Drosophila hyperplastic discs encodes an E6-AP type ubiquitin-protein ligase involved in cell proliferation control

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Abstract

The product of the *hyperplastic discs (hyd)* gene of *Drosophila*, HYD, is required for regulating imaginal disc cell proliferation and pattern formation. Sequence comparisons revealed that HYD contains a region similar to the putative catalytic domain of E6-AP, a cellular protein that associates with a human papillomavirus protein, E6. As evidence that HYD is functionally similar to E6-AP, we show that HYD associates with a *Drosophila* ubiquitin conjugating enzyme encoded by the *bendless* gene and that HYD forms a thioester with ubiquitin in a reaction dependent on this E2 enzyme.

Key Words : Hyperplastic discs, Ubiquitin-protein ligase, Cell proliferation

Introduction

The imaginal discs of *Drosophila melanogaster* provide an excellent system for examining the consequence mutations in genes that are required for normal cell proliferation and pattern formation. Imaginal discs consist of a single-cell layer epithelium that arises as an infolding of the embryonic ectoderm; they grow during the three larval instars by proliferation of their diploid cells. After an exponential increase in cell number, cell division terminates in a spacially and temporally defined pattern across the disc. More than 50 *Drosophila* genes have already been identified by loss of function mutations that cause a failure to terminate cell division leading to an imaginal disc overgrowth phenotyoe[1)]. This overgrowth is termed neoplastic if the overgrown imaginal discs lose their epitherial morphology and their ability to differentiate characteristic adult structures or hyperplastic if the overgrown imaginal discs retain their epitherial morphology and their ability to differentiate characteristic adult structures. Since the overgrowth phenotype caused by these mutations is the consequence of the loss of a gene function, these genes are considered to be tumor suppressor genes. The nucleotide sequences of several of these genes have revealed predicted proteins which may not have been expected as the products of tumor suppressor genes. As examples, the *fat* gene encodes an enormous cadherin-like transmembrane molecule that is probably involved in cell adhesion[2)]; the *expanded* gene encodes a protein with a potential SH-3 binding domain that may function by binding proteins that are in a signal transduction pathway[3)]; the *fused* gene encodes a serine/threonine protein kinase that is implicated in signaling initiated by the *hedgehog* gene product[4)].

The *hyperplastic discs (hyd)* gene has been categorized as one in which mutations hyperplastic imaginal disc overgrowth[1)]. Although wing and haltere imaginal discs in *hyd* hypomorphic mutant larvae are very overgrown, all of the imaginal discs in *hyd* null mutant larvae are smaller than normal. The *hyd* gene encodes a 9.5 kb transcript, which is present throughout development; the HYD protein is about 280 kDa[5)]. Databank searches have revealed strong similarity to the RAT 100-kDa PROTEIN, whose function is unknown[6)]. The C terminus of HYD and of RAT 100-kDa PROTEIN are similar to the C-terminus of several proteins from different eukaryotic organisms including the C-terminus of E6-AP, a protein with ubiquitin ligase activity. This domain was called HECT domain because it is **h**omologous to **E**6-AP **c**arboxy **t**erminal region[7)]. E6-AP is a 100 kDa cellular protein that interacts with the E6 protein of the cancer-associated human papillpmavirus type 16 and 18. The E6/E6-AP complex binds to and targets p53 for ubiquitin-mediated proteolysis[8-12)]. Ubiquitin is a highly conserved polypeptide found in all eukaryotes; its major function is to target

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proteins for complete or partial degradation by a multisubunit complex called the proteosome. Conjugation of ubiquitin to proteins destined for degradation proceeds in three-steps[13]. Initially, the C-terminal glycine of ubiquitin is activated by ATP to a high energy thioester intermediate in a reaction catalyzed by a ubiquitin-activating enzyme, E1. Following activation, E2 proteins (ubiquitin carrier proteins or ubiquitin conjugating enzymes) transfer ubiquitin from E1 to a substrate that may be bound to a ubiquitin-protein ligase or E3 protein. Then an isopeptide bond is formed between the activated C-terminal glycine of ubiquitin and an ε-NH_2 group of a lysine residue of the substrate. As E3 enzymes specifically bind protein substrates, they probably play an important role in selection of proteins for degradation. E6-AP is classified as a novel type of E3 ubiquitin protein ligase in that it accepts ubiquitin from an E2 ubiquitin conjugating enzyme and catalyzes isopeptide bound formation between ubiquitin and a targeted substrate[14]. The HYD protein has another sequence motif, called the ubiquitin-associated (UBA) domain, which occurs in a subset of the E2, E3 and UBP (ubiquitin carboxy-terminal hydrolase) superfamilies (Fig. 1A)[15]. This domain is predicted to have an alpha-helical arrangement, and to confer target specificity to multiple enzymes of the ubiquitination system.

Here, we described the function of the HYD protein as an ubiquitin-protein ligase, required for targeting substrate proteins for ubiquitination.

Experimental procedures

GST fusion proteins, far-western blot and binding assay

The expression of GST-HYD fusion proteins in *E. coli* (DH5α) and their purification on glutathione-Sepharose was carried according to the manufacturer's instructions (Pharmacia). GST-HYD fusion proteins were resuspended in 20 μl of SDS-PAGE sample buffer [2%(w/v) SDS, 4%(v/v) 2-mercaptoethanol, 30%(v/v) glycerol, and 0.04%(w/v) Bromphenol blue]. After heating at 100℃ for 5 min, proteins in each sample were fractionated by SDS-10% polyacrylamide gel electrophoresis. Proteins in the separating gel were electrophoretically transferred onto a nitrocellulose membrane. Generation of ^{32}P-labeled *Drosophila* E2 proteins and far-western blot analysis were carried out as described in Kaelin et al. (1992). Binding assays between HYD GST-fusion proteins and ^{32}P-labeled *Drosophila* E2 proteins was carried out as described in Hayashi et al. (1994).

In vitro translation of Flag tagged HYD proteins and ubiquitination assay

^{35}S-Methionine-labeled proteins were synthesized *in vitro* in a rabbit reticulocyte lysate with a coupled transcription/translation kit (Promega). Aliquots of the reaction mixture (10μl) were mixed with bacterially expressed *Drosophila* E2 enzymes (Bendless and Dhr6) and incubated at 30℃ for 60 min. Flag tagged HYD proteins were then immunoprecipitated with anti-Flag M5 monoclonal antibody (Kodak), and dissolved into SDS-PAGE loading buffer [62 mM Tris, pH 6.8, 2%(w/v) SDS, 10%(v/v) glycerol, 0.001% bromphenol blue] with either did or did not contain dithiothreitol (DTT; final concentration, 100 mM). Reactions that received DTT-containing loading buffer were heated at 100℃ for 5 min prior to loading onto SDS-10% polyacrylamide gel. Reactions that did or did not receive DTT were incubated at room temperature for 20 min before loading. Proteins in the separating gel were electrophoretically transferred onto Immobilon-P (Millipore) and immunostained with anti-ubiquitin antibody (Sigma).

Native HYD protein ubiquitination

Drosophila S2 cells were homogenized in RIPA buffer [50 mM Tris, pH 7.5, 1%(v/v) NP-40, 0.1%(w/v) sodium deoxycholate, 0.15 M NaCl, 10 μg/ml aprotinin] and a 10,000 x g supernatant obtained. HYD proteins were immunoprecipitated with HYD specific antibody from the supernatant, dissolved into SDS/PAGE loading buffer which either did or did not contain dithiothreitol (DTT; final concentration, 100 mM) and treated as above. The proteins were immunoblotted with HYD specific antibody[5] or anti-ubiquitin antibody (Sigma).

Generic interaction analysis

All stocks are grown at 20℃ on standard cornmeal agar medium supplemented with live yeast. The genotype of mutant stocks used in this study are *hyd^{14} red/TM3, e Ser*; *ben^{F107}/Binsc, oc ptg, cn1 P{ry$^{+t7.2}$=PZ}l(2)03405^{03405}/Cyo*; *$Pros26^{1}$ pb pTM3, y ri p sep Sb Ubx e Ser*.

Results

The presence of both HECT and UBA domains suggested that the HYD protein functions as an

E6-AP type E3 ubiquitin-protein ligase in *Drosophila*. If HYD is a ubiquitin-protein ligase, then it should bind to E2 ubiquitin conjugating enzymes. To examine this prediction, we tested whether the *Drosophila* E2 enzymes DHR6[16] or Bendless[17] could bind directly to specific fragments of the HYD protein (Fig. 1b).

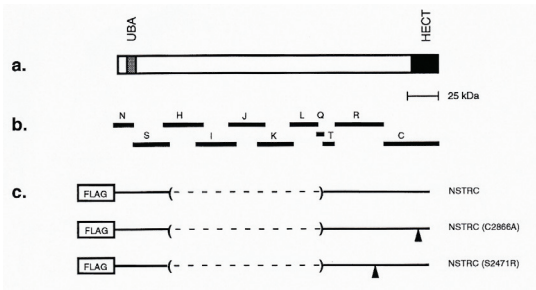

Figure 1. Fragments of HYD protein used in this study. **a,** Schematic diagram of HYD protein. UBA is ubiquitin associated domain; HECT is domain homologous to E6-AP carboxy terminal region. **b,** Schematic representation of GST-HYD expression constructs. **c**, Schematic representation of Flag tagged *hyd* cDNA constructs.

A series of gluthatione S-transferase (GST)-HYD fusion proteins, which collectively include the entire HYD protein, were expressed in bacteria, partially purified, fractionaled by SDS-PAGE and transferred onto a nitrocellulose filter. The filter was incubated with either ^{32}P-labelled DHR6-GST or Bendless-GST fusion proteins, washed and autoradiographed. DHR6 did not bind any of the HYD fragments (data not shown). However, Bendless strongly interacts with the T-region of the HYD protein (Fig. 2a). To confirm these results, purified recombinant Bendless or DHR6 protein, was labeled with ^{32}P, suspended in binding buffer with purified GST-HYD T fusion protein immobilized onto glutathione Sepharose beads and the material that bound to GST-HYD T-glutathione Sepharose was fractionated by SDS-PAGE. The Bendless protein but not DHR6 bound to GST-HYD T fusion protein *in vitro* (Fig. 2b). Neither protein bound to GST alone (Fig. 2b) or to glutathione Sepharose beads alone (data not shown).

If HYD behaves like E6-AP, then in the presence of a *Drosophila* E2 enzyme, it should form a thioester bound with ubiquitin. To examine this prediction, we constructed a partial *hyd* cDNA which includes the predicted functional regions an a Flag epitope tag[18] as shown in Figure 1c. We also made two mutant versions of this partial cDNA. C2866A is a point mutation of a conserved cysteine which in E6-AP is likely to be the site of ubiquitin thioester formation[7]. S2471R is a point mutation that is not expected to affect thioester formation. These 3 partial cDNAs were expressed in a coupled *in vitro* transcription/translation system in the

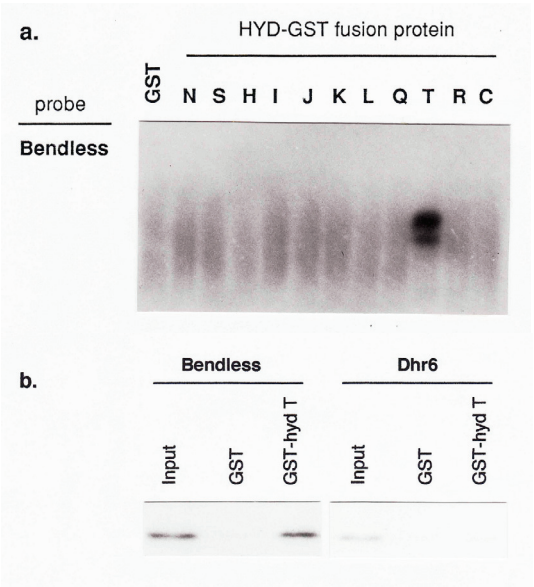

Figure 2. The *Drosophila* E2 enzyme encoded by *bendless* directly interacts with HYD *in vitro*. **a,** The T fragment of HYD binds to Bendless. GST-HYD fusion proteins were fractionated by SDS-PAGE and blotted with ^{32}P-GST-bendless. **b,** Bendless but not DHR6 binds to the T fragment of HYD. The *Drosophila* E2 enzymes encoded by *bendless* or *Dhr6* were labeled *in vitro* and subjected to GST pull-down analysis with GST-HYD(T).

presence of ^{35}S methionine, incubated with or without bacterially expressed *Drosophila* E2 enzymes, and then immunoprecipitated with a monoclonal antibody that detects the Flag epitope. Immunoprecipitated proteins were suspended in loading buffer, with or without DTT, fractionated by SDS-PAGE and then transferred onto a membrane. The non-mutant and mutant Flag tagged HYD proteins were recovered equally well in the presence or absence of DTT (Fig. 3, lane 1-6). The HYD proteins incubated in the presence of *Drosophila* E2 enzymes (Fig. 3, lanes 13-18) but not in their absence (Fig. 3, lane 7-12) were ubiquitinated as detected by antibodies directed against ubiquitin. Although in the previous assays we did not detected direct binding of DHR6 to HYD (Fig. 2), in this assay DHR6 was somewhat

effective in facilitating the ubiquitination of HYD. We do not yet know the reason for this difference.

The binding of ubiquitin to HYD is likely to be via a thioester bound because it is not detected if incubation is done in the presence of DTT (data not shown). This binding does not represent multi-ubiquitination, since the molecular mass of the ubiquitinated HYD protein was not greatly increased (compare Fig. 3 lanes 1-6 to lanes 13-18). These results indicate that the HYD protein, like E6-AP, can form a thioester bound with ubiquitin. These results also suggest that the conserved cysteine residue in the HECT domain is not the only cysteine in the HYD protein that can serve as an ubiquitin acceptor site, since the C2866A mutant protein also shows thioester ubiquitination which is dependent on E2 enzymes (compare lanes 13 to 15).

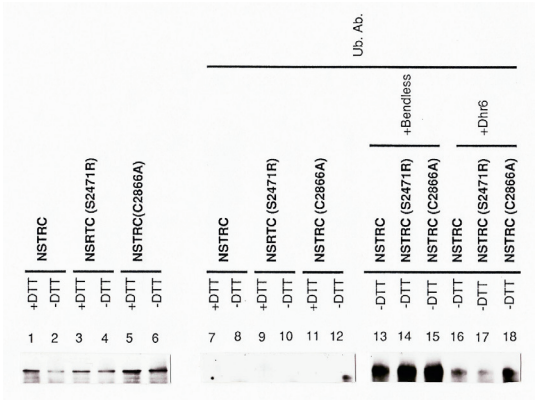

Figure 3. In the presence of E2 enzymes HYD is ubiquitinated *in vitro*. **Lanes 1-6,** Flag tagged, *in vitro* translated, non-mutant or mutant *hyd* cDNA fragments NSTRS labeled with ^{35}S-methionine can be immunoprecipitated with anti-Flag M5 monoclonal antibody. **Lane 7-12,** Flag tagged, *in vitro* translated, non-mutant or mutant *hyd* cDNA fragments incubated in the absence of E2 enzymes are not detected by blotting with anti-ubiquitin antibody after immunoprecipitation with M5 and SDS-PAGE. **Lane 13-18,** Flag tagged, *in vitro* translated, non-mutant or mutant *hyd* cDNA fragments incubated in the presence of E2 enzymes Bendless or Dhr6 are detected by blotting with anti-ubiquitin antibody after immunoprecipitation with M5 and SDS-PAGE.

To demonstrate that endogenous HYD protein, as well as a fragment of HYD protein, can form a DTT-sensitive, i.e. thioester bound, with ubiquitin, proteins were immunoprecipitated from extracts of *Drosophila* S2 cells with HYD specific antibodies[5], fractionated by SDS-PAGE, and immunoblotted with antibodies that recognize either HYD or ubiquitin. The full-length HYD protein was detected in the absence of DTT by both antibodies (Fig. 4) demonstrating that the endogenous 280 kDa HYD protein is ubiquitinated via a thioester bound. If the biological function of HYD depends upon its role in ubiquitin dependent protein degradation,

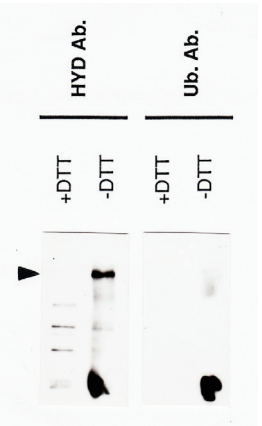

Figure 4. HYD is ubiquitinated in vivo. HYD protein was immunoprecipitated from an S2 cell extract, incubated in the presence or absence of DTT, fractionated by SDS-PAGE and immunoblotted with antibodies that detect either HYD or ubiquitin.

then the phenotype of *hyd* mutants should be enhanced by mutations in genes that encode other components of the ubiquitin dependent protein degradation pathway. To test this prediction, we examined the adult phenotype of double heterozygotes of a null mutation in *hyd* and mutations in an ubiquitin activating enzyme, in *bendless*, which encodes an E2 enzyme, and in *Pros26* which encodes a proteasome subunit[19]. Three percent of adult flies heterozygous for the null allele hyd^{14} have extra wing veins (Table 1). Characteristic fractions of adult flies heterozygosis for mutations in the ubiquitin activating enzyme, *bendless*, and *Pros26* also have extra wing veins (Table 1). However, adults doubly heterozygous for hyd^{14} and these other mutations have highly significantly increased penetrance of the extra wing vein phenotype (Table 1, Photo 1). These genetic data support our interpretation that the biological function of HYD depends upon its role in ubiquitin dependent protein degradation.

Discussion

Based on the sequence similarity between HYD and E6-AP, it was initially hypothesized that HYD had ubiquitin ligase activity. We have presented

three lines of evidence in support of this hypothesis. One; a small fragment of HYD (HYD-T) binds to Bendless, a *Drosophila* ubiquitin conjugating enzyme. Two; like E6-AP, a HYD fragment can be ubiquitinated *in vitro* in the presence of a ubiquitin conjugating enzyme. Three; a fraction

Table 1. Enhancement of *hyd* phenotype by mutations that affect components of ubiquitination pathway

GENOTYPE				
hyd	other gene	No. of flies examined	No. with extra veins	% with extra veins
+/+	+/+	200	0	0
hyd^{14}/+	+/+	256	8	3.1
+/+	+/ben^{F107}	50	23	3.1
hyd^{14}/+	+/ben^{F107}	64	51**	79.7
+/+	+/*Duba*	130	2	1.5
hyd^{14}/+	+/*Duba*	252	39**	15.5
+/+	+/*Pros26*	107	2	1.9
hyd^{14}/+	+/*Pros26*	56	11**	19.6

Wild type (Canton-S = +/+), single heterozygote, and double heterozygote adult flies were examined for presense of extra veins. **P<0.01 that observed difference is due to chance according to chi-square test.

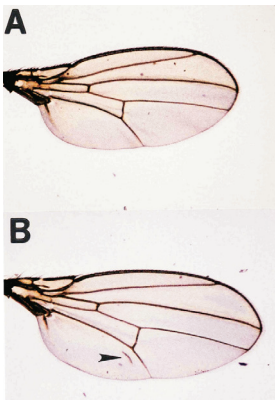

Photo 1. Mutations in *hyd* show genetic interactions with mutations in genes that encode *Drosophila* E2 enzyme. Wild-type wing venation **(A)** is compared to that in ben^{F107}/+;hyd^{14}/+ double trans-heterozygote fly **(B)**. Seventy-nine percent of animals exhibit an ectopic vein (arrow) stemming from the posterior cross-vein or posterior side of fifth longitudial vein.

of full length HYD protein is ubiquitinated *in vivo*. Finally we have presented genetic evidence that the ubiquitin ligase activity of HYD is an essential part of its normal function. Mutations in genes that encode a ubiquitin activating enzyme, a ubiquitin conjugating enzyme, and a proteasome component each enhances the phenotype of a *hyd* mutation.

Ubiquitin-protein ligases are required for targeting substrate proteins for ubiquitination. The identity of E3 ubiquitin protein ligases are a central issue in ubiquitin dependent protein degradation because they are potential regutlators of ubiquitination timing and substrate selection. Functional studies of E2 enzymes have shown that they can be clustered in two major groups. Most E2s catalyze transfer of ubiquitin to small amines or basic proteins, such as histones, in mono-ubiquitin derivatives that do not serve as proteolysis intermediated. The second group of E2 enzymes is involved in multiple ubiquitinations that target protein substrates for degradation. It is assumed that the E2 enzymes involved in proteolysis recognize distinct species of E3's. However, they do not appear to have specific recognition sites for the substrates. This task is accomplished, most probably, by the different E3 ubiquitin-protein ligases. The formation of E2/E3 complexes probably facilitates the transfer of activated ubiquitin from E2 to the protein substrate bound to the ligase.

Ubiquitin-dependent selective protein degradation serves to eliminate abnormal proteins and provides controlled short half-lives to certain cellular proteins, including proteins of regulatory function such as phytochrome, p53 and cyclines.

The *hyd* gene is the first E6-AP type, E3 ubiquitin-protein ligase gene in which mutations have been recovered. Genetic interaction analysis between *Drosophila* E1 and E2 enzymes support the idea that the specificity for substrate binding depends on the combination of E2 enzymes *in vitro*. Therefore, we interpret the abnormal proliferation of imaginal discs in *hyd* mutants as a consequence of the failure to degrade protein targets of the ubiquitination pathway. The identification of HYD dependent protein targets of ubiquitination will be the next step to understanding cell proliferation control and pattern formation that is dependent on HYD.

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