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Regulation of Apterous activity in *Drosophila* wing development

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SUMMARY

Apterous is a LIM-homeodomain protein that confers dorsal compartment identity in *Drosophila* wing development. Apterous activity requires formation of a complex with a co-factor, Chip/dLDB. Apterous activity is regulated during wing development by dLMO, which competes with Apterous for complex formation. Here, we present evidence that complex formation between Apterous, Chip and DNA stabilizes Apterous protein in vivo. We also report that a difference in the ability of Chip to bind the LIM domains of Apterous and dLMO contributes to regulation of activity levels in vivo.

Key words: Apterous, dLMO, Homeodomain, LIM domain, Wing development

INTRODUCTION

apterous (*ap*) is the selector gene responsible for the establishment of dorsal (D) and ventral (V) compartments in the developing *Drosophila* wing (Diaz-Benjumea and Cohen 1993; Blair et al., 1994). Apterous protein is expressed in dorsal cells in the wing disc, where it has three functions. First, Apterous is responsible for forming the boundary of cell lineage restriction that separates D and V compartments (García-Bellido et al., 1973). Second, Apterous is responsible for establishing the signaling center at the DV boundary. This depends on localized activation of the Notch signaling pathway in cells adjacent to the DV boundary. Apterous protein controls Notch activation through regulation of the Notch ligands Serrate and Delta, and through Fringe, which controls the sensitivity of Notch for its ligands (Irvine, 1999). Third, Apterous acts via the homeodomain protein Msh to specify dorsal cell identity (Milán et al., 2001).

Apterous protein (Ap) is a LIM-homeodomain transcription factor. LIM-domains are cysteine-rich zinc-finger domains that mediate protein-protein interactions (Jurata and Gill, 1998). Evidence has been presented that the LIM domains block DNA-binding activity of the homeodomain. The *Xenopus* Ldb1 protein was identified as a cofactor for the LIM-homeodomain Xlim1 by virtue of binding to the LIM domains (Agulnik et al., 1996). The *Drosophila* homolog of Ldb1 is called dLDB or Chip (Morcillo et al., 1997; Fernandez-Funez et al., 1998). Chip provides both a self-interacting dimerization domain and a LIM-interaction domain that binds to the LIM domains of Ap and other proteins (Jurata et al., 1998). A dimer of dLDB/Chip has been shown to bridge two Ap molecules to form a tetrameric complex, which is active in vivo (Milán and Cohen, 1999; van Meyel et al., 1999; Rincon-Limas et al., 2000).

Ap activity levels are temporally regulated during wing development (Milán and Cohen, 2000). Ap negatively regulates its own activity by inducing the expression of the *Beadex/dLMO* gene during third instar (Milán et al., 1998; Shoresch et al., 1998; Zeng et al., 1998). *Beadex/dLMO* encodes a LIM-domain containing protein of the LIM-only type, called dLMO. dLMO protein contains two LIM domains with sequences highly similar to the LIM domains of Ap. dLMO has been shown to bind to Chip, and to compete for binding between Chip and Ap, thereby inactivating Ap (Milán et al., 1998; Milán and Cohen, 1999; van Meyel et al., 1999).

The spatially and temporally restricted expression of dLMO limits the time window during which the induction of the DV organizer can take place. Early in development, Apterous induces expression of the *fringe* and *Serrate* genes and represses *Delta* in D cells (Irvine, 1999). Apterous subsequently induces expression of dLMO, which reduces Ap activity levels and leads to downregulation of *Serrate* and *Fringe*, and allows dorsal expression of *Delta*. Therefore, loss of dLMO leads to overexpression of *Serrate*, to reduced expression of *Delta* and to concomitant defects in differentiation and cell survival in the wing primordium (Milán and Cohen, 2000). All of these phenotypes have been shown to be due to excess Ap activity. Thus dLMO serves as an important regulator of Ap activity during wing development.

The model that dLMO acts as a competitive inhibitor of Ap suggests that dLMO should displace Ap from Chip. We present evidence that Ap protein is destabilized in cells expressing dLMO. Interestingly, Ap appears to be destabilized in situations where it is unable to bind to DNA in active tetrameric complexes with Chip. Previous studies have suggested that dLMO competes effectively with Ap for binding to Chip, whereas Ap competes poorly with dLMO. A possible explanation for this behavior is an intrinsic difference in the affinities of the LIM domains from Ap and dLMO proteins for Chip. We addressed this possibility by replacing the LIM domains of Ap with those of dLMO, to generate an Ap-dLMO fusion protein. This fusion protein has Ap activity and unlike the endogenous Ap protein, competes effectively with dLMO

in vivo. These observations support the view that dLMO is a potent inhibitor of Ap activity because it binds Chip more effectively and therefore provide an explanation for the long-standing puzzle that overexpression of Apterous cannot cause an increase in Apterous activity in vivo.

MATERIALS AND METHODS

Cell culture expression experiments

Ap-myc was generated by PCR with 5′ TCCGAATTCCACACA-TGGGCGTCTGCACC 3′ and 5′ CTGCTCGAATTCGTCCAAG-TTAAGCGG 3′ using Ap cDNA (Cohen et al., 1992) as a template, cut with *Eco*RI and cloned into pRmHa3-myc that had been produced by excising the fringe ORF from pRmHa3-fng-myc (Brückner et al., 2000) with *Eco*RI. dLMO-myc was generated by PCR with 5′ CAATGAATTCATATATGATGACTATGGAC 3′ and 5′ CTGCTCG-AATTCGCTGGACGCTCCTAG 3′ using dLMO cDNA as a template (Milán et al., 1998), cut with *Eco*RI and cloned into pRmHa3-myc. Chip-myc was made by PCR with 5′ GATGAATTCCAGGCATGA-ATCGTAGGGGT 3′ and 5′ CTGCTCGAATTCTTGAGATACAA-TGGG 3′ using Chip cDNA as a template (Morcillo et al., 1997), cut with *Eco*RI and cloned into pRmHa3-myc. ChAp-myc was made by PCR using the primers 5′ GATGAATTCCAGGCATGAATCGTA-GGGGT 3′ and 5′ CTGCTCGAATTCGTCCAAGTTAAGCGG 3′ using pUAST-ChAp as a template (Milán and Cohen, 1999), cut with *Eco*RI and cloned into pRmHa3-myc. *Drosophila* Schneider cells (Di Nocera and Dawid, 1983) were grown in *Drosophila* Schneider's medium with 10% fetal bovine serum under standard conditions. Transfections were performed using Lipofectin (Gibco). A total of 10 μg of DNA was transfected and empty vector (pRmHa3) used to equalize the total amount. Cells were transfected overnight in serum-free medium and the following morning serum was supplied. Expression from the pRmHa3 vectors was induced by adding 0.7 mM $CuSO_4$ for 36-48 hours, after which the cells were harvested. After boiling in SDS-sample-buffer lysates were subjected to SDS gel electrophoresis (10-12%) and transferred to nitrocellulose membranes. Transfer efficiency and equal protein loading were verified by Ponceau S staining of the filters. For the DNA-binding site competition experiment, pGSU-Adh-CAT was used, which carries tandem repeats of Lhx2 binding sites from the α-glycoprotein subunit promoter, that have been shown to bind Ap (Rincon-Limas et al., 2000).

Imaginal disc culture

Wild-type mid-third instar larvae were incubated in *cl-8* cell medium for three hours before fixation and staining. For treatment with MG-132, discs were cultured in medium containing 10 μM MG132 (experimental discs) or an equal volume of DMSO (control discs).

Drosophila expression constructs

UAS-dLAp-flag was prepared by PCR, generating three overlapping pieces: the N-terminal Ap part (amino acids 1-148) was amplified using primers (Ap N-ter top) 5′ TCCGAATTCCACACATGGGCG-TCTGCACC 3′ and (Ap N-ter bottom) 5′ GCCGCAGCCTGCACA-GTCGTCGAGGTTTCG 3′ from an ap cDNA template; the LIM-domains of dLMO (amino acids 92-192) were amplified using (dLMO top) 5′ CGAAACCTCGACGACTGTGCAGGCTGCGGC 3′ and (dLMO bottom) 5′ CTGGTCTCCCTTTGTGAATCTGTGGTTACA 3′; and the C-terminal part of Ap (246-469) was generated by PCR with (Ap C-ter top) 5′ TGTAACCACAGATTCACAAAGGGAGA-CCAG 3′ and (Ap C-ter bottom) 5′ GTCGCGGCCGCCGTCCAAGT-TAAGTGGTGG 3′. All three pieces were mixed and used as a template for PCR with the primers Ap N-ter top and Ap C-ter bottom. The resulting product was cut with *Eco*RI and *Not*I and cloned into pUAST (Brand and Perrimon, 1993) carrying the flag epitope. pUAST-flag was generated by annealing the oligos 5′ GGCC-GCGACTACAAGGACGACGATGACAAGTAACCTAC 3′ and 5′ CTTACTTGTCATCGTCGTCCTTGTAGTCGC 3′ and inserting them between the *Not*I and *Kpn*I sites of pUAST. The dLAp-flag ORF was entirely sequenced to verify correct synthesis.

Drosophila strains

ap^{Gal4} has been described previously (Calleja et al., 1996). ap^{UGO35} and ap^{rk568} (*apterous*-lacZ) have been described previously (Cohen et al., 1992). hdp^{R590}, *MS1096* and UAS-dLMO have been described previously (Milán et al., 1998). UAS-ChAp and UAS-ChipΔLID have been described previously (Milán and Cohen, 1999). $dLMO^{\Delta 39}$was generated by imprecise excision of the Gal4 element of *MS1096* as described previously (Milán et al., 1998). $dLMO^{\Delta 39}$ is a hypomorphic allele of dLMO that does not affect cell survival in mutant clones (as opposed to $dLMO^{R590}$). $Chip^{e5.5}$ has been described previously (Morcillo et al., 1997).

Genotypes of crosses

$dLMO^{\Delta 39}$ loss-of-function clones: armlacZFRT18/armlacZFRT18; hsFlp/hsFlp × $dLMO^{\Delta 39}$ FRT18/Y
$dLMO^{\Delta 39}$ clones in *ap-lacZ* background: UbiGFPFRT18/FM6; hsFlp/CyO × $dLMO^{\Delta 39}$ FRT18/Y; ap^{rk568} /+
dLMO gain-of-function clones: hsFlp/Y; uas-dLMO/SM6-TM6 × Act>CD2>Gal4 uas-GFP/ Act>CD2>Gal4 uas-GFP
$Chip^{e5.5}$ loss-of-function clones: hsFlp/hsFlp; FRT42 armlacZ/FRT42 armlacZ × FRT42 $Chip^{e5.5}$/CyO

Antibodies

Guinea-pig anti-Ap was generated using recombinant Ap protein produced in *E coli*, as described (Milán et al., 1998). Although it was raised against the whole Ap protein, this antibody does not recognize the fusion protein ChAp. As the homeodomain is highly conserved across species, we speculate that is does not constitute a very efficiently recognized epitope and therefore only the LIM domains or other parts of the protein, which are conserved to a lesser extent, were antigenic. Rat anti-Serrate and rat anti-dLMO were produced using recombinant proteins produced in *E coli*. Mouse anti-Wg was from Brook and Cohen (Brook and Cohen, 1996). Rabbit anti-β-galactosidase (Cappell). Rabbit anti-Ci was from C. Schwartz.

RESULTS AND DISCUSSION

Post-transcriptional regulation of Apterous protein levels in vivo

Apterous activity levels are spatially and temporally regulated in the wing disc by expression of dLMO. Comparing expression of Ap protein and mRNA in the wing imaginal disc suggested that Ap might be subject to post-transcriptional regulation (Fig. 1A,B). *ap* mRNA is expressed at similar levels in the presumptive wing hinge and wing pouch. By contrast, Ap protein levels are considerably lower in the wing pouch than in the hinge region. The region where Ap levels are low coincides with the region in which dLMO is expressed (Fig. 1C,D). This suggests that the difference in Ap protein levels reflect a post-transcriptional consequence of dLMO expression. To ask whether dLMO is responsible for reducing Ap levels where the two proteins are co-expressed, we produced genetic mosaics in which dLMO activity was removed from clones of cells. Ap protein was more abundant in cells homozygous mutant for $dLMO^{\Delta 39}$(Fig. 2A). The increased level of Ap protein in the clone was similar to the level detected in the hinge. Clones of $dLMO^{\Delta 39}$ mutant cells

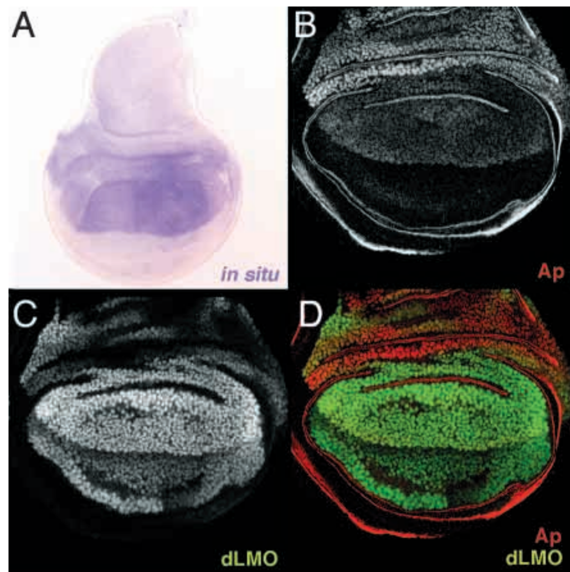

Fig. 1. Ap protein levels differ between the dorsal wing pouch and the hinge region. (A) *ap* mRNA levels visualized in a wild-type disc by in situ hybridization with an *ap* antisense RNA probe. (B) Third instar wing imaginal disc labeled with anti-Ap. Ap levels were higher in the hinge region than in the dorsal wing pouch. The difference in Ap protein levels between pouch and hinge is not reflected by a difference in transcript levels. (C) Anti-dLMO staining of the disc in B. (D) Overlay of B and C. Low Ap levels coincide with high dLMO levels.

were also generated in larvae carrying an *ap-lacZ* reporter gene. β-Gal expression by the reporter gene reflects the level of *ap* transcription. Ap protein was increased in the mutant clones, but β-gal expression was unaffected (Fig. 2B). This confirms that the effect of removing dLMO on Ap protein level is not due to increased expression of *ap* transcript.

These observations suggest that dLMO protein is responsible for the reduced level of Ap protein in the dorsal wing pouch. To further test this possibility, we generated clones of cells overexpressing dLMO and assessed Ap protein levels. As expected from the loss-of-function data, dLMO expression reduced Ap levels in the hinge region, where Ap levels are usually high (Fig. 2C, long arrow). The lower level of Ap in the dorsal pouch was further reduced by elevated dLMO expression (short arrow). We therefore conclude that dLMO reduces Ap levels in third instar imaginal wing discs. To determine whether Ap protein might be degraded in dLMO-expressing cells by a proteasome-dependent mechanism, we incubated wing discs with the proteasome inhibitor MG-132. As a control we examined Ci protein, which was stabilized in anterior cells as reported previously (Wang and Holmgren, 2000). Ap protein levels were increased in the wing pouch relative to the levels in the hinge in drug treated but not in control solvent treated discs (Fig. 3). This suggests that Ap protein is more susceptible to proteasome-mediated degradation in cells expressing dLMO.

Complex formation with Chip and DNA stabilizes Ap

As dLMO competes with Ap for binding to Chip, we considered the possibility that Ap protein may be protected when it is in a complex with Chip. To test this, we generated *Chip*$^{e5.5}$ mutant clones, which lack Chip protein and therefore lack Ap activity (Morcillo et al., 1997). Ap protein levels were reduced in *Chip* mutant clones (arrows, Fig. 4A), and increased in the wild-type twin spots which contain a higher level of Chip protein (arrowheads). To verify that reduced Chip activity does not affect *ap* mRNA levels we examined *ap-lacZ* reporter gene expression in discs expressing the dominant negative form of Chip, ChipΔLID. Ap protein levels were reduced in cells expressing ChipΔLID but *ap-lacZ* levels were unaffected (Fig. 4B). Thus, loss of Chip leads to reduced levels of Ap protein. We note that *Chip* mutant clones also lack dLMO expression (Milán and Cohen, 2000). Thus, loss of Ap protein in *Chip* mutant clones does not correlate with expression of dLMO, as in wild-type cells. Rather, reduction of Ap levels correlates with the availability of Chip as a binding partner. This suggested that binding to Chip contributes to stabilization of Ap.

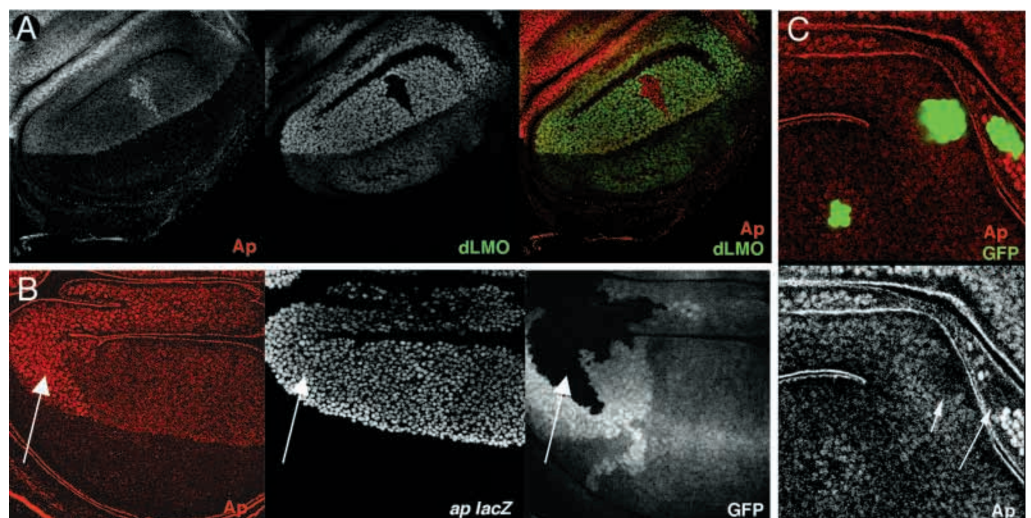

Fig. 2. dLMO decreases Ap levels in the dorsal wing pouch. (A) Wing disc with a clone of *dLMO*$^{\Delta 39}$ mutant cells. dLMO protein was not detectable in the mutant clone (center). Ap levels were higher in the dLMO mutant cells (left). Overlay of Ap and dLMO staining (right). (B) Region of an *ap-lacZ* wing disc with a large clone of *dLMO*$^{\Delta 39}$ mutant cells (arrow). The clone was labeled by the absence of GFP (right). Ap protein increased in the clone (left), but *ap-lacZ* transcript levels reflected by anti-β-gal did not differ between the clone and the surrounding tissue (center). (C) Disc with clones of dLMO-expressing cells (labeled by co-expression of GFP, green). The reduction of Ap is most obvious in the hinge where the endogenous protein level is higher (long arrow). dLMO also reduced Ap levels in the wing pouch (note the loss of nuclear label in the clone near the short arrow). The residual signal in the clone reflects nonspecific background produced by the antibody (as in the ventral compartment in B).

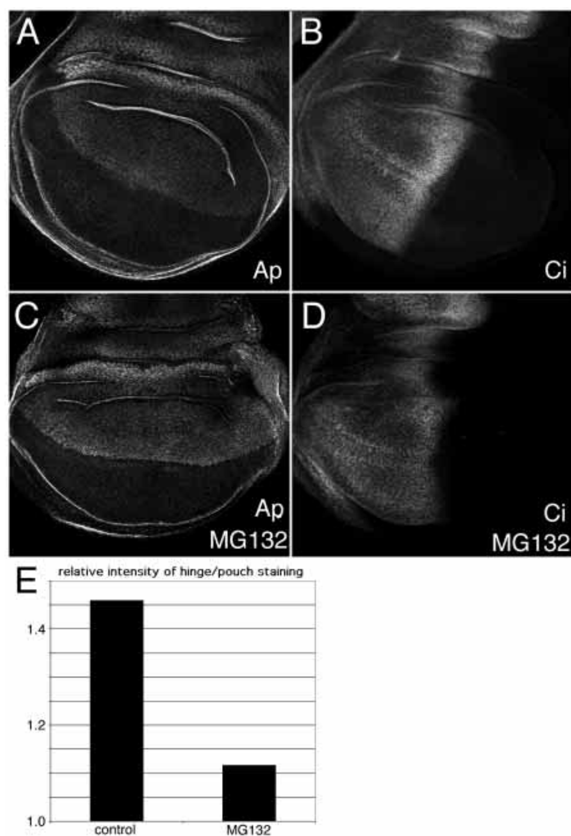

Fig. 3. Ap is degraded by a proteasome-mediated mechanism in the wing pouch. (A,B) Wild-type, DMSO-treated discs stained with anti-Ap (A) and anti-Ci (B) antibodies. (C,D) Wild-type discs treated with MG-132 for 3 hours. Ap staining was more intense in the dorsal wing pouch after treatment with the proteasome-inhibitor (D) when compared with the hinge (C). In D, the previously reported stabilization of Ci is evident. (E) Ratio of Ap staining intensity in the pouch and hinge region (averaged over three areas of the wing pouch in the discs in A,C). The level of Ap in the pouch and hinge are more similar in the MG132-treated disc.

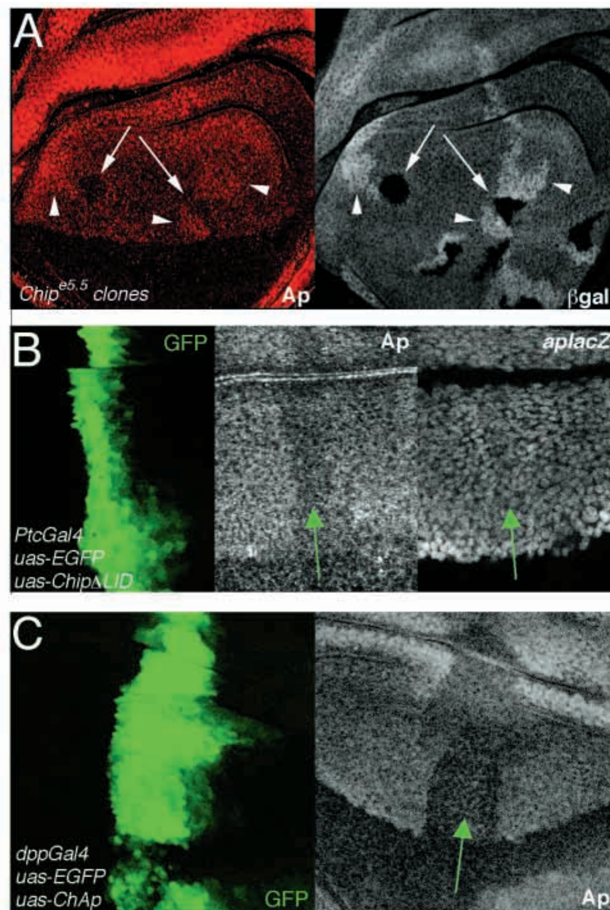

Fig. 4. Complex formation with Chip is required to stabilize Ap protein. (A) Wing imaginal disc with homozygous *Chip*$^{e5.5}$ mutant clones. Clones were marked by the absence of β-gal (right). Ap levels decreased in mutant clones, compared with the surrounding tissue (left panel, arrows). Ap levels were higher in homozygous wild-type twin spots, which contain two copies of the *Chip* gene, than in the tissue heterozygous for Chip (arrowheads). (B) *ptcGal4 uas-EGFP; uas-ChipΔLID* wing disc. ChipΔLID overexpression was visualized by co-expression of GFP (left panel). Ap expression was reduced (anti-Ap shown in white, center). *ap-lacZ* transcript levels reflected by anti-β-Gal did not differ (right). (C) *dppGal4 uas-EGFP; uas-ChAp* wing disc. ChAp and GFP were co-expressed (left; GFP shown in green). Endogenous Ap protein was reduced to background levels in these cells (right panel, arrow). Note the anti-Ap antibody does not recognize ChAp.

ChipΔLID is capable of binding to full-length Chip through its dimerization domain, but cannot bind to Ap (illustrated in Fig 8A,B). Consequently, ChipΔLID leads to formation of trimeric complexes and thereby blocks Ap activity in vivo (Milán and Cohen, 1999; van Meyel et al., 1999). The observation that ChipΔLID led to reduced Ap stability without affecting *ap-lacZ* expression suggested that stabilization might require formation of tetrameric complexes between Chip and Ap. The tetrameric form of Chip and Ap is thought to be the active DNA-binding complex. Overexpression of ChipΔLID does not decrease the availability of LIM-binding sites in wild-type Chip, but does compete for tetramer formation. This raised the possibility that Ap stability might depend on whether it is able to form a DNA-binding complex with Chip.

If Ap stability decreases when it is unable to bind DNA, we reasoned that providing additional binding sites might stabilize the protein. To test this possibility we turned to a cell culture system in which we could vary the number of Ap-binding sites by transfection. We first verified that co-expression of dLMO would decrease Ap stability in transfected cells. A constant amount of a plasmid directing expression of a Myc-tagged Ap protein was co-transfected with varying amounts of a plasmid directing expression of myc-tagged dLMO (Fig. 5A). As observed in the wing disc, overexpressed dLMO reduced Ap protein levels in S2 cells. We note that high levels of dLMO are required to reduce Ap levels. The relative levels of the two proteins can be directly compared in this assay by virtue of the myc-epitope tag. Comparison of relative levels of the endogenous proteins is not possible.

To test the effect of Ap-binding sites on Ap protein stability, a constant amount of a plasmid directing expression of a myc-tagged Ap protein was co-transfected with varying amounts of a plasmid carrying nine tandem repeats of a binding-site for the mammalian Ap-homolog Lhx2. This DNA sequence has been shown to bind *Drosophila* Ap in S2 cells (Rincon-Limas

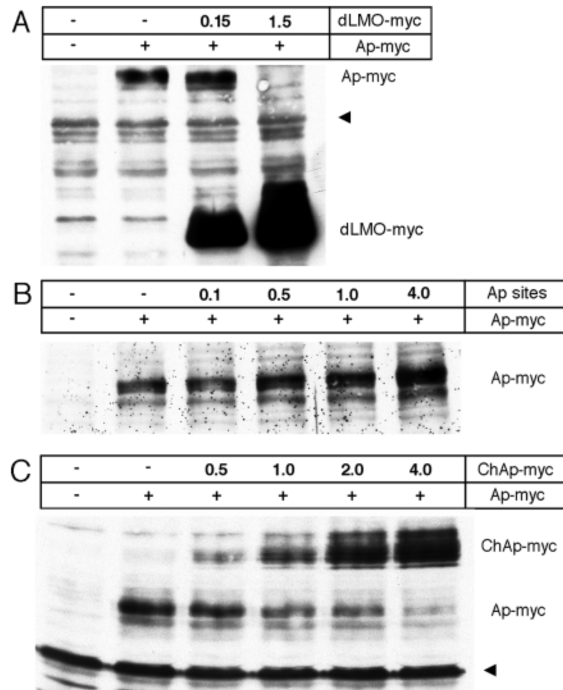

Fig. 5. Complex formation with DNA and Chip stabilizes Ap protein. (A) Immunoblot of S2 cell lysates transfected with constructs to express myc-tagged Ap and myc-tagged dLMO. Cells were transfected with a constant amount of Ap-myc (+) and increasing amounts of dLMO-myc as indicated. Total levels of transfected DNA were held constant using empty vector to compensate for alteration in the level of dLMO-myc plasmid. Both proteins were visualized with anti-myc. The arrowhead indicates a nonspecific band to serve as a loading control. (B) Immunoblot of S2 cell lysates transfected with a plasmid to express myc-tagged Ap and with plasmids containing additional Ap-binding sites. Cells were transfected with a constant amount of Ap-myc (+) and increasing amounts of plasmid containing additional Ap-binding sites (competitor). Total DNA levels were held constant in the transfection by addition of empty vector. Ap protein levels increased with increasing copies of the binding site construct. (C) Immunoblot of S2 cell lysates transfected with constructs to express myc-tagged Ap and myc-tagged ChAp. Cells were transfected with a constant amount of Ap-myc (+) and increasing amounts of ChAp-myc as indicated. Both proteins were visualized with anti-myc. Increasing amounts of ChAp decreased levels of Ap-myc protein. We tested whether the endogenous dLMO gene was induced in ChAp transfected cells by immunoblotting with anti-dLMO, and were unable to detect it (not shown). The level of dLMO that was needed to reduce Ap-myc protein in S2 cells was readily detectable by immunoblotting, so we conclude that the effect of ChAp-myc was not due to increased levels of dLMO.

et al., 2000). The total amount of DNA in the transfection assay was held constant by varying the ratio of plasmid containing the binding sites and empty vector. Increasing the ratio of the plasmid containing the binding sites resulted in dose-dependent stabilization of Ap-myc protein (Fig. 5B). This observation supports the idea that availability of binding sites limits the amount of Ap protein that is stable in the cell when mRNA levels are held constant.

Another means to test this possibility is by competition between Ap and a related protein for a fixed number of binding sites. For these experiments we made use of a fusion protein between Chip and Ap (called ChAp). In this protein the dimerization domain of Chip mediates dimerization of the DNA-binding domains of Ap (illustrated in Fig 8C). Thus, ChAp dimers should compete with endogenous Chip:Ap tetramers for DNA-binding sites. Use of the Myc tag versions of both proteins allowed direct comparison of their relative levels in co-transfected cells. Using this assay we verified that increasing the level of ChAp-myc decreased the level of co-transfected Ap-myc in a dose-dependent manner (Fig. 5C). Expression of Chip-myc as a control had little effect on Ap-myc levels (not shown). Note that the level of Ap-myc construct was held constant in all samples. ChAp-myc and Chip-myc expression levels were controlled by varying the ratio of the expression constructs to the empty expression vector in the transfections.

We next asked whether competition for DNA-binding sites would affect Ap stability in the wing disc. Fortuitously, the antibody raised against Ap does not recognize ChAp. This allows us to measure the level of the endogenous Ap protein in cells expressing ChAp. ChAp expression under dpp^{Gal4} control led to a decrease in the level of endogenous Ap protein (Fig. 4C). Together, these observations suggest that Ap protein is unstable in vivo unless bound to DNA as part of an active complex with its co-factor Chip (Fig. 8A; for simplicity, the illustration depicts selective degradation of Ap monomers; it is equally plausible that Ap:Chip complexes of any stoichiometry are more susceptible to degradation when they are not bound to DNA).

Intrinsic differences in the LIM domains of dLMO and Ap

dLMO has been proposed to act as a competitive inhibitor of Ap in vivo (Milán and Cohen, 1999; van Meyel et al., 1999). This model suggests that overexpression of Ap should be able to produce phenotypes similar to those caused by reduced levels of dLMO; however, this has not been observed. Overexpression of Ap in its endogenous domain does not produce alterations in the wing comparable with those caused by loss of dLMO activity. By contrast, expression of fusion proteins between Chip and Ap, which are insensitive to repression by dLMO, produce the expected phenotypes (Milán and Cohen, 1999; van Meyel et al., 1999). This suggests that Ap does not compete effectively with dLMO for interaction with Chip, even when overexpressed. These observations could be explained by an intrinsic difference in the affinities of the two LIM domain proteins for Chip. To test this possibility, we constructed a fusion protein that contains the LIM domains of dLMO (100 amino acids) but otherwise consists entirely of Ap sequences (Fig. 6A). We call this protein dLAp to indicate LIM-domains of dLMO in Ap. To distinguish dLAp from endogenous Ap and dLMO proteins, a C-terminal flag tag was included.

Two assays were performed to test the functionality of dLAp. First, we made use of the fact that ap^{Gal4} is a mutant allele of *ap*. ap^{Gal4}/ap^{UGO35} larvae have reduced *ap* activity and fail to develop normal wings. Expression of Ap protein under ap^{Gal4} control rescues wing development in this mutant combination (O'Keefe et al., 1998). Expression of dLAp was able to replace endogenous *ap* and rescue the ap^{Gal4}/ap^{UGO35} mutant (Fig. 6C,D). Second, ectopic expression of dLAp induced the formation of an ectopic DV boundary, as revealed

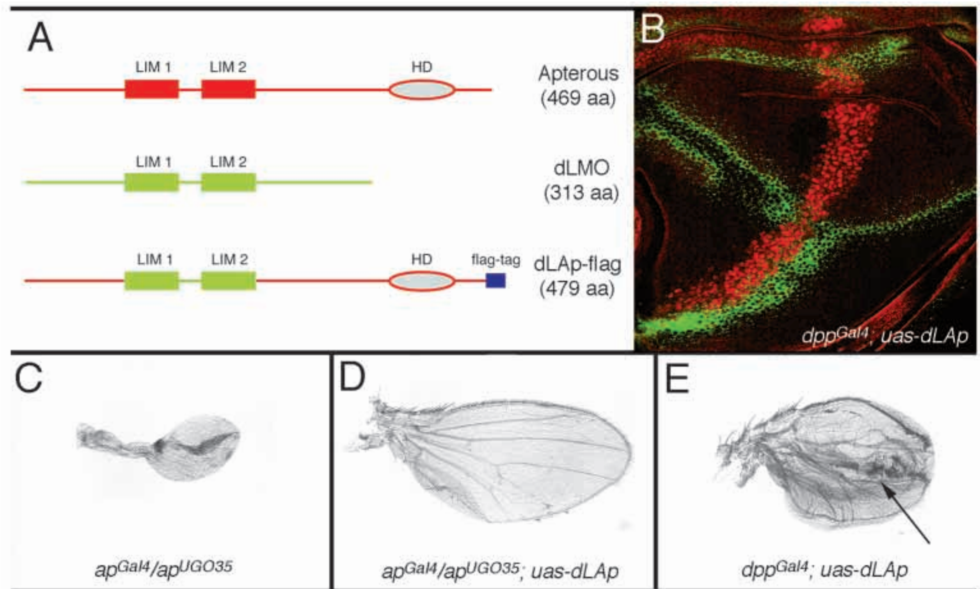

Fig. 6. The Ap-dLMO fusion protein dLAp is functional. (A) Domain organization of Apterous, dLMO and dLAp-flag. For the fusion protein dLAp-flag, the LIM-domains of Apterous were replaced by the LIM-domains of dLMO. In addition, dLAp contains a C-terminal flag-tag to make it distinguishable from the endogenous Ap and dLMO proteins. (B) Confocal image of a third instar wing imaginal disc of the genotype *dpp*^{Gal4}*; uas-dLAp-flag* stained with anti-Ap (red) and anti-Wg (green). Endogenous Ap protein barely detectable as faint red label in the dorsal compartment. The intense red stripe reflects overexpression of dLAp in the *dpp*^{Gal4} domain. The Wg stripe follows the border between dLAp-expressing and non-expressing ventral cells. (C) Cuticle preparation of an *ap*^{Gal4}*/ap*^{UGO35}wing. The heteroallelic combination *ap*^{Gal4}*/ap*^{UGO35} shows strongly reduced Ap activity but retains Gal4 expression in the Ap domain. (D) Cuticle preparation of an *ap*^{Gal4}*/ap*^{UGO35}*; uas-dLAp-flag* wing. Expression of the uas-dLAp-flag transgene in this domain is able to support wing development, indicating that it can provide Ap function. (E) Cuticle preparation of a *dpp*^{Gal4}*; uas-dLAp-flag* wing. Overexpression of dLAp along the AP boundary by *dpp*^{Gal4} leads to the formation of ectopic wing margin in the ventral compartment (arrow).

by ectopic wing margin formation and ectopic Wingless expression (Fig. 6E,B). Ectopic Wingless is induced wherever Ap-expressing cells and non-expressing cells are juxtaposed (Diaz-Benjumea and Cohen, 1993; Milán et al., 1998). These results indicate that dLAp has Ap activity.

To ask whether overexpression of dLAp in dorsal cells would compete effectively with dLMO to produce a net increase in Ap activity levels, we compared wing development in flies expressing dLAp or Ap under *ap*^{Gal4} control. Ap overexpressing wings were normal (not shown). In *ap*^{Gal4}*/+; uas-dLAp/+* flies we observed defects in wing veins, especially in the posterior crossvein and vein 5, and a held up wing phenotype (Fig. 7A,B). These defects resemble the *dLMO* mutant phenotype, which has been shown to be due to excess Ap activity (Shoresch et al., 1998; Milán et al., 1998). Another feature of *dLMO* mutant wings is overexpression of Serrate in the D compartment. Overexpression of wild-type Ap under *ap*^{Gal4} control did not cause abnormal Serrate expression (Fig. 7C); however, expression of dLAp in *ap*^{Gal4}*/+; uas-dLAp/+* wing discs induced ectopic Serrate in the dorsal compartment and caused mild reduction of the D compartment (Fig. 7D). Similar, though somewhat stronger effects were obtained by

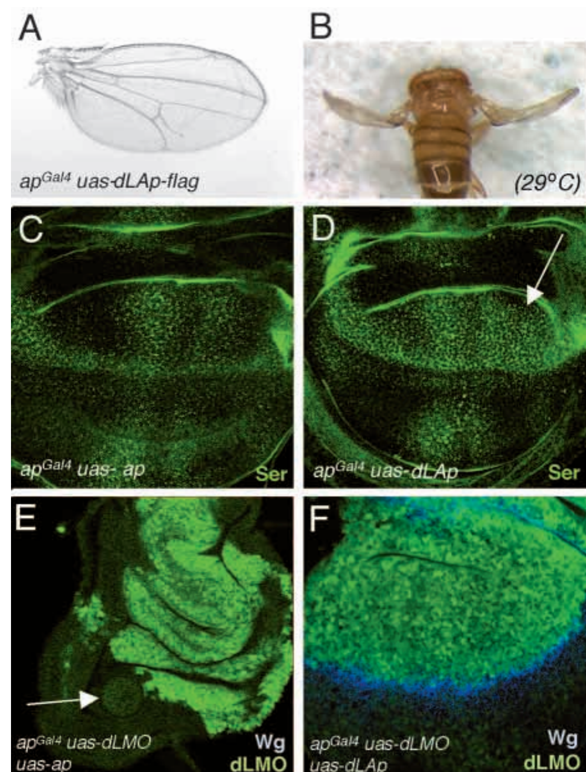

Fig. 7. Overexpression of dLAp-flag causes an *ap* gain-of-function phenotype. (A) Cuticle preparation of an *ap*^{Gal4}*; uas-dLAp-flag* wing. Overexpression of dLAp-flag caused extra vein tissue between veins 4 and 5 and other vein defects. (B) *ap*^{Gal4}*; uas-dLAp-flag* fly showing the abnormal wing posture and upward curvature of the wing caused by the reduced size of the D compartment. (C) Serrate protein expression (green) in a disc overexpressing *uas-ap* in the *ap*^{Gal4}domain. The pattern of Serrate staining is the same as in wild-type discs and shows elevated expression along veins 3 and 4 and on both sides of the DV boundary. (D) Expression of *uas-dLAp-flag* caused overexpression of Serrate in the D compartment. Ectopic Serrate staining can be seen in intervein regions (e.g. arrow). Note the reduced size of the D compartment. (E) dLMO and Wg protein expression in an *ap*^{Gal4}*; uas-dLMO; uas-ap* third instar wing disc. Anti-dLMO (green) and Anti-Wg (blue). Note the small wing pouch (arrow) and the absence of Wg expression at the interface between D (green) and V (not green) cells. (F) dLMO and Wg protein expression in an *ap*^{Gal4}*; uas-dLMO; uas-dLAp* third instar wing disc. Note that Wg expression is restored along the DV boundary and growth of the wing pouch is restored.

overexpression of the Chip/Ap fusion protein ChAp, which is insensitive to competition by dLMO (Milán and Cohen, 1999). Thus, dLAp expression can increase Ap activity to levels above normal in the presence of dLMO.

Ap activity can be abolished by overexpression of dLMO under ap^{Gal4} control in the wing disc. Providing additional Ap protein by co-expression of Ap did not overcome the inhibitory effects of dLMO. Wingless was not expressed at the interface between D and V cells and the wing pouch was very small (arrow, Fig. 7E). By contrast, co-expression of dLAp restored Wingless expression along the DV boundary and wing pouch growth (Fig. 7F). This indicates that dLAp is able to restore Ap activity in the presence of dLMO. Taken together, these observations indicate that dLAp competes efficiently with dLMO for binding to Chip, whereas Ap does not (illustrated in Fig. 8D). As the only difference between Ap and dLAp is in the LIM domains, we attribute their different behavior to an intrinsic property of the LIM domains.

Competition for Chip in formation of different complexes

In this report we have addressed the problem of asymmetry in the competition between dLMO and Ap. The simplest model for competitive inhibition by dLMO would suggest that Ap should compete effectively with dLMO for binding to Chip when overexpressed. However, overexpression of Ap does not produce an excess of Ap activity. dLMO competes effectively for Ap activity, but the reverse is not true. Our finding that swapping the LIM domains of Ap for those of dLMO produces a functional Ap protein that is able to compete effectively with dLMO may provide an explanation for the non-reciprocal properties of Ap and dLMO. We attribute the effectiveness of dLMO as an inhibitor of Ap activity to an intrinsic difference in the ability of the LIM domains of these two proteins to bind to Chip. We consider it likely that the LIM domains of dLMO bind the LID of Chip with higher affinity than the LIM domains of Ap. However, we have not been able to produce these proteins in soluble form at adequate concentrations and so were unable to determine the affinities of these binding interactions directly.

Other proteins might also contribute to stabilization of Chip-dLMO complexes or to destabilization of Chip-Ap complexes in vivo. Interactions involving Ap, Chip and other proteins have been reported. For example, Pannier interacts with Chip and competes with Apterous for patterning of the thorax (Ramain et al., 2000). In this model, Chip is found in a complex with Pannier and dLMO, which promotes dorsal thorax formation. Chip is also found in a complex with Ap. The level of Chip is not in great excess, so competition occurs between Ap and Pannier for formation of Chip complexes, despite the fact that Pannier and Ap do not bind to Chip in the same way. We noted that overexpression of dLAp-flag appears to interfere with Pannier complex formation, because it causes the formation of a cleft in the thorax, resembling a *pannier* loss-of-function mutant phenotype (data not shown). Comparable overexpression of Ap does not do so. This suggests that dLAp competes more effectively than Ap for binding to Chip and so is more effective at sequestering Chip from Pannier-containing complexes. The relative affinity of these proteins appears to play an important role in maintaining the proper balance of complex formation in vivo. Numerous LIM-HD proteins have been found to play important roles in development of a number of species (Hobert and Westphal, 2000). It seems likely that other LIM-homeodomain transcription factors will be regulated in similarly complex ways.

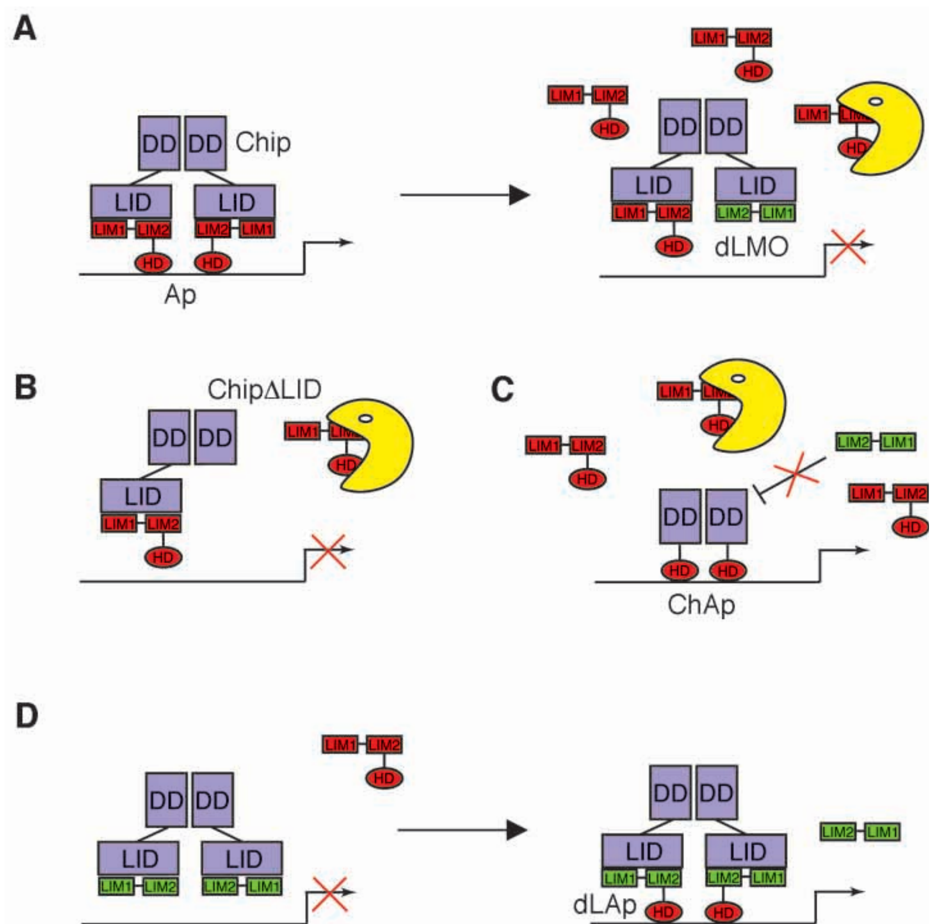

Fig. 8. Model. (A) Ap activity regulation under wild-type conditions. Once dLMO is expressed, Ap detaches from Chip and is degraded. Transcriptional activation of its target genes is abolished. For simplicity we depict degradation of Ap monomer. It is also possible that any form of incomplete Ap;Chip complex is degraded. (B) Inhibition of Ap:Chip tetramer formation by ChipΔLID. (C) ChAp binds to Ap target sites on DNA and displaces endogenous Ap. dLMO cannot interfere. Ap is degraded. (D) Inhibition of Ap activity by dLMO can be reverted by providing the fusion protein dLAp. dLAp competes with dLMO and activates Ap-dependent transcription.

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