A Unique Protection Signal in Cubitus interruptus Prevents Its Complete Proteosomal Degradation†∗

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The limited proteolysis of Cubitus interruptus (Ci), the transcription factor for the developmentally and medically important Hedgehog (Hh) signaling pathway, triggers a critical switch between transcriptional repressor and activator forms. Ci repressor is formed when the C terminus of full-length Ci is degraded by the ubiquitin-proteasome pathway, an unusual reaction since the proteasome typically completely degrades its substrates. We show that several regions of Ci are required for generation of the repressor form: the zinc finger DNA binding domain, a single lysine residue (K750) near the degradation end point, and a 163-amino-acid region at the C terminus. Unlike other proteins that are partially degraded by the proteasome, dimerization is not a key feature of Ci processing. Using a pulse-chase assay in cultured Drosophila cells, we distinguish between regions required for initiation of degradation and those required for the protection of the Ci N terminus from degradation. We present a model whereby the zinc finger region and K750 together form a unique protection signal that prevents the complete degradation of Ci by the proteasome.

The Hedgehog (Hh) family of secreted signaling molecules plays an important role in developmental patterning and organogenesis in animals from Drosophila to human (9), including anterior-posterior patterning of Drosophila appendages and vertebrate limbs, dorsal-ventral patterning of the vertebrate spinal cord, and the regulation of stem cell self-renewal versus differentiation in many contexts. Mutation of components of the Hh pathway can cause inherited developmental disorders, and inappropriate activation of the pathway later in life can lead to initiation or maintenance of many common cancers (34).

Hh acts primarily by regulating gene expression in target tissues. In Drosophila, Hh signaling is mediated by the zinc finger transcription factor Cubitus interruptus (Ci) (9). Ci activity is regulated in multiple ways. In the absence of Hh, full-length Ci (Ci-155) is processed by limited proteolysis, leaving the N terminus (Ci-75) intact (1). Ci-75 acts as a repressor of some Hh-target genes (1, 19). In the presence of Hh, this processing is blocked, and Ci-155 accumulates and is further activated to become a more potent transcriptional activator (19, 22). Gli2 and Gli3, two vertebrate homologues of Ci, are proteolyzed in a similar manner to Ci (27, 43), yielding in the case of Gli3 a repressor that is important in patterning the limb and neural tube (29, 33).

Several lines of evidence suggest that Ci-155 is processed by the proteasome to yield Ci-75. First, proteasome inhibitors prevent Ci-75 formation in cultured Drosophila wing disc cells (3). Second, supernumerary limbs (Slimb), Cullin 1, and Roc1a, components of an SCF E3 ubiquitin (Ub) ligase (SCFSlimb), are required for Ci processing (11, 21, 25). Slimb binds directly to Ci but only when Ci has been phosphorylated on multiple sites by protein kinase A (PKA), glycogen synthase kinase 3 (GSK3), and casein kinase I (CK1), explaining the requirement of these kinases and their phosphorylation sites in Ci for Ci processing (5, 10, 38). A mutant in which these phosphorylation sites are replaced with a single high-affinity Slimb binding site is processed normally to Ci-75 (10, 38), suggesting that Ci is a direct target of ubiquitination. Kinesin-like microtubule binding protein Costal-2 (Cos2) is also required for Ci-75 formation. It is thought to act as a scaffold for Ci and the kinases and is required for the efficient phosphorylation, and thus proteolysis, of Ci in vivo (37, 51). In addition to this partial degradation of Ci, activated full-length Ci is also the target of complete degradation by the proteasome, mediated by an E3 Ub ligase containing Cullin 3 (Cul3) and roadkill/Hh-induced MATH and BTB-containing protein (HIB) (13, 25, 50).

The role of the Ub-proteasome system in the limited proteolysis of Ci is surprising since this pathway usually leads to complete degradation of substrates. However, several exceptions besides Ci have been reported, including the p105 precursor to the NF-kB p50 subunit and its two distant homologues in Saccharomyces cerevisiae, MGA2 and SPT23 (8, 26, 32). Each of these proteins contains in its N-terminal half an immunoglobulin-like plexin transcription factor (IPT) domain, which mediates dimerization and in NF-kB is part of the larger Rel homology domain (RHD). Like Ci, the IPT-containing proteins are processed in a way that leaves the N-terminal half of the protein intact.

Several regions of these IPT proteins are important for processing. A short glycine-rich region (GRR) in p105 located between the RHD and the C-terminal end of p50 is required for p50 formation (17). Originally, the GRR was proposed to be a signal for the initiation of an endoproteolytic cleavage event, based on the detection of a stable C terminus in some
model substrates (17), but was later suggested to form a “stop” signal that protects p50 from degradation (23). The IPT dimerization domain is also required for processing of p105 (16) and SPT23 (32). The substrate for processing is thought to be a dimer of full-length molecules (in the case of SPT23 and MGA2) or of nascent polypeptide chains being synthesized by a polysome (in the case of cotranslational production of p50 from p105), leaving a heterodimer of a full-length molecule and the short form as the product (16, 32).

A clearer understanding of the mechanism of partial proteolysis came with a study by Matouschek and coworkers (42). Their in vitro assays led to a model whereby a tightly folded domain (like the RHD) and a sequence of low complexity (like the GRR) together form a protection signal that prevents further progress by the proteasome. Additionally, they suggest that the Ci zinc fingers fill the role of the tightly folded region and that a region encompassing the degradation end point (amino acids 612 to 760), previously shown to be required for processing, fills the role of the simple sequence in Ci (42).

We have examined more closely the mechanism of Ci processing and whether Ci-75 formation takes place with a mechanism similar to that of the IPT-containing proteins. In this paper, we use delletion analysis to show that several regions of Ci are required for correct processing in addition to the previously characterized phosphorylated region: at least one of two Cos2 binding sites (though a small amount of cleavage occurs in the absence of both sites), a 163-amino-acid region at the very C terminus of Ci, the zinc finger region, and a 48-amino-acid region near, but C-terminal to, the degradation end point. We find no evidence that the zinc fingers are required as a dimerization domain in Ci processing, but, instead, they are required because of their tightly folded tertiary structure. We also show that the block on Ci-75 formation by deletion of the 48-amino-acid region can be mimicked by mutation of a single residue (K750), and we postulate that it is a ubiquitination site required for limited proteolysis. Using a pulse-chase assay in Kc cells to distinguish between mutants that do not form Ci-75 because they are completely degraded or because they are not the target of the proteasome at all, we show that, together, the zinc finger region and K750 form a protection signal that prevents the complete degradation of Ci by the proteasome and leads to formation of Ci-75.

MATERIALS AND METHODS

DNA constructs. All Ci deletion mutants and fusions used in this work were derived from the plasmid pGEMCi wild type by using existing restriction sites or by introducing convenient restriction sites or by introducing convenient restriction sites by PCR. Constructs were subcloned into pUAST (a pCaSpeR3-based vector with five tandem GAL4-upstream activation sequence [UAS] sites upstream of the hsp70 TATA box) with three copies of HA. For pCaSpeR3-based vector with five tandem GAL4-upstream activation sequence inserted cDNAs; alternatively, constructs were subcloned into pUAST (a (HA) epitope tag or six copies of the epitope tag at the N terminus of

300 μl of extract buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1 mM EDTA) supplemented with protease inhibitors (Complete Mini; Roche). For dimerization assays, cells were lysed with 300 μl of Harlow buffer (4) supplemented with protease inhibitors. The lysates were then centrifuged at a relative centrifugal force of 13,000 for 15 min at 4°C.

RNA interference (RNAi) in Kc cells was performed as described previously (48). The double-stranded RNAs (dsRNAs) used were the following (numbers indicate the nucleotides starting from ATG): EGFP, 1 to 720; Coe2, 1 to 3600; Slimb, 1 to 1533; and Cul3, 579 to 1072.

IP and Western blotting. Lysates from Kc cells were incubated with 1.4 μg of anti-HA antibody (12CA5; CRUK monoclonal antibody service) or 1.4 μg of anti-myc antibody (9E10; Santa Cruz) for 1 h at 4°C. Samples were combined with 25 μl of protein G-Sepharose beads and incubated for 2 h on a rocker at 4°C. Beads were washed three times with 500 μl of extract buffer and then boiled in 30 μl of sodium dodecyl sulfate loading buffer. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% or 12% gels, transferred to polyvinylidene difluoride membranes, blotted with the primary antibody for 2 h and with the secondary antibody for 1 h (for anti-myc blots), and then visualized by ECL Plus (Amersham Life Science). Primary antibodies and dilutions used were hereditary peroxidase-conjugated 3F10 antibody (1:1500), Roche) and 9E10 antibody (1:250). The in vivo ubiquitination assay was performed as described previously (44), using pMT-HAUb (provided by Isaac Edery, Rutgers University). Band intensities were quantified from autoradiographs of Western blots using ImageJ software (NIH).

Drosophila experiments. For embryo extracts, transgenic females with wild-type or mutant Ci under the control of GAL4-UAS sites were crossed to ptc females. Embryos were collected for 3 h on apple juice plates at room temperature and then incubated for 5 h at 29°C to obtain stage 9 to 13 embryos. Extracts were prepared as described previously (30) and used directly in Western blot analysis. Wing disc experiments were done as described previously (31).

Luciferase reporter gene assays. Cells in 24-well plates were cotransfected with 0.04 μg of ptc-luciferase (3), 0.04 μg of copia-Remilia (18), 0.1 μg of ACTCi (wild-type or mutants), and 0.02 μg of either actin 5C (Act5C)-Hs or act5C empty vector. After 48 h, cells were harvested, and luciferase activity was measured using a Dual Luciferase Reporter assay system (Promega). Firefly luciferase activities were normalized to cotransfected Remilia activities and are shown as a relative value to that of the unstimulated wild-type Ci (set to 100).

CHX chase experiments. Cycloheximide ([CHX] final concentration, 10 μM; Calbiochem) was added to cells 40 h after transfection. Subsequently, approximately 4 × 10⁶ cells were harvested at each time point. Cell lysates were prepared in 60 μl of extract buffer, and Western blotting was performed on 5 to 7 μl of lysate. The half-lives of proteins were calculated by linear regression analysis of log of the total density per band against time.

Mathematical modeling of the generation of Ci-75. A mathematical model of the amount of Ci-75 in CHX chase assay was built based on the following assumptions: (i) Ci-75 has the same half-life as Ci612Stop (Ci mutant encoded by a gene with a stop codon introduced after the codon for amino acid 612); (ii) the degradation of Ci-155 and Ci-75 follows exponential decay; (iii) Ci-155 can be processed to Ci-75 or completely degraded. At any given time t, the amount of Ci-75 equals:

\[ x_T = \frac{A_k e^{-k_1 t}}{k_2} + B e^{-k_2 t} \]

where \( x \% \) is the percentage of Ci-155 that is processed to Ci-75, \( A_k \) is the starting amount of Ci-155, \( k_1 \) is the rate constant of Ci-155 degradation, \( B \) is the starting amount of Ci-75, \( k_2 \) is the rate constant of Ci-75 degradation, and \( k_1 \) does not equal \( k_2 \).

RESULTS

Kc cells are a good model for Ci-75 formation. First, we further characterized Kc cells as a model for studying CI proteolysis. We used an immunoprecipitation (IP)-Western blotting assay with transiently transfected Ci that has a triple HA tag at the N terminus (HACi) so that both Ci-155 and Ci-75 can be detected. Using a similar assay, it was previously shown that Ci-155 is processed to Ci-75 in Kc cells and that this limited proteolysis is dependent on the PKA phosphorylation sites in the C-terminal half of Ci (4) (Fig. 1A and B, lanes 1 to 3). Similar to what is found in Drosophila embryos and wing
discs (31), the GSK3 and CKI sites primed by three of the five C-terminal PKA sites (Fig. 1A) are also required for Ci-75 formation: neither CiNm (in which the two GSK3 sites have been changed to alanine) nor CiCm (in which the three CKI sites have been changed to alanine) are cleaved to form Ci-75 in Kc cells (Fig. 1B, lanes 4 and 5).

It has been shown in *Drosophila* that phosphorylation and proteolysis of Ci require Cos2 (37, 51). Consistent with this, we find that Cos2 RNAi blocks Ci-75 formation (Fig. 1C, lane 3). Cos2 binds directly to two domains in Ci, one N-terminal to the zinc fingers (amino acids 346 to 440) and the other in the C-terminal half of Ci (amino acids 941 to 1065) (45, 46), though Ci-75 formation in mutants lacking these domains has never been tested. Deletion of either the N-terminal Cos2 binding site (along with amino acids 1 to 345; Ci/H9004N) or the C-terminal Cos2 binding site (Ci/H9004Cos2) had only a small effect on processing (Fig. 1D, lane 1, and 2B, lane 7). However, when both Cos2 binding sites are deleted (Ci/H9004 Δ Cos2), Ci-75 formation is significantly reduced (Fig. 1D, lane 3). These results suggest that the Cos2 binding sites act redundantly to regulate Ci proteolysis as well as Ci subcellular localization (45, 46).

Multiple regions of Ci are required for its processing. Next, we determined which regions of Ci are required for Ci-75 formation. Previously, a deletion in Ci spanning the presumptive degradation end point (amino acids 612 to 760; referred to as Ci/H9004Δ612–760 or Ci/H9004Δ) was shown to abolish cleavage in Kc cells and in vivo, while a smaller deletion (amino acids 612 to 712) reduced cleavage but did not completely block it (19).
We tested whether the small region of difference between these two deletions (amino acids 712 to 760) was responsible for the difference in processing of the two mutants. We find that Ci/H9004712-760 is not processed to Ci-75 in Kc cells (Fig. 2A, lane 4), suggesting that elements critical for cleavage are located within this short 48-amino-acid region located C-terminal to the degradation end point (i.e., in the part of Ci that is degraded).

Deletion of the region from amino acid 761 to 829, just N-terminal to the phosphorylated region (Ci/H9004761-829), has no effect on Ci processing (Fig. 2B, lane 6), nor do small deletions adjacent to the zinc fingers (Ci/H9004Δ442-452 and Ci/H9004Δ605-611) (Fig. 2B, lanes 3 and 5). However, deletion of amino acids 1067 to the C terminus of Ci (Ci/H9004Δ1067C) blocks Ci-75 formation almost completely (Fig. 2B, lane 8). We therefore divided this region into two smaller deletions, CiΔ1067-1234 and CiΔ1235C; amino acids 1067 to 1234 are dispensable for Ci-75 formation, but amino acids 1235 to 1397 are required (Fig. 2C, lanes 1 to 4).

Next, we examined the role of the five Zn finger DNA binding domains by deleting either zinc finger 1; zinc finger 2; zinc fingers 1 and 2 together; zinc fingers 3, 4, and 5 together; or all five zinc fingers. We find that Ci-75 is not formed in any of these mutants (Fig. 2B, lane 4, and D, lanes 3 to 6), in agreement with previous work suggesting that the zinc finger region is important for Ci-75 formation (42).

In summary, our data show that, in addition to the PKA, GSK3, and CKI phosphorylation sites in the C-terminal half of Ci/H9004, multiple domains of Ci are required for its processing.

FIG. 2. Multiple domains of Ci are required for its processing. (A) Western blot of immunoprecipitates from Kc cells transfected with HACi wild-type (lane 1) and the indicated HACi mutants. In this and subsequent panels, the ratio of processed form to full-length form, normalized to that of HACi, is shown in the box below the lane numbers. (B) Western blot of immunoprecipitates from Kc cells transfected with HACi wild type (lane 1) and the indicated mutants. (C) Western blot of immunoprecipitates from Kc cells transfected with HACi wild-type (lane 1) and the indicated mutants. (D) Western blot of immunoprecipitates from Kc cells transfected with HACi wild type (lane 1), HACi3m (lane 2), and HACi with deletions of the indicated zinc finger(s): ΔZn1, deletion of zinc finger 1; ΔZn2, deletion of zinc finger 2; ΔZn12, deletion of zinc fingers 1 and 2; ΔZn345, deletion of zinc fingers 3, 4, and 5. (E) Schematic drawing of the domains required for Ci proteolysis, with green indicating domains not required for Ci-75 generation, red indicating domains absolutely required for Ci-75 generation, yellow indicating domains required for the optimal processing, and orange indicating the domains together are required for optimal Ci-75 generation.
Ci, multiple regions of Ci are absolutely required for Ci-75 formation: the zinc finger DNA binding domain, amino acids 612 to 760, and amino acids 1235 to 1397. At least one of the two Cys2 binding domains and the previously characterized amino acids 612 to 712 (19) are required for optimal cleavage.

**Dimerization is not a determinant in Ci-75 formation.** Because Gli2 and Gli3 dimerize via their first two zinc fingers (20) and because dimerization is required for processing of IPT-containing proteins, we tested whether the reason the zinc fingers are important for Ci processing is their potential ability to mediate dimerization. We replaced the zinc fingers with either the GAL4 dimerization domain (CiZGAL4) (7) or enhanced green fluorescent protein (EGFP) A206K (a mutant form which prevents the weak dimerization of wild-type EGFP [CiZEGFP206K]) (49) (Fig. 3A) and coexpressed myc- and HA-tagged versions of these mutants in Kc cells. Using a co-IP assay, we find that the GAL4 substitution mutant homodimerizes, but neither the EGFP A206K fusion protein nor wild-type Ci shows detectable homodimerization (Fig. 3C, top panel; compare lane 3 with other lanes). In the proteolysis assay, CiZGAL4 does not form a Ci-75-like product (Fig. 3B, lane 3, and C, lane 3, middle and lower panels), but the monomeric CiZEGFP206K mutant leads to strong formation of a Ci-75-like band of the expected size (Fig. 3B, lane 4, and C, lane 1, middle and lower panels). These results suggest that dimerization is not a critical factor for formation of Ci-75 and that EGFP is providing a function for processing that the GAL4 dimerization domain cannot.

**Tightness of folding of N-terminal domain correlates with Ci-75 production.** Because dimerization does not appear to be instrumental for Ci processing, the zinc finger region might act as a tightly folded domain important for protection of the unproteolysed region, as previously suggested (42). This may explain the difference in processing between CiZGAL4 and CiZEGFP206K: the GAL4 dimerization domain consists of three short (12 residues or less) helices which form a coiled-coil (7), while EGFP folds as an 11-stranded beta barrel with a coaxial helix (24). To test whether the zinc finger domain is important because of its stable tertiary structure and to determine whether there is a threshold of folding stability for Ci-75 production, we made substitutions of the zinc finger region with wild-type and mutant forms of barnase (35), a bacterial RNase which has been used in many protein folding studies. A mutant in which the Ci zinc fingers were replaced by wild-type barnase (CiZBar) is processed to a Ci-75-like product, albeit to a lesser extent than wild-type Ci (data not shown). To boost the amount of Ci-75 formed, we cotransfected a constitutively active PKA catalytic subunit (mC*) with the barnase-Ci fusion proteins (Fig. 3D, lanes 1 to 5). When the PKA sites are changed to alanine in the wild-type barnase-Ci fusion (CiZBar3m), the cleavage product is no longer formed (Fig. 3D, lane 2), suggesting that CiZBar is processed similarly to wild-type Ci. Replacing the zinc fingers with three different barnase mutants that have free energies of unfolding, relative to wild-type barnase, of $\sim$1.66 to $\sim$3.44 kcal/mol (where negative numbers indicate greater stability than wild-type) showed that the amount of Ci-75-like product formed increases when the stability of the barnase mutant used in the fusion protein increases (Fig. 3D, lanes 1 and 3 to 5, and E). Our data definitively show that the salient feature of the Ci zinc fingers in Ci-75 formation is that they serve as a tightly folded domain. Our results also show that there is not a steep threshold in stability for this protection effect: the amount of N-terminal product formed is roughly directly proportional to the relative stability of this domain.

**Simple sequences contribute to, but are not absolutely required for Ci-75 formation.** The latest models for partial proteolysis by the proteasome suggest that, in addition to the tightly folded domain, the protection signal requires a simple, low-complexity sequence located between the well-folded domain and the degraded part of model substrates. In NF-kB p105 this simple sequence is a 37-amino-acid GRR. Amino acids 612 to 760 have been suggested to play the role of the simple sequence of Ci: deletion of this region blocks processing (19, 42) (Fig. 2A, lane 4), and replacement of this region by two copies of the GRR sequence from p105 rescued Ci-75 formation (42). The SEG algorithm (47) reveals two spans of sequence with low complexity (i.e., with complexity values less than 2.5) between amino acids 615 and 760: a serine-rich region (amino acids 657 to 670; complexity value, 1.95) and an acidic region (amino acids 699 to 712; complexity value, 2.26) (Fig. 4A). Deletion of either region alone (CiΔ612–689 or CiΔ690–712) or both regions (CiΔ612–712) only partially blocks Ci processing (Fig. 4B, lanes 3 to 5). These results suggest that the two simple sequences are required to achieve optimal processing of Ci-155 but are not absolutely required for Ci-75 formation. In contrast, although amino acids 712 to 760 do not contain any low-complexity sequences that can be identified by the SEG algorithm, this domain is absolutely required for Ci processing (Fig. 2A, lane 4, and 4B, lane 6), suggesting that something other than a simple sequence is an absolute determinant in the process of Ci-75 generation.

**K750 is required for Ci-75 formation in Kc cells.** If amino acids 712 to 760 are not of low complexity, why are they required for Ci-75 formation? This region contains a single lysine residue, the amino acid to which Ub is covalently attached by Ub-conjugating enzymes. We changed this lysine (K750) to arginine to determine whether it is necessary for Ci-75 formation. Two nearby lysines (K652 and K662) were changed to arginines as a control. Mutation of K652 and K662 together had little or no effect on Ci processing (Fig. 5A, lane 4), but mutation of K750 to arginine blocked Ci-75 formation (Fig. 5A, lane 3). Furthermore, the spacing between this lysine and the zinc finger region is important for processing: insertion of a 75-amino-acid lysine-less spacer after amino acid 712 blocked Ci-75 formation (Fig. 5B, Ci712Spacer).

In light of this result, we reexamined our interpretation of the results for the deletions of the simple sequences between amino acids 657 and 712 (CiΔ612–689, CiΔ690–712, and CiΔ612–712) because the distance between K750 and the zinc finger region is reduced in these mutants. Our conclusion that the simple sequences are not absolutely required for Ci-75 formation still holds, but are simple sequences required when K750 is at its naturally occurring distance from the zinc fingers? To determine this, we made substitutions of the simple sequences (amino acids 605 to 689 or amino acids 605 to 712) with the appropriate lengths of two different unstructured regions of normal sequence complexity to reestablish the wild-type distance between K750 and the zinc fingers without introducing simple sequences (Fig. 5C). We find that in one case, in
FIG. 3. A well-folded domain, but not dimerization, is required for Ci-75 formation. (A) Schematic representation of Ci and Ci-fusion proteins. (B) Western blot of immunoprecipitates from Kc cells transfected with the indicated HACi constructs. The filled arrowhead indicates full-length proteins, and the open arrowhead indicates Ci-75 or corresponding processed products. The ratio of processed form to full-length form, normalized to that of HACi, is shown in the box below the lane numbers. (C) Western blot of immunoprecipitates (top two panels) or lysates (bottom panel) from Kc cells expressing the indicated proteins. Antibodies used for Western blotting (WB) and IP are shown to the left of the panels. The filled arrowheads indicate full-length proteins. The open arrowheads indicate Ci-75 or corresponding processed products. (D) Western blot of immunoprecipitates from Kc cells transfected with mC* and the following HACi chimeras in which the zinc fingers have been replaced by wild-type barnase or the indicated barnase mutant, with the folding stability relative to wild-type shown in parentheses: HACiZBar, wild-type barnase; HACiZBarAm, barnase with three mutations (ΔΔG\text{folding} = -1.66 kcal/mol); HACiZBarACm, barnase with five mutations (ΔΔG\text{folding} = -2.56 kcal/mol); HACiZBarABCm, barnase with six mutations (ΔΔG\text{folding} = -3.44 kcal/mol). (E) A plot of the relative folding strength of barnase mutants versus the ratio of processed product to the full-length form when the barnase mutants are substituted for the zinc finger region of Ci.
which 85 or 108 amino acids from the protein CG15031 (14) are substituted into Ci, no Ci-75 is formed (Fig. 5D, lanes 2 and 3). However, in the other case, in which 85 or 108 amino acids from mouse 4EBP1 (6) are substituted into Ci, Ci-75-like proteins are formed (Fig. 5D, lanes 4 and 5). These results suggest that when K750 is in its normal location, there are some additional sequence requirements for processing, but sequence complexity is apparently not the only determinant.

Finally, we tested whether any lysines near the usual location of K750 are required when two copies of the GRR are substituted for amino acids 615 to 760. When all the lysines near K750 (Ci residues 782, 792, 795, and 822) and an additional lysine introduced with the two copies of GRR in CiGRR are changed to arginine (CiGRRK5R), the amount of Ci-75-like protein formed is similar to the level of wild-type CiGRR (Fig. 5E, lane 4). However, in the other case, in which 85 or 108 amino acids from the protein CG15031 (14) are substituted into Ci, no Ci-75 is formed (Fig. 5D, lanes 2 and 3). Thus, we conclude that K750 is absolutely required for Ci-75 formation in vivo.

**K750 is required for Ci-75 formation in vivo.** Next, we tested whether the K750R mutant is processed in Drosophila, using transgenic flies expressing HACiK750R or HACiΔ712–760 (HACi wild-type and HACi3m lines [31] were used as positive and negative controls) under the control of GAL4-UAS sites. We crossed these flies with ptc-GAL4 to drive expression in Drosophila embryos in cells where Ci is usually expressed. Western blots on extracts from stage 9 to 13 embryos show that neither of these mutants is processed to Ci-75 in vivo (Fig. 6A, lanes 3 and 4), suggesting that amino acids 712 to 760 and K750 play the same role in Ci processing and leading to an increase in expression of target genes such as patched (ptc). Repressor formation in the A compartment away from the A/P border is necessary to repress hh and decapentaplegic (dpp) expression (9). To assay Ci mutants without interference from endogenous wild-type Ci, Ci transgenes are ectopically expressed in the P compartment. Hh signaling is active here, so smo mutant clones (marked by the absence of CD2) are induced to analyze Ci transgenes in cells with and without Hh signaling (19). Our results suggest that CiK750R is not processed in wing imaginal discs: in smo mutant P compartment cells ectopically expressing CiK750R, hh-lacZ expression is not repressed (Fig. 6D), while those ectopically expressing wild-type Ci do repress hh-lacZ (Fig. 6C). CiK750R behaves in this sensitive assay similarly to other Ci mutants that form very little or no Ci-75 (19, 31, 38, 39).

In a similar assay, we show that CiK750R is transcriptionally active only in response to Hh. Like wild-type Ci (Fig. 6E) and CiΔ172–760 (19), when CiK750R is ectopically expressed in the posterior compartment, ptc-lacZ is expressed in smo mutants, but not smo mutant cells (Fig. 6F). Ci phosphorylation site mutants, however, have some activity in the absence of Hh signaling (31). Finally, assays in Kc cells with an Hh-responsive reporter also show that the K750R mutant increases its transcriptional activity in response to Hh (Fig. 6B). Taken together, these results suggest that this mutation blocks Ci-75 formation but not Ci transcriptional activity in response to Hh.

**K750 may act as a polyubiquitination site.** We originally mutated K750 because lysine is the residue modified by Ub. Is K750 a site of ubiquitination? In the context of the full-length protein and in shorter fragments, mutation of this site had no discernible effect on Ci ubiquitination (data not shown) (Fig. 5F, compare lanes 2 and 5), suggesting that either K750 is not a ubiquitination site or that it is one of several, making the change in ubiquitination between wild-type and the K750R mutant undetectable. Slmb RNAi significantly blocks ubiquitination of both the wild-type Ci fragment and the K750R mutant (Fig. 5F, lanes 3 and 6), providing evidence that when K750 is in its normal location, there are some additional sequence requirements for processing, but sequence complexity is apparently not the only determinant.

**FIG. 4.** A simple, repetitive sequence is not absolutely required for Ci-75 formation. (A) Amino acid sequence of Ci from 605 to 760. The low-complexity sequences (according to the SEG algorithm with parameters 12/2.2/2.5) are highlighted in yellow, and the lysine K750 is highlighted in red. (B) Western blot of immunoprecipitates from Kc cells transfected with the indicated triple-HA-tagged proteins. The ratio of processed form to full-length form, normalized to that of HACi, is shown in the box below the lane numbers.
FIG. 5. A single lysine residue is necessary for Ci-75 formation. (A and B) Western blot of immunoprecipitates from Kc cells expressing the indicated HACi wild-type or mutant proteins. In panel A and subsequent panels, the ratio of processed form to full-length form, normalized to that of HACi, is shown in the box below the lane numbers. Ci712Spacer refers to Ci with an insertion of a 75-amino-acid lysineless spacer after amino acid 712. (C) Schematic representation of residues 453 to 760 of wild-type Ci and the corresponding region of the Ci fusion proteins that are used in panel D. Green, red, and yellow regions are as described in the legend of Fig. 2E. Blue indicates amino acids from CG15031; pink indicates residues from mouse 4EBP1. CiCG85Ins and CiCG108Ins refer to Ci with 85 or 108 amino acids, respectively, substituted from CG15031; Ci4EBP85Ins and Ci4EBP108Ins, Ci with 85 or 108 amino acids, respectively, substituted from mouse 4EBP1. (D) Western blot of immunoprecipitates from Kc cells expressing the indicated HACi wild-type or mutant proteins. The open arrowheads indicate the Ci-75-like processed.
that there are Slimb-dependent ubiquitination sites in Ci even when K750 is mutated.

As an alternative means of addressing whether K750 is a ubiquitination site, we replaced amino acids 713 to 760 of Ci (thus deleting K750) with either two or four in-frame copies of Ub G76V (Ci2xUb and Ci4xUb, respectively) (Fig. 5G). The Ub G76V mutant is resistant to cleavage by Ub C-terminal hydrolases (2, 12). It has been shown that four and, to a much lesser extent, two tandem copies of Ub G76V create high-affinity sites for Ub ligases that efficiently catalyze the formation of polyubiquitin chains (40). Ci4xUb forms a Ci-75-like protein in Kc cells (albeit smaller in size), while Ci2xUb forms very little (Fig. 5H, lanes 2 and 3), providing evidence that K750 may be a site of polyubiquitination required for formation of Ci-75. Processing of Ci4xUb to this Ci-75-like protein is blocked when the first two zinc fingers are deleted (Fig. 5H, lane 4), suggesting that it is processed similarly to wild-type Ci.

Inhibition of processing versus complete degradation.

There are at least two scenarios where particular Ci mutants will not form Ci-75. One occurs when the proteasome does not recognize the mutant as a target, and thus proteolysis is never initiated. We would predict this scenario, for example, for phosphorylation-site mutants, which do not bind Slimb and are thus not thought to be ubiquitinated. The other scenario oc-
curs when the proteasome initiates proteolysis and then completely degrades the mutant substrate instead of leaving the N-terminal portion intact. This scenario is predicted for mutants lacking all or part of the protection signal, which are otherwise good targets for the proteasome. In order to distinguish between these two scenarios for our uncleavable Ci mutants, we have developed a pulse-chase assay in Kc cells. After allowing tagged wild-type or mutant Ci to accumulate for 40 h, we treat the cells with CHX to inhibit new protein production. We then determine the amount of tagged protein after various time points (0 to 6 h). In the first scenario, we would expect the Ci mutant to have a longer half-life than wild-type Ci, while in the second scenario, the mutant will have a similar half-life to wild-type Ci.

First, we characterized wild-type Ci in this assay. Wild-type Ci-155 has a half-life of 1.9 ± 0.3 h in Kc cells (Fig. 7A) and is stabilized to a half-life of ~5 h by Slimb RNAi (Fig. 7B). We do not see the expected increase in Ci-75 levels as Ci-155 is processed (Fig. 7A). One possible reason for this is that Ci-75 has a short half-life and is thus degraded as fast as it is made. When we engineer a stop codon near the presumptive C-terminal end of Ci-75 (Ci612Stop), we find that this protein has a half-life of ~2.2 h (Fig. 7A). The short half-life of Ci-75 alone, however, cannot account for Ci-75 levels (Fig. 7A, purple dashed line). An additional reason for constant Ci-75 levels may be that only a fraction of degradation initiation events in Ci-155 leads to Ci-75 production, with the remaining leader to complete degradation. In fact, if only 20 to 40% of degradation initiation events lead to Ci-75 formation, Ci-75 levels would be roughly constant (Fig. 7A, green dashed line).

Next, we examined uncleavable Ci mutants in this assay. Ci3m has a much greater half-life than wild-type Ci (≥6 h), as predicted, since this mutant should not be a proteasome target (Fig. 7C). CiΔZincAll also has a long half-life (Fig. 7C), suggesting that it, too, is defective for initiation of degradation.

Surprisingly, Ci with a deletion of all five zinc fingers (amino acid residues 453 to 604 [CiΔZincAll]) has a half-life similar to Ci3m and CiΔZincAll (Fig. 7C) and thus apparently is not efficiently targeted by the proteasome, obscuring a possible role for the zinc fingers in protecting Ci-75 from proteasomal degradation. When we enhance initiation of degradation of CiΔZincAll by addition of mC* in order to uncover a potential role for the zinc finger region in the protection signal, we find that the half-life of the mutant is reduced to ~3 h (Fig. 7D) although no Ci-75 is formed (data not shown), consistent with a role for the zinc fingers in protecting the N terminus from complete degradation. Moreover, we find that the half-life of a destabilized zinc finger mutant, Ci CS80 585A, in which two zinc-binding residues in the fifth finger are altered, is similar to wild-type Ci (Fig. 7C) but forms no Ci-75 (similar to in vitro results [42]), suggesting that initiation of degradation is normal in this mutant and that the proteasome continues degrading the entire N terminus. Our results are consistent with the idea that the zinc finger domain is a protection signal and reveal a second role for the zinc fingers in initiation of proteasomal degradation.

Next, we used the pulse-chase assay to analyze CiK750R mutant. Since in-frame fusion of four copies of Ub, an effective polyubiquitination signal, can substitute for K750, it is possible that the K750R mutation blocks initiation of degradation. However, we find that CiK750R has a half-life similar to wild-type Ci-155 (~2 h) (Fig. 7C) even though no Ci-75 is formed. Like wild-type Ci, CiK750R is stabilized when Slimb is knocked down by RNAi (Fig. 7E). This result suggests that proteasomal degradation of CiK750R is initiated normally via Slimb-dependent ubiquitination sites but that degradation is complete; i.e., K750 is part of the protection signal that spares Ci-75 from complete proteasomal degradation.

Finally, in addition to using the pulse-chase assay to classify uncleavable Ci mutants, we have used it to further characterize the Ci-barnase fusion proteins. Like the CiΔZincAll mutant, the barnase substitution mutants have similar half-lives to Ci3m that are reduced to ~3 h when PKA activity is increased (Fig. 7F), not entirely surprising since these mutants are also lacking the zinc finger region (though see Discussion). Wild-type barnase and a stabilized mutant have the same half-lives (Fig. 7F) but generate different amounts of Ci-75 (Fig. 3D and E). The short forms of these Ci-barnase fusion proteins (made by introducing a stop codon near the presumed degradation end point) have nearly identical stability, regardless of the relative folding strength of the barnase domain (Fig. 7G). Together, these results suggest that proteolysis in the full-length Ci-barnase fusion proteins is initiated similarly and that the differences in the amount of Ci-75-like product formed is due to differing capacities to protect the N terminus from cleavage (owing to their different folding stabilities).

**DISCUSSION**

We have carried out a structure-function analysis of Ci to determine the mechanism of partial proteolysis of Ci by the proteasome. We demonstrate that several regions of Ci are required for Ci-75 formation and find that while Ci processing shares some features of partial proteolysis of the IPT-containing proteins NF-κB p105, SPT23 and MGA2, Ci has a unique protection signal. Specifically, we find the following: (i) a domain in the C terminus of Ci-155 is required for Ci-75 formation because it is required for initiation of degradation, (ii) in contrast to IPT-containing proteins, dimerization of Ci is not required for processing; (iii) the zinc finger region is required both for initiation of degradation of full-length Ci and for protection of Ci-75 from complete degradation; (iv) a tightly folded domain confers progressive protection on the N terminus of Ci rather than being a binary switch between complete and partial degradation; (v) unlike NF-κB p105, a low-complexity sequence is not an absolute requirement for Ci-75 formation, though it is required for optimal processing; and (vi) K750, a lysine required for the generation of Ci-75, acts not as a degradation signal but as part of the protection signal preventing degradation of Ci-75.

**Regions of Ci required for Ci-75 formation.** Our structure-function analysis, together with previous work, shows that a large portion of Ci is required for optimal processing, including at least one of two Cos2 binding sites, the zinc finger region, a lysine (K750) located near but C-terminal to the degradation end point, a phosphorylated region between amino acids 830 and 940, and a 163-amino-acid region at the C terminus (Fig. 2E). Our experiments show that in Ci processing, Cos2 acts primarily (though not exclusively) through two redundant...
FIG. 7. Determination of the half-lives of Ci and its mutants. (A) The half-lives of wild-type Ci-155 and Ci612Stop are determined by CHX chase in Kc cells (see Materials and Methods). Data points were plotted as the percentage of the amount of protein present at the beginning of the chase, except for Ci-75, where data points were plotted as the percentage of the amount of Ci-155 present at the beginning of the chase. The theoretical curves were calculated based on the assumption that either 30% or 100% of degradation initiation events generate Ci-75 (see Materials and Methods). (B and E) CHX chase of wild-type Ci-155 (B) and CiK750R (E) from Kc cells treated with the indicated dsRNAs. (C) CHX chase of wild-type Ci-155 and the indicated mutants. (D) CHX chase of wild-type Ci-155 and Ci\Delta ZnAll with or without coexpression of mC\* as indicated. (F) CHX chase of CiZBar, CiZBarABCm (CiZBar with six mutations in barnase), and CiZBar3m with coexpression of mC\*. (G) CHX chase of CiZBarStop and CiZBarABCmStop (CiZBar and CiZBarABCm, respectively, each of whose genes has a stop codon introduced after the codon for residue 612).
ZincAll, increasing PKA activity does not/H9004 wild-type Ci and Ci/roadkill, is not involved in Ci-75 formation (13, 50). Unlike of Ci, perhaps by binding to a factor required for one of these processes. The only protein known to bind this region, HIB/roadkill, is not involved in Ci-75 formation (13, 50). Unlike wild-type Ci and CiΔZincAll, increasing PKA activity does not appear to decrease the half-life of this mutant (data not shown) though the reason is unclear. It is also interesting that this region shares some homology with Gli family proteins (51% identity and 69% similarity to Gli3 in the final 39 amino acids) although this region of Gli3 is apparently not required for processing (28) and in amphioxus Gli is required for full transcriptional activity (36).

The half-life of a mutant lacking the zinc finger region shows that this region is also required for initiation of proteasomal degradation. It is not clear how the zinc fingers contribute to initiation of degradation though the fifth finger is not required for this activity since degradation is initiated normally in a mutant in which the fifth finger is destabilized. Increasing PKA activity decreases the half-life of CiΔZincAll, showing that this mutant can be phosphorylated and may have a defect other than poor phosphorylation. Intriguingly, we find that replacing the zinc finger region with other domains in some cases (the GAL4 dimerization domain [data not shown] and EGFPα206K) rescues the degradation initiation defect of CiΔZincAll but in other cases (barnase) does not. The domains that support normal degradation initiation do not have anything obvious in common that is lacking in barnase; thus, we do not understand the significance of this result.

Dimerization and a simple sequence are not part of the Ci protection signal. Current models for partial degradation by the proteasome, based largely on NF-κB p105 and model substrates, suggest that features important for the protection of the N terminus of substrates are the dimerization and/or tightness of folding of the RHD and a simple, low-complexity sequence, the GRR. We find that, unlike p105 and SPT23, dimerization is not a key feature in Ci-75 production. Because of the insensitivity of our assay, we cannot fairly conclude that Ci does not dimerize. However, the results with the GAL4 dimerization domain and monomeric EGFP substitution mutants clearly show that even if Ci does dimerize, this is not required for Ci-75 formation. The lack of dimerization as a critical feature of Ci processing highlights one difference in the mechanism of partial proteolysis of Ci and the IPT proteins. It is possible that in IPT-containing proteins, dimerization is required for the necessary stability of this domain to protect against complete degradation.

Our results with the simple sequences in Ci also conflict with the model suggesting such a region is critical for protection of the N terminus of partially degraded substrates. Precise deletion of the simple, repetitive sequences in Ci shows that these regions are not absolutely required for Ci-75 formation. Instead, we find that the region comprising amino acids 712 to 760, which does not contain a simple repetitive sequence, is absolutely required. It is true that this region can be dispensed with when certain simple sequences (two copies of GRR or a serine rich region) are substituted in its place (42); however, the processing mechanism of these substitution mutants appears to differ from that of wild-type Ci since the PKA sites and lysines near the cleavage site are no longer absolutely required for processing. Amino acids 612 to 712 do seem to be required for optimal processing; however, it is not clear that low sequence complexity is their key feature: when these sequences are replaced by some, though not all, unstructured regions of normal sequence complexity, Ci-75 processing can be rescued. Clearly, there are sequence requirements for processing between the zinc finger region and K750 that we do not yet understand. The lack of a simple sequence like the GRR of NF-κB p105 as an absolute determinant for partial proteolysis highlights a second difference between Ci and NF-κB processing.

The role of the zinc fingers in the protection signal. Our results using the pulse-chase assay suggest that Ci is completely degraded when the zinc finger region is missing, at least when PKA activity is increased, or that it is not properly folded. Thus, in addition to its role in initiation of degradation, we conclude that the zinc finger region also protects the Ci N terminus from complete cleavage, as previous in vitro studies had shown. We find that Ci fusion proteins in which the zinc fingers are replaced with increasingly stable forms of barnase generate increasing amounts of Ci-75, consistent with the observation that folding stability is a salient feature of this part of the protection signal (42). Additionally, this result shows that protection ability is graded depending on folding strength. Even in wild-type Ci, we do not envision that the protection signal completely protects the Ci N terminus from degradation. We estimate that only 20% to 40% of degradation initiation events in Ci-155 lead to Ci-75 production, with the remaining events leading to complete degradation. Of course, as pointed out previously (42), domains of higher folding stability than the zinc fingers may form more Ci-75 because of their greater ability to prevent N-terminal destruction. This may explain why we usually see greater Ci-75 formation with CiEGFPα206K than wild-type Ci (Fig. 3B).

The role of K750 in the Ci protection signal. Amino acids 712 to 760 are absolutely required for Ci-75 formation. We show that mutation of a single residue (K750) in the region mimics the deletion of the region. We find that mutation of K750 leads not to stabilization of Ci but to complete degradation of the Ci N terminus, suggesting that it is part of the protection signal. The spacing between the zinc finger region and K750 is crucial; thus, we propose that these two elements together form a unique protection signal for Ci-75 formation, different from the IPT proteins.

In Drosophila wing disc cells, this mutant lacks transcriptional activity in the absence of Hh signaling, similar to wild-type Ci and CiΔ1 (CiΔ612–760) and unlike Ci phosphorylation site mutants, which has some activity in the absence of Hh signaling (31, 38, 39). This lack of activity of CiΔ1 in the absence...
of Hh has been attributed to its low full-length levels, which are similar to wild-type Ci (since the degradation initiation of Ci\textsuperscript{L} is normal) but unlike stabilized phosphorylation site mutants (39). Our results with Ci\textsuperscript{K750R} are consistent with this interpretation, and we would additionally argue that the reason Ci\textsuperscript{L} behaves this way is that it lacks K750.

What role is K750 playing in Ci processing? Like wild-type Ci, in the absence of Slimb, ubiquitination of Ci\textsuperscript{K750R} is reduced, and the protein is stabilized, suggesting that Slimb-mediated ubiquitination at other sites is responsible for engagement with the proteasome. Thus, cleavage is still initiated in this mutant, and K750, whether a ubiquitination site or not, is not required for recognition of full-length Ci by the proteasome. Since lysine is the residue to which E2 enzymes transfer Ub, we think K750 may be a site of ubiquitination, and the processing of Ci4xUb is consistent with this idea. If this is true, ubiquitination (possibly polyubiquitination) of this site plays a counterintuitive role in that it prevents cleavage by the proteasome of regions N-terminal to it. Matouschek and colleagues have speculated that the GRR lowers the affinity of the substrate for the proteasome while the tightly folded domain slows its progress (42). It again seems counterintuitive that polyubiquitination of K750 would perform a similar role. Of course, the ε-amino group on lysine is the site of other modifications, such as sumoylation, and we have not yet ruled out the possibility that K750 acts as a site of nonubiquitin modification.

While specific lysines in human NF-κB and Gli3 have been shown to be required for processing to the short form of both proteins (23, 41), it is not clear if these are involved in initiating proteasomal degradation or in protecting the N terminus from complete cleavage. Intriguingly, changing V620 and E621 of Gli2 to KR, the residues in the comparable location in Gli3 (amino acids 685 and 686, a different lysine from the ones mentioned above), confers increased processing on Gli2 in HEK293 cells. However, mutating these amino acids in Gli3 did not reduce its processing (28), suggesting that Gli3 K685 is not acting like Ci K750. Therefore, K750 in Ci is the first lysine residue demonstrated to be part of a protection signal. It will be interesting in the future to determine whether there is a comparable lysine in Gli3 or other partially degraded proteasomal substrates.

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