A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets

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Eukaryotic genomes encode large families of deubiquitinating enzymes (DUBs). Genetic data suggest that Fat facets (Faf), a Drosophila DUB essential for patterning the compound eye, might have a novel regulatory function; Faf might reverse the ubiquitination of a specific substrate, thereby preventing proteasomal degradation of that protein. Additional genetic data implicate Liquid facets (Lqf), a homolog of the vertebrate endocytic protein epsin, as a candidate for the key substrate of Faf. Here, biochemical experiments critical to testing this model were performed. The results show definitively that Lqf is the key substrate of Faf in the eye; Lqf concentration in the absence of functional Faf protein, (2) Lqf is ubiquitinated in vivo and deubiquitinated by Faf, and (3) Lqf and Faf interact physically.

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UBiquitin (Ub) is a highly conserved 76-amino-acid polypeptide, whose main role in eukaryotic cells is to target proteins for degradation by a proteolytic complex called the proteasome [Weissman 2001]. Ub becomes covalently attached to an internal lysine residue of a substrate protein via an isopeptide bond, in a series of reactions requiring several enzymes and ATP. Through its C-terminal glycine residue, another Ub monomer may be linked to an internal lysine residue of the first one. Several rounds of Ub addition result in an isopeptide-linked Ub chain; a chain of four or more Ub residues constitutes a degradation tag. In contrast, monoubiquitination can regulate protein function, in a manner analogous to phosphorylation [Hicke 2001].

Deubiquitinating enzymes (DUBs) cleave Ub-protein bonds, DUBs cleave Ub from peptide-linked translational fusion proteins and/or isopeptide-linked Ub chains [Wilkinson and Hochstrasser 1998]. All DUBs fall into one of two classes: Ub-C-terminal hydrolases (Uch) or Ub-specific processing proteases (Ubps). Although they may have overlapping functions, Uchs and Ubps have distinct, conserved catalytic regions [Wilkinson and Hochstrasser 1998]. Ubps, the larger class of DUBs, have characteristic Cys and His domains, which center around a key catalytic cysteine residue and two catalytic histidine residues [Baker et al. 1992; Huang et al. 1995; Wilkinson and Hochstrasser 1998]. Large families of DUB proteins have been revealed by functional screening [Baker et al. 1992] and genome sequencing [Wilkinson and Hochstrasser 1998; Chen and Fischer 2000], but the biological roles of only a few are well understood. The DUBs with known functions play housekeeping roles in the Ub-mediated protein degradation pathway; they generate Ub monomers by processing peptide-linked precursors [Ub polymers or Ub-protein translational fusions] or by recycling isopeptide-linked Ub chains [Wilkinson and Hochstrasser 1998].

The notion was advanced years ago that some DUBs may remove Ub chains from specific protein targets, and thus may act as substrate-specific regulators of ubiquitination and proteolysis [Hershko et al. 1980; Ellison and Hochstrasser 1991]. Several DUBs could potentially perform such regulatory roles, as they have been shown to be associated with the control of a wide variety of biological processes, including tumor suppression, transcription, cell growth, chromosome condensation, neural pathfinding, and memory storage [Wilkinson and Hochstrasser 1998; Wilkinson 2000; Weissman 2001]. However, in none of these cases has it been possible to identify a specific ubiquitinated substrate for a DUB or to determine its role in the Ub pathway.

In contrast, in the case of Fat facets (Faf), a Drosophila DUB required for patterning the compound eye, there is compelling genetic evidence that Faf activity antagonizes both ubiquitination and proteasomal proteolysis, and Faf was therefore hypothesized to cleave a degradation tag from a specific targeted protein [Huang et al. 1995; Wu et al. 1999]. Subsequently, genetic experiments identified Liquid facets (Lqf), an endocytic protein homologous to vertebrate epsin, as a candidate for the critical substrate of Faf in the eye [Fischer et al. 1997; Chen et al. 1998; Cadavid et al. 2000]. Four genetic observations are consistent with a model in which the function of Faf is to prevent Lqf degradation: (1) lqf loss-of-function mutations are strong dominant enhancers of the faf mutant eye phenotype, (2) faf and lqf loss-of-function mutations have similar mutant eye phenotypes, (3) the faf+ and lqf+ genes are required in the same group of cells in the eye, and (4) one extra copy of the lqf+ gene obviates the need for the faf+ gene in the eye.

The genetic data show definitively that lqf+ functions downstream of faf+ in a common pathway. Here, we report the results of critical biochemical tests of the model wherein Faf is a substrate-specific regulator of ubiquitination and proteolysis, and its key substrate is Lqf. We generated an antibody to Lqf and used it to show: (1) there is less Lqf protein in the developing Drosophila eye in the absence of functional Faf protein, (2) Lqf is ubiquitinated in developing eyes and deubiquitinated by Faf, and (3) Lqf and Faf interact physically.

Results and Discussion

Lqf protein colocalizes with endocytic proteins in Drosophila eye discs

To detect Lqf protein levels in developing eyes, we generated an antibody to Lqf (see Materials and Methods). Eye discs were double-labeled with anti-Lqf and antibo-
ies to the endocytic protein Shibire (Shi; Estes et al. 1996), which shows that Lqf and Shi colocalize at cell membranes; Lqf and Shi are concentrated apically in cells within the morphogenetic furrow, an indentation that marks the onset of differentiation [Wolff and Ready 1993], and also in developing photoreceptors where their membranes meet (Fig. 1A–J). Similar results were obtained with antibodies to two other endocytic proteins [Dap160 and α-Adaptn [α-Ada]], and with phalloidin, which labels f-actin at cell membranes (data not shown).

Faf activity increases the level of Lqf protein in Drosophila eye discs

One prediction of the hypothesis that Faf activity prevents the degradation of Lqf is that in the developing eyes [larval eye discs] of faf null mutant flies, there should be less Lqf protein than in wild-type eyes. We expected there to be less Lqf protein, as opposed to no Lqf protein, because the lqf null mutant eye phenotype is much more severe than the faf null mutant eye phenotype [Fischer et al. 1997; Cadavid et al. 2000].

To test whether the level of Lqf is affected by faf gene activity, first, using confocal microscopy, we compared the levels of Lqf in adjacent groups of faf− and faf+ cells in the eye disc. We generated clones of homozygous faf− cells in faf+/faf− heterozygous eye discs, marked by the absence of β-galactosidase (β-gal) expression [see Materials and Methods]. The eye discs containing clones were triple-labeled with antibodies to β-gal [to outline the clones], to Lqf [to detect the level of Lqf protein], and to Shi [as a negative control]. We found that throughout the eye disc, the level of Lqf protein, reflected in the strength of the signal from antibody labeling, is lower within the faf− clones than in the faf+/faf− heterozygous cells surrounding them [Fig. 2]. In contrast, the levels of Shi protein are the same within and outside the clone boundaries [Fig. 2].

To quantify the difference in Lqf protein levels in faf+ and faf− cells, we assayed the levels of Lqf in eye disc protein extracts prepared from wild-type and faf− flies in Western blot experiments [see Materials and Methods]. We used homozygotes for two different mutant faf alleles that behave genetically as strong loss-of-function mutations: fafβX4 is an inversion that makes no functional Faf protein, and fafP188 encodes an Faf protein with histidine residue 1986, which is critical for DUB catalytic activity, changed to tyrosine [Fischer-Vize et al. 1992; Huang et al. 1995; Chen and Fischer 2000]. We found that there is two- to threefold less Lqf in eye disc protein extracts of faf− homozygotes than in wild-type extracts [Figs. 3A, 4A]. We also performed the experiment by adding faf+ gene function back to faf− flies [Figs. 3B, 4B]. A transgene containing faf− genomic DNA [P[gaf+]], which when introduced into faf− homozygotes complements the mutant eye phenotype [Fischer-Vize et al. 1992; Huang et al. 1995], results in a two- to threefold increase in Lqf protein level in eye disc extracts [Figs. 3B, 4B]. A nearly identical transgene [P[gaf+P188]] that fails to complement the faf− mutant phenotype because it has a point mutation in the codon for cysteine 1677, which is critical to the DUB activity of Faf [Huang et al. 1995], fails also to increase the level of Lqf protein in eye disc extracts [Figs. 3B, 4B]. We conclude that faf+ activity results in an increase in the level of Lqf protein.

We asked whether the effect of faf+ gene activity on the level of Lqf is specific to Lqf by performing the Western blot assay using antibodies to four other endocytosis complex proteins: Shi, α-Ada, Dap160, and Lap [see Materials and Methods]. The levels of these proteins are unaffected by the absence of faf+ activity [Fig. 3C].

Lqf is ubiquitinated in eye discs and deubiquitinated by Faf

A second prediction of the model wherein Faf prevents proteolysis of Lqf by deubiquitinating it, is that there should be Lqf protein linked to Ub chains present in eye discs. Ubiquitinated proteins are usually detected on Western blots as ladders of protein bands of higher molecular weight than the protein in question, in increments of ∼8 kD; each “rung” on the ladder represents a
protein species with a Ub chain that is one Ub residue longer than the previous rung. Proteins with Ub chains are rapidly degraded, and thus difficult to detect; usually, inhibition of proteasome and/or DUB activity is required to detect them. Here, inhibition of the DUB activity of Faf, genetically, stabilizes ubiquitinated forms of Lqf.

In all of the Western blot experiments described above, which we performed to quantify Lqf levels in faf + and faf − eye discs, a ladder of higher-molecular-weight forms of Lqf is present consistently in the faf − eye disc protein extracts (Fig. 4A [faf kΔ], 4B [faf kΔ and faf kΔ + P[gal C1677S]]). In contrast, higher-molecular-weight forms of Lqf are not detected in extracts prepared from faf + eye discs (Fig. 4A [wild-type], 4B [faf kΔ + P[gal C1677S]]). Three lines of evidence indicate that the ladders represent ubiquitinated forms of Lqf. First, consistent with the idea that the higher molecular forms of Lqf have degradation tags, the presence of the ladders in the extracts correlates with a decrease in Lqf protein level; the faf + protein extracts, which show two- to threefold lower than wild-type levels of Lqf protein (Fig. 3A,B), are the ones that contain the higher-molecular-weight forms of Lqf. Second, the incremental size differences between wild-type Lqf protein and each of the rungs of the ladder are consistent with the size of a Ub monomer (~8 kDa; Fig. 4A,B, see legend). Third, the higher molecular forms are stabilized in the absence of Faf protein with its catalytic cysteine and histidine residues intact; we have shown previously that Faf deubiquitinates synthetic Ub–protein substrates in bacteria, and that this ability depends on its cysteine residue 1677 (Huang et al. 1995).

We conclude that in eye discs, Lqf is ubiquitinlated, and subsequently either deubiquitinlated by Faf or degraded. The observation that considerable amounts of nonubiquitinlated Lqf protein remain in faf − eye discs indicates either that only a fraction of the Lqf protein in the eye disc is ubiquitinlated, and/or that DUBs other than Faf also deubiquitinlate some Lqf protein.

Faf and Lqf interact physically

A third prediction of the model wherein Lqf is the substrate of Faf is that the proteins should, either directly or indirectly, interact. We used anti-Lqf to immunoprecipitate Lqf from protein extracts prepared from embryos, and tested for the presence of Faf in the immunoprecipitates on Western blots (see Materials and Methods). Embryos were used because sufficient protein could not be obtained from eye discs. In addition, to facilitate detection of Faf, the embryos were transformed with a P[hs-myc-faf] transgene, which expresses a fully functional, myc-tagged Faf protein upon heat shock, that can be detected on Western blots with antimyc (Huang et al. 1995; Huang and Fischer-Vize 1996). myc-Faf was detected in the anti-Lqf immunoprecipitate of the protein extract from heat-shocked transformant embryos, but not in the immunoprecipitates from non-heat-shocked embryos or from heat-shocked embryos when preimmune serum or no antibody was used instead of anti-Lqf (Fig. 4C). We conclude that myc-Faf and endogenous Lqf proteins interact physically in Drosophila embryos. Bacterially produced or in vitro translated partial Faf and full-length Lqf proteins do not bind to each other in GST pull-down assays (Cadavid 2000). One possible explanation is that only full-length Faf can bind to Lqf in these assays. Alternatively, Faf and Lqf may require other proteins for their interaction.

Conclusions

The experiments presented here provide critical biochemical evidence for a model in which a DUB called Faf specifically deubiquitinlates Lqf protein, thereby preventing its proteolysis. We have shown that there is less Lqf protein in the developing eye in the absence of catalytically functional Faf protein, that Lqf is ubiquitinlated and subsequently deubiquitinlated by Faf, and that Faf and Lqf interact physically. Taken together with previous genetic evidence that provides strong support for the model, we conclude that Faf is a substrate-specific regulator of ubiquitination, a novel function for a DUB.

The eyes of faf null or lqf hypomorphic mutants have more than the normal complement of eight photoreceptor cells in each facet, owing to the failure of a cell communication pathway early in eye development (Fischer-
The Faf/Lqf interaction is essential in only a small number of cells in the eye disc, which must be particularly sensitive to the levels of Lqf, and in these cells, Lqf presumably controls the frequency or specificity of endocytosis. Although the precise mechanism of epsin function is unknown, vertebrate epsin binds to the endocytosis complex and also to PIP2 at the cell membrane, and is required for endocytosis [Chen et al. 1996; Itoh et al. 2001]. Apparently, appropriate endocytosis in this small group of cells is essential for successful communication with their neighbors; increased Lqf levels either enable these cells to send a signal to their neighbors that inhibits neural determination, or else prevents them from sending their neighbors a positive differentiation signal.

Through a variety of mechanisms, endocytosis is proposed to regulate ligand/receptor interactions during development [Cagan et al. 1992; Moline et al. 1999; Entchev et al. 2000; Dubois et al. 2001; Greco et al. 2001]. How Lqf and endocytosis regulate faf-dependent cell signaling remains to be determined. As faf has vertebrate homologs (Jones et al. 1996; Wood et al. 1997), this mode of regulation is likely to be conserved. The finding that Lqf is the key substrate of Faf in the Drosophila eye shows not only that a DUB can regulate ubiquitination and thus proteolysis, but also that an endocytosis complex protein can be a target for the control of a cell communication event critical to cell determination.

Materials and methods
Lqf antibodies
A polyclonal antibody to Lqf was generated in guinea pigs (Cocalico Biologicals) from a bacterially produced partial Lqf protein, containing the region of Lqf1C-terminal to the ENTH domain (Cadavid et al. 2000). A DNA fragment encoding the C-terminal portion of Lqf1 was ligated into pET-28α (Novagen), and the resulting plasmid was used to transform...
BL-21 cells [Stratagene]. Protein expression was induced and the protein purified using a Ni^{2+} column according to the procedures recommended by Novagen. Western blots of protein extracts prepared from eye disc extracts or from 0-20 h embryos, labeled with anti-Lqf serum, showed only one or two bands, which correspond to the expected sizes of Lqf1 (∼68 kD) and/or Lqf2 (70-70 kD), Cadavid et al. 2000). Labeling with pre-immune serum resulted in no signal on Western blots of bacterially produced antigen, eye disc, or embryo protein extracts, nor in eye disc whole mounts.

**Generation of fat mutant clones in the eye disc**

Clones of homozygous fat[ null mutants fat^{f2} or fat^{f5}; Fischer-Vize et al. 1992, Chen and Fischer 2000] cells were induced in fat/ fat[ larval eye disc using FTY; hs-neo; FLRT] (Xu and Rubin 1993), P[coDNA96A] (Tio and Moses 1997), and P[ey-FLP]D12 (Newsome et al. 2000) chromosomes. Eye discs were dissected from third instar larval embryos of the genotype P[FLP]/+; P[VFRT]82B fat/ P[VFRT]82B P[coDNA96A].

**Antibody labeling of eye discs**

For Figure 1, wild-type third instar larval eye discs were immunostained essentially as described previously, using PLP fixation and 0.1M NaPO/0.2% saponin wash and incubations solutions (Fischer-Vize et al. 1992). The primary antibodies were guinea pig anti-Lqf at 1:1000, rabbit anti-Shi at 1:200, rabbit anti-Elav (mAb7E8A10; Developmental Studies Hybridoma Bank, DSHB) undiluted, and mouse anti-Cut (mAb2B10; DSHB) at 1:10. The secondary antibodies used (Molecular Probes) were Alexa488-anti-mouse (1:100), TexasRed anti-guineapig (1:400), and Alexa555-anti-rabbit (1:500). Stained eye discs were mounted in Vectashield (Vector Laboratories)mounting medium. Images were produced using a Leica TCS 4D confocal microscope, and processed using Adobe Photoshop.

**Western blot analysis of eye disc protein extracts**

To generate eye disc protein extracts, five pairs of eye discs, dissected from third instar larvae, were homogenized in 50 µL of 2× Laemmli buffer in a microtube with a Teflon pestle, and then centrifuged at 13,000 rpm at 4°C for 5 min. Four aliquots of the supernatant (15 µL, 10 µL, 5 µL, and 5 µL) were distributed into microtube tubes and their volumes increased to 15 µL with 2× Laemmli buffer. Each aliquot was boiled for 5 min and then subjected to SDS-PAGE. The proteins were transferred to nitrocellulose (Bio-Rad), then labeled with primary and secondary antibodies (see below), and the standard procedures. The HRP signal was detected with ECL Renaissance reagents (NEL). The signals were quantified using NIH Image 1.62 Software (http://svb.info.nih.gov/nih-image).

To obtain measurements within the linear range of detection of the software, we used exposures of the images such that measurements of the signals in at least three of the four lanes gave the same value for [Lqf]/ [tubulin], only Lqf2 was measured. The primary antibodies used were mouse anti-tubulin (mAbE7; DSHB) at 1:10, guinea pig anti-Lqf at 1:4000, rabbit anti-Shi (Estes et al. 1996) at 1:1000, rabbit anti-α-Ada (Gonzalez-Gaitan and Jackle 1997) at 1:1000, rabbit anti-Dap160 (Roos and Kelly 1998) at 1:200, guinea pig anti-Lap (Zhang et al. 1998) at 1:200, and mouse anti-Arm (monoclonal N27A1; DSHB) at 1:50. The secondary antibodies used were HRP-anti-tubulin (Jackson) at 1:4000-20,000, HRP-anti-mouse [Santa Cruz] at 1:500, and HRP-anti-rabbit [Santa Cruz] at 1:500.

**Immunoprecipitation**

Anti-Lqf (2 µL) was added to 30 µL of protein A-agarose [Bio-Rad], the mixture was incubated on ice for 1 h, and the soluble protein extract from 150 µL of heat-shocked embryos was added in 150 µL [Embryo extract preparation and heat shock conditions were as described previously. Huang et al. 1995, Huang and Fischer-Vize 1996. The mixture was incubated at 4°C on a nutator for 12 h, then the beads were spun down (8000 rpm in a microtube for 2 min) and the supernatant was removed. The beads were then washed 5 times for 5 min each, with 1.5 mL of buffer (15 mM Hepes-KOH at pH7.6, 10 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.5 mM EGTA, 0.5 mM sucrose), resuspended in 2× Laemmli buffer, and boiled for 5 min prior to loading on an SDS-PAGE gel. Three negative controls were performed, the immunoprecipitation reactions were carried out without the addition of anti-Lqf, with preimmune serum, or the protein extracts were prepared from P[hs-myc-fat] transformant embryos that were not heat-shocked. Western blots were prepared and developed with anti-myc (mAbE9E10; Santa Cruz) at 1:1000 as described above.

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