on the third \textit{Drosophila} chromosome. This mutation is homozygous lethal and the lethality is not complemented by three \( P \)-elements insert lines that map to the same area. Several of these lethal \( P \) insert lines by themselves also modify the \textit{pSEW-wingless(ts)} phenotype. We have excised several of these \( P \)-inserts and found that the stocks revert to viability.

These \( P \)-inserts provide a good starting point for molecular cloning of the gene. We have performed plasmid rescue experiments to retrieve flanking DNA. This flanking DNA was used to isolate more genomic DNA from the area, and to examine possible transcript. One of these clones indeed hybridizes to an RNA of 6.2 Kb. In this exercise, it will be very useful to have multiple alleles of the desired gene, preferentially alleles resulting from rearrangements; they will allow identification by restriction mapping. Such alleles can be generated by generating new \( P \)-element inserts. In the latter approach, a useful starting point could be a the \( P \)-element that is inserted nearby on the chromosome, as \( P \)-elements often transpose to nearby sites. By crossing such as strain with a strain carrying the gene for transposase (\textit{D2-3}) transposition will be initiated. The most straightforward method to prove that a transcriptional unit is indeed an allele of the gene found in the screen is to rescue flies mutant for the gene. Hence, we will construct \( P \)-elements containing the gene of interest, generate transformants and assess for rescue by appropriate crosses to the strains carrying the mutations.
It should be pointed out that the molecular cloning of newly identified *Drosophila* genes is a significant effort but will be greatly facilitated by the ongoing and expanding *Drosophila* genome project. Significant portions of the genome have been cloned in an organized way (overlapping cosmids or YAC clones).
Conclusions

Since the work started, three years ago, we have made significant progress. The main goal of the project, the identification of a wingless receptor, has been accomplished. We have no Drosophila mutants in the receptor gene, Dfz2, but we have found a number of suppressor mutations in Drosophila, one of which may correspond to the receptor gene. Further work will address the biochemical mechanism of signal transduction by the Dfz2 receptor, and the interactions between other members of the frizzled receptor gene family and the various Wnt proteins.
References


