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The goal of our study is to identify the signaling pathway by which the *Drosophila* gene *Notch* controls cell morphology, and to distinguish it from the pathway by which *Notch* controls cell identity. The specific third year goals of the project were: (1) to investigate biochemically the interaction between NOTCH protein and the axonal signaling protein DISABLED; and (2) to further characterize the genetic interaction between *Notch* and *abl* mutations as a prelude to performing a genetic screen for new elements of the *Notch/abl* pathway.

In the past year, we have demonstrated that NOTCH binds directly to DISABLED *in vitro*, and have mapped the DISABLED binding site to a well-characterized signaling domain of NOTCH. Our results also provide some suggestion that the binding of NOTCH to DISABLED may be modulated by phosphorylation, and suggest a possible mechanism by which the NOTCH-DISABLED association may coordinate axon patterning with cell identity.

In the past year, we have also investigated the genetic interactions between a large number of *Notch*, *abl* and *disabled* alleles, under a wide variety of conditions, and have identified a few allelic combinations that appear particularly promising as the basis for a screen for modifier loci.

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5. INTRODUCTION

The *Drosophila Notch* gene is the founding member of a family of ubiquitous transmembrane receptors that are conserved among all metazoans and play key roles in normal development and in disease [1, 2]. *Notch* mutations are associated with pediatric leukemias in humans [3], viral transduction of *Notch* fragments is responsible for lymphomas in cats [4], and retroviral activation of *Notch* is associated with breast cancer in mice [5]. Study of the functions of *Notch* genes in a variety of organisms has helped to explain why *Notch* should be associated with cancer, since a wealth of evidence demonstrates that *Notch* plays a central role in the control of cell identity. For example, in many cases where a cell undergoes an asymmetric cell division, *Notch* is part of the mechanism that causes the fates of the two daughters to be different: if *Notch* activity is disrupted or hyperactivated, both daughters adopt the same, rather than different identities [6]. Should this occur in the context of a stem cell lineage, the consequence can be to divert all cells into a stem cell compartment, and thus to cause massive hyperproliferation. In addition, our previous experiments have demonstrated directly that activation of *Notch* can arrest cells in a precursor-like state within a differentiated lineage, another property that is common to transformed cells [7].

As part of our efforts to understand the signaling cascade working downstream of *Notch*, we investigated the possibility that *Notch* might interact with a protein tyrosine kinase. As we reported last year, we found a synergistic genetic interaction between mutations in *Notch* and mutations in the *Drosophila* homolog of the mammalian tyrosine kinase oncogene, *abl*. This was of particular interest because, in *Drosophila*, *abl* appears not to be involved in the control of cell identity, but rather of cell morphology [8]. It is thought that *abl* acts through small GTPases of the Rho subfamily to regulate actin structure and dynamics [9-11]. Since the goal of the project supported by this grant is to determine whether *Notch* controls cell morphology directly, by a signaling pathway distinct from the one it uses to control cell identity, and since the discovery of a genetic interaction between *Notch* and *abl* provided an excellent candidate for such a pathway, we redirected our efforts towards understanding the link between *Notch* and the *abl* signaling pathway. A revised Statement of Work detailing our plans was submitted last year and approved by the USAMRMC.

Over the past year, we have performed three experiments which demonstrate that NOTCH binds directly to the ABL accessory protein, DISABLED. We have mapped the DISABLED binding site on NOTCH, and find that it is in the same part of the protein where other regulators and effectors of *Notch* function are known to bind. These results provide a potential biochemical rationale for the genetic interaction we observed between *Notch* and *abl*. Moreover, we have preliminary data suggesting that the binding of NOTCH to DISABLED may be modulated by phosphorylation. If correct, this result may suggest a mode of regulation for the *Notch/abl/disabled* interaction. Finally, we have begun to characterize the phenotypes observed for various combinations of *Notch* and *abl* alleles as a first step towards establishing a genetic screen for other members of this novel signaling pathway.

6. BODY

METHODS

Drosophila stocks

abl^1, abl^2, *Df (3L) sti*^{7} and *Df(3L)*^{std11} were obtained from Corey Goodman; P[adh^+; abl^+] on 3R was obtained from Frank Gertler and Mike Hoffman. All *abl* chromosomes were cleaned by recombining markers from *rucuca* (*ru h th st cu sr e ca*) across the entire chromosome, and then replacing them with wild type sequences from an isogenized Oregon R stock. *Df (1)*N^8, N^{55e11}, N^{ts1}, *Dp* (w^-N^+)*.Y*, and *lacZ*-marked balancers for the first and third chromosomes were obtained from Y.N. Jan. N^{ts1} was isogenized prior to use. N^{5419} was obtained from Gerold Schubiger. nd^0, nd^1 and nd^3 were obtained from the *Drosophila* Genetics Stock Center

(Bloomington, IN). Most experiments, and all quantitation of penetrance and expressivity, employed heteroallelic combinations of *abl* alleles. All genetic crosses and maintenance of flies were done by standard methods.

Cloning and expression of NOTCH and DISABLED fragments

1. Expression of NOTCH intracellular domain in bacteria

By the following sequence of steps, we constructed a plasmid (pEG203) that encoded the amino-terminal 468 amino acids of the NOTCH intracellular domain (residues 1767-2235), fused at its N-terminus to a His_6 tag for convenient purification and two copies of a protein kinase A phosphorylation site, under control of a T7 promoter. Phage containing *Notch* cDNAs were isolated from the Kauvar E7 library by hybridization, using a small PCR fragment from the *Notch* intracellular domain as a probe. Sequences encoding the complete intracellular domain of NOTCH were amplified by PCR and cloned into bacterial expression vector pEG 180. pEG180 had been prepared by synthesizing an oligodeoxynucleotide encoding two copies of a protein kinase A recognition sequence and inserting it into the BamHI site of the His_6 vector pRSET A (Invitrogen). *Notch* sequences were subcloned between the Bgl II and Kpn I sites of pEG180 to generate pEG 203. One isolate acquired an adventitious frameshift mutation at nt 7448, appending the amino acid sequence RPPT, followed by a stop codon, to residue K_{2235}; this isolate was found to make an abundant, stable and soluble fragment of NOTCH and one which appears to have complete *Notch* signalling activity as assayed by its dominant phenotypes when expressed *in vivo* [7, 12, 13]; EG unpublished]. All cloning steps were performed in the bacterial strain DH10B (Gibco/BRL) or BL21 (Novagen).

Induction, purification and kinase labelling of the NOTCH intracellular domain were performed as follows. BL21::λDE3::pLysS::pEG203 cells were grown at 37° in 100 ml LB containing 50μg/ml carbenicillin and 15 μg/ml chloramphenicol to mid-log phase (OD^{600}=0.4). IPTG was added to 1mM, cells were grown 4 hrs and then harvested by centrifugation. Induced cells were resuspended in 10 ml of lysis buffer: 8M urea, 50mM Na Phosphate, pH 7.2, 100 mM NaCl plus protease inhibitors (a 1:100 dilution of the following cocktail: leupeptin, 1 mg/ml; pepstatin, 1 mg/ml; aprotinin, 2 mg/ml; benzamidine, 10 mg/ml; PMSF, 100mM; prepared in DMSO and stored in small aliquots at -20°). After sonication for 3 - 4 bursts of 30", 1.5 ml Ni^{+2} resin (Invitrogen) was added to the bacterial lysate and allowed to bind for 2 hrs with rocking at RT. Bound protein was washed twice with lysis buffer, twice with lysis buffer containing 1M NaCl and twice more with lysis buffer. Protein was renatured by overnight dialysis, still on the beads, into 25 mM Hepes, pH 7.5, 100 mM NaCl, 0.5% NP40, 2mM β-mercaptoethanol and 0.1mM PMSF. To kinase label the purified Notch, 100μl of NOTCH-bearing beads was washed into kinase buffer (20mM Tris, pH 7.6; 100 mM NaCl; 12 mM $MgCl_2$; 1 mM DTT), then incubated in a 250 μl total reaction volume of kinase buffer containing 50 μCi γ-^{32}P-ATP and 50 units heart muscle kinase (Sigma), at 37° for 1 hr. Beads were washed extensively in NOTCH renaturation buffer (above), then eluted with renaturation buffer containing 20mM EDTA.

2. Subcloning and expression of fragments of the NOTCH intracellular domain.

Four non-overlapping fragments of the NOTCH intracellular domain were subcloned into pRSET A and expressed by coupled *in vitro* transcription/translation in a rabbit reticulocyte lysate. All fragments were PCR amplified and subcloned between the NheI and Bgl II sites of pRSET A. Thus, each of these fragments bore a His_6 tag at its N-terminus, but lacked other N-terminal fusion sequences typically associated with the RSET vectors (and present in the complete intracellular domain construct described above). The NOTCH fragments were: Ram23 region, amino acids 1766-1896; ankyrin repeats, aa 1896-2109; PEST/OPA region, aa 2262-2606; and notchoid region, aa 2612-2703. In each case, a stop codon was introduced in the 3' PCR oligonucleotide after the final amino acid. Expression of NOTCH fragments employed the Promega TNT

reticulocyte lysate coupled transcription/translation system. In each case, 1μg of supercoiled template was incubated in a 50μl reaction containing 40 μCi of ^{35}S-methionine, for 90' at 30°.

3. Subcloning and expression of the DISABLED PTB domain.

Nucleotides 1176-1619, corresponding to amino acids K_{36}-I_{184} of DISABLED, were amplified by PCR and subcloned between the BamHI and EcoRI sites of the GST vector pGEX-2T. Note that this is the numbering of the corrected *disabled* sequence [14]; published erratum: Genes Dev 1996 **10**:2234]. GST-DISABLED and the parent GST vector were transformed into bacterial strain BSJ72 and 100 ml cultures were grown to mid-late log phase at 37°. Cultures were induced with 0.5 mM IPTG, grown an additional 4 hrs and harvested by centrifugation. Cell pellets were suspended in 5 ml lysis buffer (50 mM Tris, pH 7.9; 0.5% Triton X-100, 1 mM PMSF) at 0°, and lysed by sonication with 4 bursts of 30" each. Extract was cleared by centrifugation at 10K RPM for 10', and supernatent was added to glutathione sepharose beads (Pharmacia) and rocked for 10' at RT. (Prior to binding fusion protein, beads had been blocked by 45' incubation in 25 mM Hepes, pH 7.6; 50 mM NaCl; 0.5% NP40; 5 mM β-mercaptoethanol; 0.5% nonfat dry milk, followed by washing with the same buffer without the milk.) Beads bearing GST-DAB or GST were collected by brief centrifugation, washed 3 times with lysis buffer and stored at 4° with 4 mM NaN_3.

Protein binding experiments

1. Binding of DAB to NOTCH ICD purified from bacteria.

Glutathione beads bearing GST-DAB PTB or GST alone were blocked by incubation in binding buffer containing 2% BSA for 30', followed by several washes with binding buffer alone. Binding buffer was 25 mM Hepes, pH7.5; 50 mM NaCl; 1% NP40; 0.1% SDS; 15 mM β-mercaptoethanol. Labelled, purified NOTCH intracellular domain was added to binding buffer containing 2% BSA and first incubated with blocked GST beads for 30' at 4°, at a ratio of 10 μl beads per 750 μl diluted NOTCH protein. Extract was cleared by spinning in a Sorvall centrifuge at 17Krpm for 10'. To each cleared sample, 10 μl of blocked GST or GST-DAB beads was then added. Beads and NOTCH protein were incubated with rocking 90' at 4°. Beads were pelleted by brief centrifugation (5"), washed 5 times with 500 μl of binding buffer, resuspended in 20 μl Laemmli sample buffer and boiled 4'. Protein was separated in a 7.5% SDS-polyacrylamide gel, the gel was dried-down and the NOTCH protein visualized by autoradiography.

2. Binding of DAB to fragments of the NOTCH ICD translated *in vitro*.

Beads were prepared as above, except that binding buffer for experiments using *in vitro* translated NOTCH protein contained 0.5% NP 40 and no SDS. *in vitro* transcription/translation reactions prepared as described above were stopped by dilution in 700 μl binding buffer. Protocol for clearing of extract, binding to beads and washing was the same as for protein purified from bacteria. Beads were boiled in 20 μl Laemmli sample buffer for 4' and electrophoresed through a 12% SDS-polyacrylamide gel. Gel was washed 3 times for 30' in 30% methanol; 10% acetic acid; 5% glycerol, then for 45' each in solution A and solution B of Entensify (NEN). Gel was dried-down and labelled proteins were visualized by autoradiography.

3. Binding of DAB to NOTCH in a crude embryo lysate.

Total embryo lysate was prepared as follows. 0-24 hr embryos were harvested, dechorionated with 50% bleach, washed with 0.7% NaCl, 0.3% Triton X-100, washed once with water and transferred to an ice-cold Dounce homogenizer. Embryos were then washed once with 0° lysis buffer (25 mM Hepes, pH 7.5; 300 mM NaCl; 0.5% NP40; 15 mM β-mercaptoethanol; 5 mM NaF; 10 mM Na Pyrophosphate; 25 mM β-glycerophosphate). Embryos were suspended in 3 embryo volumes of lysis buffer containing a 1: 100 dilution of our standard cocktail of protease inhibitors (see above) and homogenized on ice by 10-15 strokes with an A pestle, followed by 15-20 strokes with a B pestle. Embryo homogenate was incubated with gentle rocking for 1 hr at 4°

and cleared by centrifugation in a Sorvall centrifuge at 17Krpm for 30'. Cleared supernatent was harvested, avoiding the cloudy top (lipid) layer. If necessary, extract was briefly re-spun in a microfuge to facilitate separation of layers. GST beads were added to the lysate at a ratio of 30 µl beads per 500 µl extract, rocked for 45' and cleared by centrifugation at 17Krpm for 20'. Extract supernatent was then diluted (400 µl->1 ml) with lysis buffer lacking NaCl (referred to below as Buffer A) to reduce the NaCl to a final concentration of 125 mM. Pre-blocked GST-DAB or GST beads were added to the cleared lysate at a ratio of 30 µl beads per 1 ml diluted extract and incubated overnight at 4° with gentle rocking. Beads were collected by brief centrifugation (5"), and washed 6 times with Buffer A containing 50 mM NaCl and 0.1 mM PMSF. Beads were resuspended in 30µl Laemmli sample buffer, boiled 4', and electrophoresed through a 6% SDS-polyacrylamide gel. Gel was transferred to nitrocellulose, probed with anti-NOTCH antibodies and peroxidase-coupled secondary antibody by standard methods and visualised by chemiluminescence using the Renaissance reagent (NEN).

RESULTS

1. The *abl/disabled* signaling pathway.

In order to explain our motivation for analyzing the binding of NOTCH to DISABLED, we must digress briefly and introduce the *abl/disabled* signaling pathway. In *Drosophila*, *abl* mutations do not produce obvious defects in embryogenesis, and in fact, about a third of homozygous *abl* mutant flies survive to adulthood (the remainder die as pupae) [8, 9]. A requirement for *abl* function can be uncovered, however, by screening for mutations that interact synergistically with *abl* mutations [14]. Three loci have been characterized which have that property, of which the best understood is *disabled* (*dab*). Individuals of the genotype *abl* $^{-/-}$ *dab* $^{-/+}$ die as embryos with severe defects in axonogenesis, a phenotype that is not seen with either mutation by itself. The sequence of *disabled* suggests that the protein may be an "adaptor" protein, since it has a variety of protein-protein interaction motifs but no known enzymatic activity. *dab* includes peptides which resemble consensus *abl* phosphorylation sites, and which would be consensus binding sites for the *abl* SH2 domain if phosphorylated. DAB is indeed phosphorylated on tyrosine in cultured *Drosophila* cells [14]. There is a mammalian homolog of *dab*, called *m-dab*, and phosphorylated m-Dab does bind the Abl SH2 domain *in vitro*, and it binds the closely-related Src SH2 *in vivo* [15]. Thus, it is plausible that *Drosophila* DAB is a direct binding partner of ABL *in vivo*, though this has not been demonstrated.

In addition to its potential SH2 interactions, *dab* has its own protein binding module, a PTB (phosphotyrosine-binding) domain. Last year, we noted that the *dab* PTB was most closely related to the PTB domain of the *Drosophila* NUMB protein, a domain which is known to bind to NOTCH. We therefore tested the m-Dab PTB (generously provided by B. Howell and J. Cooper) for binding to the intracellular domain of NOTCH. We found that the m-DAB PTB binds to *Drosophila* NOTCH *in vitro*, even in the absence of NOTCH tyrosine phosphorylation. Howell and Cooper have shown that m-Dab binds to other non-phosphorylated targets [15], and it is known that the NUMB PTB can bind to targets lacking phosphotyrosine [16]. In light of these data, and of our data demonstrating a genetic interaction between *Notch* and the *abl* signaling pathway, we set out to determine whether *Drosophila* DISABLED binds to *Drosophila* NOTCH.

2. *Drosophila* DISABLED binds NOTCH *in vitro*

Three experiments demonstrated that *Drosophila* DISABLED binds NOTCH *in vitro*. First, we incubated glutathione beads bearing a GST-DAB PTB domain fusion in a total *Drosophila* embryo extract. After spinning-out the beads and washing extensively we assayed the beads for associated NOTCH protein by Western analysis. We found that the DAB PTB domain selected full-length wild type NOTCH protein out of the total embryo extract, whereas beads bearing GST alone did not bind NOTCH appreciably (Figure 1). Interestingly, we found that it was necessary to pre-incubate the embryo extract at elevated ionic strength (300 mM NaCl) prior to incubation with DAB protein in order to detect binding. We infer that either the DAB binding site

on NOTCH is quantitatively occluded by endogenous bound proteins in wild-type embryos, or that some conformational change is required in full length NOTCH protein to make the DAB binding site accessible.

We next began to localize the DAB binding site on NOTCH. We prepared His-tagged fusions of four non-overlapping fragments of the *Notch* intracellular domain, representing four distinct functional domains of the protein. These were the RAM23 region (*Notch* amino acids 1766-1896), the ankyrin repeats (amino acids 1896-2109), the PEST/OPA region (amino acids 2262-2606) and the *notchoid* region (amino acids 2612-2703). Genes encoding the four *Notch* fusions were separately translated *in vitro* in reticulocyte lysates, in the presence of ^{35}S-methionine, and assayed for binding to the DAB-PTB domain. We found that only the RAM23 region bound detectably to DAB, and none of the fusion proteins bound to GST alone (Figure 2). For comparison, the NUMB PTB domain also binds the RAM23 portion of NOTCH, but not the ankyrin repeats or PEST/OPA region [16]. Unlike DAB, NUMB also binds the *notchoid* domain of NOTCH.

The experiments above demonstrate that DAB can be found in a complex with NOTCH, but they do not prove that the interaction between DAB and NOTCH is direct. We therefore purified from bacteria a stable and soluble fragment of the NOTCH intracellular domain and assayed it for binding to beads bearing the purified DAB PTB domain. Sequences encoding amino acids 1767-2235 of NOTCH (which includes the entire RAM23 domain) were subcloned into a His-tag vector that had been altered to include two copies of a Protein Kinase A phosphorylation site. The NOTCH fusion was purified on a Ni^{+2} column, labelled with γ-^{32}P-ATP and tested for binding to DAB. We found that purified NOTCH bound to purified DAB, proving that the interaction is direct (Figure 3A). Moreover, this experiment demonstrated that tyrosine phosphorylation of NOTCH is not required for binding of *Drosophila* DISABLED.

Unexpectedly, we found a difference in the properties of the NOTCH-DAB binding interaction in the different assays described above. In all cases, the interaction was stable to modest concentrations of non-ionic detergent (NP40; 0.5 -1%). Binding of NOTCH protein purified from bacteria, however, was also stable in the presence of ionic detergent (0.1% SDS) whereas binding of *in vitro*-translated protein was severely impaired under these conditions (Figure 3B). Two simple explanations can be offered for this discrepancy. Protein made in bacteria was denatured in urea and slowly renatured, and it could be that this permitted the protein to attain a more nearly native conformation than was the case for the protein translated *in vitro*. Alternatively, the purified protein was labelled by phosphorylation with Protein Kinase A prior to assay, whereas the *in vitro* translated protein was labelled by incorporation of ^{35}S-methionine and was not phosphorylated. It could be that phosphorylation, either of the vector-introduced PKA sites or of sites in the NOTCH portion of the fusion, altered the binding properties of the protein. This is significant since it potentially offers a way to regulate the NOTCH-DISABLED interaction. Experiments are currently in progress to phosphorylate the *in vitro*-translated protein to discriminate whether it is the presence of phosphorylated residues or the manner of making the protein that is responsible for this difference in binding properties.

A manuscript presenting the detailed phenotypic analysis of the *Notch/abl* genetic interaction and the biochemical analysis of the NOTCH/DISABLED physical interaction has been submitted for publication and is currently in review.

3. Additional characterization of the *Notch/abl* genetic interaction

The central goal of this project is to test whether *Notch* controls cell morphology directly, *via* a pathway distinct from the one it uses to control cell identity. The phenotypic analysis of the *Notch/abl* interaction suggests that the *Notch/abl* signaling pathway may play just this role. The most thorough and convincing way to test this hypothesis is to identify other elements of the

Notch/abl pathway and then to ask whether they indeed define a novel signaling pathway or whether they are elements of the *Su(H)* pathway by which *Notch* controls cell identity.

In last year's progress report, we described preliminary results (obtained under the auspices of a grant from the March of Dimes) demonstrating that the *Notch/abl* genetic interaction can lead to synthetic lethality, or under a different set of conditions, to a synthetic visible adult phenotype (notched wings). We realized that these phenotypes potentially lent themselves to the development of convenient genetic screens to identify additional members of the pathway, as mutations that enhance or suppress the *Notch/abl* interaction phenotype(s).

In the past year, therefore, we have performed an extensive matrix of crosses, combining various *abl* and *dab* alleles (abl^1, abl^2, $Df(abl)^{stJ7}$, $abl^1 dab^{M54}$ and $Df(abl\ dab)^{std11}$, plus and minus complementing transposons, in different recombinant genetic backgrounds) with different *Notch* alleles ($Df(N)^8$, N^{5419}, N^{55e11}, N^{ts1}, nd^0, nd^1, nd^3) at various temperatures (18, 20, 23 and 25°), to determine which allelic combinations and growth conditions are likely to be most effective as the basis of a screen for mutations that modify the *Notch/abl* interaction. The details of the entire series of experiments are not critical, but we will here present the most relevant observations.

3.A. Viability

As reported last year, *abl* homozygotes do not survive when *Notch* activity is reduced, as in the presence of a N^{ts1} allele at semi-permissive temperature or in flies that are also heterozygous for a strong *Notch* allele. Extensive additional testing has confirmed that result. The lethal phenotype is apparently quite robust, inasmuch as almost all combinations examined gave complete lethality (except the very weakest *nd* alleles of *Notch*). Both *abl* and *Notch* duplications were capable of suppressing the lethality, thus verifying the specificity of the interaction. Together, these data suggest that the synthetic lethality of *Notch* and *abl* should provide a robust basis for a mutant screen to isolate suppressor mutations.

In light of our plans to perform a screen for suppressor mutations that rescue the lethality of flies which are N^{ts}; $abl^{-/-}$ it was important to verify that the rescued male flies would be fertile, as we would otherwise be unable to recover putative suppressor mutations that we induce. We therefore took advantage of a $Notch^+$ duplication on the Y chromosome that provides only partial *Notch* activity. We constructed male flies that were $Notch^{ts1}/Notch^+ \cdot Y$; abl^1/abl^2 (ie, *N/abl* flies that are rescued to viability by the duplication), and outcrossed them to wild-type females. We found that the genetically rescued males were indeed fertile, suggesting that male infertility of homozygous *abl* flies will not create any insuperable obstacles to a succesful screen for suppressors of the *Notch/abl* interaction.

3.B. Wing Notching

Preliminary experiments had suggested that some *Notch/abl* combinations cause wing margin defects in adults. These experiments have now been greatly extended. As reported previously, male flies that are N^{ts}; abl^1/abl^+ have notched wings at a modest frequency (14%). Decreasing *Notch* activity by increasing the temperature, for example to 21° or 25° is sufficient to cause lethality of the hemizygous (*Notch/Y*) males, but not to induce wing notching in heterozygous ($Notch^{-/+}$) females. In an equivalent genotype (N^{ts}; abl^2/abl^+) a weaker allele of *abl* causes wing notching only at very low penetrance at 18° (2%), but this can be increased by further reduction of *abl* activity (for example, by covering an *abl* null allele with a hypomorphic abl^+ transposon). In aggregate, these experiments show that, in the background of a given *Notch* allele (N^{ts1} at 18°), the frequency of wing notching varies simply as a function of *abl* activity. These data support the idea that wing notching is a useful assay for the severity of the *Notch/abl*

interaction. In experiments with the N^{ts1} allele, varying the *Notch* activity by varying the temperature changes which genotypic combinations have the right range of residual *Notch/abl* activity to survive and to display wing notching, but none of these combinations was significantly more convenient or reliable than development at 18°. Similarly, the use of hypomorphic *nd* alleles of *Notch* in analogous experiments offered no striking advantages: allelic combinations that were sufficiently active to be viable displayed little or no wing notching, while more extreme mutant combinations were lethal.

In the experiments described above, the *abl* chromosome was marked with two recessive, viable mutations to facilitate identification of genotypes from the various crosses. We noted, however, that one of these marker mutations (cu^-) itself appeared to enhance the *Notch/abl* wing notching phenotype. Upon performing additional meiotic ecombinations to remove the *cu* allele from the abl^1 chromosome, we found that N^{ts1}; cu^+abl^1/abl^+ flies did not display the wing notching phenotype described above for N^{ts1}; cu^- abl^1/abl^+ flies. This observation validates the approach of using wing notching as a screen for interacting loci, since it demonstrates directly that there exists at least one second site mutation that can induce wing notching in *Notch/abl* flies. Unfortunately, the molecular identity of *cu* has not been determined, so the physical basis of its interaction with *Notch* and *abl* is not immediately apparent.

3.C. Eye Defects

In addition to assaying the phenotypes produced by different combinations of *Notch* and *abl* alleles, we also asked whether the *Notch/abl* interaction was affected by mutations in *disabled*. *N/+ abl/+ dab/+* triply heterozygous flies were viable, and they did not display obvious wing notching. With very high penetrance, however, they had a severe rough eye phenotype. This has been observed with pairwise combinations of two strong *Notch* alleles with two unrelated *abl dab* double mutant chromosomes. This observation is significant for two reasons. First, we have postulated the existence of a signaling pathway that includes *Notch, abl and disabled*, and the observation of eye defects in triply heterozygous flies demonstrates directly the existence of a three-way genetic interaction among these genes. Second, it alerts us to another potential phenotype of loci that modify the *Notch/abl* interaction. Until we have performed a pilot genetic screen, we will not know which phenotype, wing notching or eye defects, is more useful for identifying enhancers of the *Notch/abl* genetic interaction. Conversely, it may be that we will be able to use rescue of these eye defects as a screen for suppressors of the *Notch/abl/disabled* interaction. We are currently extending our phenotypic analysis of various combinations of *Notch*, *abl* and *dab* alleles to determine whether any allelic combination yields defects that are sufficiently penetrant to serve as the basis for a genetic suppressor screen. We are also performing additional genetic controls to verify the authenticity of the phenotype.

DISCUSSION

Under the revised Statement of Work for this project, the specific goals for the current year were to perform a biochemical analysis of the physical interaction of the NOTCH and DISABLED proteins, and to test various allelic combinations of *Notch* and *abl* mutations to determine their suitability for a screen for additional interacting loci in the coming year. As described above, we have achieved both of these goals.

1. NOTCH-DISABLED interaction *in vitro*

What might be the biochemical basis of the *Notch/abl* genetic interaction? A good deal is known about the biochemistry of ABL and its interactions with a variety of binding partners [10, 17], and there are no sequences in NOTCH that resemble known binding sites for ABL protein. In contrast, the PTB domain of DISABLED resembles the domain of NUMB protein which is known to bind directly to NOTCH. Last year, we found that purified *Drosophila* NOTCH intracellular domain was capable of binding to purified mouse Disabled PTB domain *in vitro*. We have now extended those experiments to show that *Drosophila* NOTCH binds to *Drosophila* DISABLED.

First, we found that the DISABLED PTB domain can select NOTCH out of a total embryo lysate. Second, we verified that the interaction is specific by mapping the DAB binding site to a small (130 amino acid) signaling domain of NOTCH . This is the same domain that is recognized by the closely-related PTB domain of NUMB. Finally, we showed both that the NOTCH-DISABLED interaction is direct and that it is not dependent on tyrosine phosphorylation by showing that the two proteins bind *in vitro*, even under quite stringent conditions (1% NP40; 0.1% SDS), after expression and purification from bacteria.

Unexpectedly, we also observed that purified NOTCH protein bound to DAB more avidly than did NOTCH protein that was translated *in vitro*. This may simply be an artifact of the two different expression systems. Alternatively, it may reflect the different phosphorylation states of the two proteins: while neither is phosphorylated on tyrosine, the protein made in bacteria was labelled by phosphorylation *in vitro* at protein kinase A recognition sequences. It is interesting to note that there is a cluster of phylogenetically conserved serine, threonine phosphorylation sites in NOTCH, including recognition sequences for Protein Kinase A, and that these are present in the construct we used for our binding experiments. Should the difference in binding strength indeed be a consequence of protein phosphorylation, this would potentially provide a mechanism for regulation of the NOTCH-DAB signaling pathway. We are currently testing the binding properties of the *in vitro*-translated NOTCH protein, with and without PKA phosphorylation, to distinguish whether it is the expression system or the phsophorylation state that is responsible for the difference in binding properties in these assays.

The data presented here beg the question, of course, of whether there is a genetic interaction between *Notch* and *disabled* mutations. The direct experiment to test this possibility, analysis of axon extension in embryos that are $Notch^{-/+}$; $dab^{-/-}$, is unfortunately not currently feasible: all of the existing *dab* alleles are on chromosomes that also bear mutations in *abl*, which is too closely linked to separate by recombination. In the future, we hope to generate new *dab* alleles to examine this question. We have, however, examined the phenotype of animals that are triply heterozygous for *Notch*, *abl* and *dab*. Preliminary results suggest the existence of a three-way genetic interaction among mutations in these genes, since the triply heterozygous adult flies show severe defects in eye morphology (as discussed above). Additional genetic tests will be required to verify the authenticity of this phenotype and to determine its cellular basis.

2. Potential genetic screens for modifiers of the *Notch/abl* genetic interaction

Much of our effort over the past year has been directed towards a thorough characterization of the *Notch/abl* genetic interaction, in order to facilitate the development of an effective screen for additional elements of this novel genetic pathway. Our results demonstrate the following properties of the *Notch/abl* pathway:

1. Decrease of *Notch* activity is reliably lethal in a background that is homozygous mutant for *abl*, viability of affected flies can be rescued by restoration of *Notch* function or *abl* function and flies that are genetically rescued from *Notch/abl* lethality are sufficiently fertile to permit recovery of the mutated locus. The most convenient starting genotype to screen for suppressor mutations is likely to be N^{ts1}; *abl/abl*, at 18°. All heteroallelic combinations of abl^1, abl^2 and $Df(abl)^{stj7}$ appear to be equally useful for the proposed screen.

2. The *Notch/abl* interaction can be enhanced to give rise to a synthetic wing-notching phenotype, and at least one extragenic dominant enhancer has already been identified ($cu^{-/+}$). For this genetic screen, the most appropriate genotype is likely to be N^{ts1}; abl^1/+ males, at 18°. Analysis of males that are N^{ts1}; abl^2/+ males is also possible, and will constitute a more stringent screen (since the abl^2 allele is less severe than abl^1).

3. The *Notch/abl* interaction can be enhanced to give rise to a severe rough eye phenotype. We are still testing various genotypic combinations, but it appears that the most convenient will be $Df(N)^{8}/+$; $abl^{-}/+$. The *abl* alleles abl^{1} and $Df(abl)^{stj7}$ appear to be equally applicable for this screen.

4. It is not yet clear whether the *Notch/abl/dab* synthetic eye defect is sufficiently robust to serve as the basis of a suppressor screen, but we should know this shortly. If so, it would have three significant advantages over a screen for restoration of viability. First, it would be less stringent than demanding restoration of viability and thus would permit us to obtain a different class of suppressor mutations. Second, flies that are triply heterozygous for *Notch*, *abl* and *dab* are substantially healthier than flies which are *Notch; abl/abl*, reducing the technical difficulty of the experiment. Third, since *abl* and *dab* are closely linked on a single chromosome it would save us from the difficulty of having to control three chromosomes simultaneously, Thus, the mechanics of the screen would be significantly simpler.

7. CONCLUSIONS

In the past year, we have characterized the physical interaction between the NOTCH and DISABLED proteins, and we have further characterized the genetic interactions between mutations in *Notch* and *abl*. Since DISABLED is a good candidate for an ABL-binding protein, the finding of a direct physical interaction between NOTCH and DISABLED suggests a potential biochemical basis for the *Notch/abl* genetic interaction. This idea is further supported by the observation of a three-way synthetic genetic interaction between *Notch*, *abl* and *disabled*: flies that are triply heterozygous for strong mutations in these genes suffer severe defects in eye development. Together these data suggest that a heterotrimeric complex of the NOTCH, DISABLED and ABL proteins may form the backbone of this novel signaling pathway. Our *in vitro* investigation of NOTCH-DISABLED binding, moreover, have turned-up two results that may shed light on the mechanism of *Notch-abl* signaling and its interaction with other *Notch* signaling pathways, as we now discuss.

Preliminary experiments suggest that NOTCH-DISABLED binding affinity may be modulated by Protein Kinase A-dependent phosphorylation of NOTCH. This potentially provides a way for *Notch/abl* signaling to be modulated by a wide array of other cellular signaling pathways, including those mediated by G-protein coupled receptors or by the Hedgehog signaling system (both of which signal through PKA). This idea is of particular interest in light of the evidence that axon guidance decisions require summation of multiple guidance cues [18]. To date, nothing is known to suggest how such signal integration might occur; our result is consistent with the NOTCH-DISABLED interaction being a direct target of other guidance cascades. In the future, we can test this model by extending our *in vitro* studies of the Notch-DISABLED binding interaction, and by asking whether mutations in *PKA* affect *Notch/abl* signaling *in vivo*.

We have also mapped the DISABLED binding site on NOTCH to the same part of the protein where the NUMB and SU(H) proteins are known to bind. It may be that the interaction of NOTCH with each of these proteins is restricted either temporally or spatially, so that the signaling systems do not interact. Alternatively, it may be that the binding of each of these proteins is competitive with the other two. It is tempting to speculate that such a competition could be involved in coordinating the neuron's identity with its axonal projection pattern. For example, binding of DAB to NOTCH could both make the neuron competent to extend an axon along a DELTA-expressing substratum and simultaneously inhibit that neuron from taking on an alternative *Notch*-dependent cell fate *via* activation of *Su(H)* -- such as a fate that involves axon growth on another substratum. To test this model, it will be important to determine whether DISABLED can compete with NUMB or SU(H) for binding to NOTCH, or alternatively whether binding of these proteins is independent of one another.

Finally, the key to a complete understanding of the *Notch/abl* signaling pathway will be isolation of additional components of this system. Our characterization of the *Notch/abl* genetic interaction suggests several simple and direct screens for modifiers of this interaction. Performing such a screen will be a major focus of our efforts over the coming year.

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9. FIGURE LEGENDS

Figure 1. Binding of the DISABLED PTB domain to wild type NOTCH.
Western analysis of DISABLED binding to wild type NOTCH in a total *Drosophila* embryo extract. Crude embryo extract was incubated with glutathione beads bearing either a fusion of the DISABLED PTB domain to GST, or else GST alone. Beads were washed and boiled in Laemmli buffer and associated proteins were analyzed by PAGE and Western blotting with anti-NOTCH antibodies. Gel lanes: "DAB" indicates material bound to the DISABLED PTB domain, "GST" indicates material found in association with GST. "Extract" shows the pattern of immunoreactive bands found in the starting embryo extract. Large arrowhead indicates full-length NOTCH protein (~300 Kd). Migration of molecular weight markers is indicated by numbers to the right of the gel.

Figure 2. Mapping the DISABLED binding site on NOTCH.
Autoradiograph of a gel analyzing the binding of the DISABLED PTB to various functional domains of NOTCH. Four fragments from the NOTCH intracellular domain were expressed by coupled *in vitro* transcription/translation in the presence of ^{35}S-methionine and assayed for binding to glutathione beads bearing either GST-DISABLED PTB or GST alone. NOTCH domains tested were the Ram23 region (Ram), ankyrin repeats (ank), PEST/OPA region (opa) and notchoid region (nd); details of the constructions and the precise limits of the domains are described in the text and the Methods. The left four lanes ("input") show the crude material from the in vitro translation reactions. The right eight lanes are arranged as pairs, showing material bound to GST-DAB (indicated as "DAB") or GST alone (indicated as "GST"), for each of the four protein fragments. Binding was observed only for the Ram23 fragment incubated with the DISABLED PTB domain.

Figure 3A. Direct binding of purified DISABLED and NOTCH proteins.
Autoradiograph of a gel assaying the binding of the purified NOTCH intracellular domain to the purified DISABLED PTB domain *in vitro*. The amino-terminal half of the NOTCH intracellular domain was expressed in *E. coli*, fused to a His_6-tag and two copies of a protein kinase A recognition sequence. The NOTCH fragment was purified on a Ni^{+2} column, kinase labelled with ^{32}P and incubated with beads bearing either GST-DISABLED PTB, or GST alone. The beads were washed, and bound proteins were eluted by boiling in Laemmli buffer and analyzed by PAGE and autoradiography. DAB indicates protein bound to the DISABLED PTB; GST indicates material bound by GST alone. Large arrowhead indicates the position of the major purified NOTCH fragment (M_r ~70Kd for the phosphorylated species, including vector sequences and tags). Similar results were obtained in parallel experiments assaying binding of the full-length NOTCH intracellular domain to the DISABLED PTB (data not shown). Labelled band at ~45 Kd is an anonymous bacterial protein that copurifies with the NOTCH fragment and associates non-specifically with beads.

Figure 3B. Binding of unphosphorylated, *in vitro* translated NOTCH to DISABLED.
Autoradiograph of a gel analyzing the effect of 0.1%SDS on binding of the DISABLED PTB domain to *in vitro* translated NOTCH protein. The same NOTCH fragment used for the experiment of Figure 3A was prepared by *in vitro* transcription/translation and assayed for binding to DISABLED either under the conditions described in the legend to Figure 2 (-SDS) or the legend to Figure 3 (+SDS). Inclusion of SDS in the binding and washing buffers seriously compromised binding of *in vitro* translated NOTCH, whereas it had no effect on binding of NOTCH purified from bacteria. Binding of NOTCH to GST alone was negligible in either condition.

Figure1

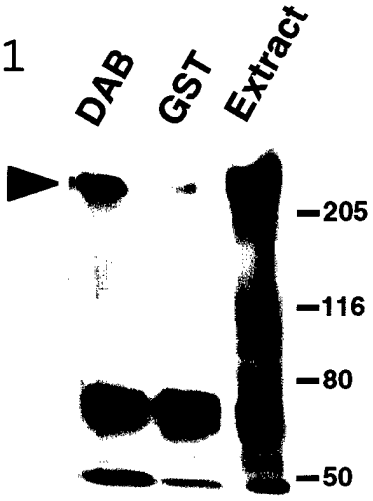

Figure2

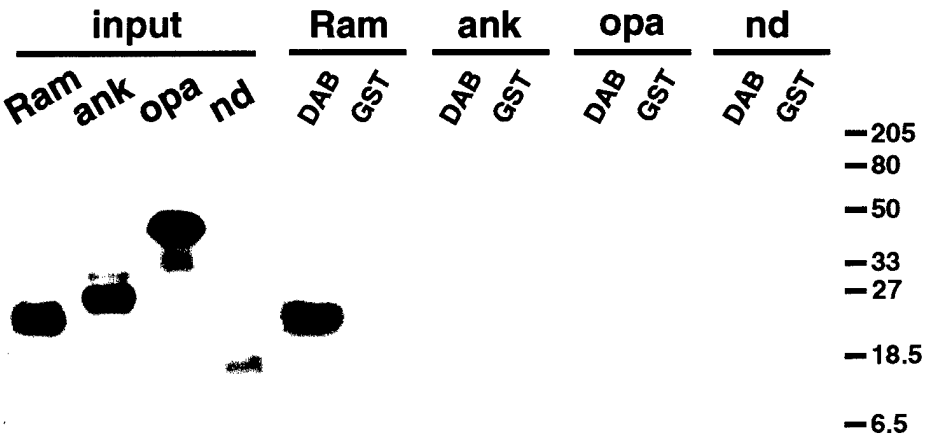

Figure3