Cactus protein degradation mediates *Drosophila* dorsal–ventral signaling

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Dorsal–ventral patterning in the *Drosophila* embryo relies on a signal transduction pathway that is similar to a signaling pathway leading to the activation of the mammalian transcription factor NF- κ B. Stimulation of this *Drosophila* pathway on the ventral side of the embryo causes the nuclear translocation of Dorsal, the *Drosophila* NF- κ B homolog. Cactus, like its mammalian homolog I κ B, inhibits nuclear translocation by binding Dorsal and retaining it in the cytoplasm. We show that Cactus, like I κ B, is rapidly degraded in response to signaling. More importantly, signal-dependent degradation of Cactus does not require the presence of Dorsal, indicating that Cactus degradation is a direct response to signaling, and that disruption of the Dorsal/Cactus complex is a secondary result of Cactus degradation. Mutant alleles of *cactus* that encode more stable forms of the protein block signaling, showing that efficient degradation is necessary for signaling. We find that Cactus protein stability is regulated by two independent processes that rely on different regions within the protein: signal-dependent degradation requires sequences in the amino terminus or ankyrin repeats, whereas signal-independent degradation of free Cactus requires the carboxy-terminal region of the protein that includes a PEST sequence.

[Key Words: Cactus; I κ B; NF- κ B; protein degradation; dorsal–ventral patterning]

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Signal transduction pathways that mediate the responses of eukaryotic cells to extracellular signaling molecules generally involve covalent modifications of cytoplasmic proteins. Protein phosphorylation, mediated either by receptor tyrosine kinases or by cytoplasmic kinases that associate with activated receptors, is the best understood of these signal-induced modifications. Recent data have suggested that a different kind of covalent change, protein degradation, may play an important role in at least one kind of signal transduction pathway, the pathway that leads to the activation of the transcription factor NF- κ B.

NF- κ B, a mammalian transcription factor originally identified in B cells, but subsequently found in several nonimmune cell types, is activated rapidly by a post-translational mechanism in response to a variety of extracellular stimuli (Blank et al. 1992; Grilli et al. 1993). Inactive NF- κ B is retained in the cytoplasm by its inhibitor, I κ B, and migrates into the nucleus when the NF- κ B/I κ B complex dissociates in response to several cytokines, including interleukin-1 (IL-1) and tumor necrosis factor (TNF α) (Sen and Baltimore 1986; Bauerle and Baltimore 1988; Beg et al. 1993). I κ B family members share a block of ankyrin repeats that mediate binding to NF- κ B

and mask the NF- κ B nuclear localization signal (Ganchi et al. 1992; Henkel et al. 1992; Beg and Baldwin 1993), providing an explanation for why NF- κ B/I κ B complexes remain cytoplasmic while free NF- κ B is nuclear.

Recent work using mammalian tissue culture cells has shown that I κ B is rapidly degraded in response to signaling, and that this degradation correlates with NF- κ B activation (Beg et al. 1993; Henkel et al. 1993; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994). Several calpain and proteasome inhibitors stabilize I κ B and block NF- κ B activation, indicating that I κ B degradation is necessary for activation of NF- κ B (Miyamoto et al. 1994; Traenckner et al. 1994; Lin et al. 1995). In some of these experiments, the stabilized form of I κ B is a more highly phosphorylated form, suggesting that phosphorylation of I κ B in the complex in response to signaling precedes its degradation.

The *Drosophila* dorsal–ventral signal transduction pathway is comprised of 12 known maternal effect genes. The end result of the activity of this pathway is the nuclear localization of the transcription factor Dorsal on the ventral side of the embryo. Three components of this pathway, Toll, Cactus, and Dorsal, are homologous to members of the IL-1/NF- κ B pathway. The cytoplasmic domain of Toll, a transmembrane receptor protein, is homologous to the cytoplasmic domain of the mammalian IL-1 receptor (Hashimoto et al. 1988; Schneider et al. 1991). Dorsal and Cactus are homologous

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gous to NF- κ B (Steward 1987) and I κ B (Geisler et al. 1992; Kidd 1992), respectively. Localized ventral activation of the Toll receptor causes disruption of the Dorsal/Cactus complex and the subsequent nuclear localization of Dorsal, just as activation of the IL-1 receptor leads to the disruption of the NF- κ B/I κ B complex and the nuclear localization of NF- κ B. Neither signal-induced phosphorylation nor signal-dependent degradation of the *Drosophila* I κ B homolog Cactus has been observed previously (Whalen and Steward 1993). However, given the homology that exists between the *Drosophila* and mammalian pathways, it seems likely that degradation of Cactus could also be linked to the activation of Dorsal.

The early *Drosophila* embryo provides a unique opportunity to dissect the *in vivo* response of Cactus and Dorsal to signaling. By activating the pathway at a precise time by microinjection of the activating extracellular ligand, Spätzle, we show that Cactus, like I κ B, is rapidly degraded *in vivo* in response to signaling. Taking advantage of *Drosophila* mutants, we show that signal-induced Cactus degradation can occur in the absence of Dorsal, indicating that degradation of the Cactus protein is a direct result of signaling, rather than a secondary consequence of separation of the Dorsal/Cactus complex. We find that mutant alleles of Cactus that increase its stability block signaling, confirming that destruction of Cactus is necessary for signaling. These mutant alleles also reveal that Cactus protein turnover is regulated by two independent processes: signal-independent degradation of free Cactus, which requires the carboxy-terminal PEST region of the protein, and signal-dependent degradation, which is mediated by more amino-terminal regions of the protein.

Results

Signaling causes rapid degradation of wild-type Cactus

To test whether Cactus, like I κ B, is degraded in response to signaling, we activated the dorsal-ventral signaling pathway at a controlled time by injecting embryos with processed Spätzle, the putative Toll ligand (Morisato and Anderson 1994; Schneider et al. 1994; Fig. 1C). An activated, processed form of the Spätzle protein can activate Toll when it is injected into the extracellular (perivitelline) space of early embryos (Schneider et al. 1994). Therefore, to stimulate the pathway, we injected activated Spätzle protein into the perivitelline space of syncytial blastoderm embryos. In the 1- to 1.5-hr embryos used, all of the components required for signaling are present, and localized endogenous signaling is just beginning. After a short incubation, the embryonic cytoplasm was harvested and the Cactus protein was analyzed by Western blot. We observed rapid degradation of Cactus in response to injection of activated Spätzle (Fig. 1A); >75% of the Cactus protein was degraded in the first 15 min after injection. In control injections with water, Cactus was not detectably degraded in the 30 min following injection (data not shown).

To test whether the Cactus degradation that we observed was specific to the dorsal-ventral pathway, we repeated these injections into embryos from *Toll*⁻ (*TI*⁻) females. Because activated Spätzle is postulated to bind and activate the Toll receptor, it should not stimulate signaling in an embryo lacking Toll. Figure 1B shows that injection of activated Spätzle into *TI*⁻ embryos did not cause degradation of Cactus. Therefore, the signal-dependent degradation of Cactus that we observe in

Figure 1. Degradation of Cactus in response to signaling. Response of 1.5- to 2-hr-old embryos from wild-type (A) or *TI*⁻, *tub*⁻, and *pelle*⁻ females (B). (C) Schematic diagram of the order of action of genes in the pathway. For A and B, embryos were injected in the perivitelline space with an activated Spätzle preparation. Embryonic cytoplasm was collected 5 or 15 min after injection and analyzed by Western blot, using an anti-Cactus antibody. The lanes labeled Uninj contain cytoplasm from uninjected embryos; the lanes marked 5' and 15' contain cytoplasm collected 5 and 15 min after injection of Spätzle protein. The arrows point to the Cactus protein. In wild-type embryos, Cactus protein disappeared rapidly after Spätzle injection (A). In mutant embryos that lack Toll, Tube, or Pelle, there was no degradation of Cactus in response to Spätzle injection (B). The high molecular weight band in each lane is a crossreacting band that can be used as an internal loading control. Control injections with water caused no degradation of Cactus (data not shown). The blot in A resolves the two forms of Cactus: the predominant phosphorylated form (arrow), and the less abundant, more rapidly migrating unphosphorylated form. The upper band collapses to the lower band after treatment with calf intestinal phosphatase (Whalen and Steward 1993).

wild-type embryos is specific to the dorsal–ventral pathway. We also tested whether this degradation required the dorsal group genes *tube* and *pelle*, which act downstream of *Toll* and upstream of *dorsal* and *cactus* (Fig. 1C). The sequence of *tube* does not suggest a biochemical function (Letsou et al. 1991); *pelle* appears to encode a cytoplasmic serine/threonine kinase (Shelton and Wasserman 1993). We found that in embryos from *tube*[−] (*tub*[−]) and *pelle*[−] (*pll*[−]) females, Cactus does not degrade in response to signaling (Fig. 1B). Therefore, signal-dependent degradation of Cactus is mediated by all of the genetically defined components of the pathway that act between *Spätzle* and *dorsal/cactus* in the pathway.

Cactus is rapidly degraded in response to signal even in the absence of Dorsal

When the rapid degradation of I κ B in response to TNF α is blocked by calpain inhibitors, I κ B becomes phosphorylated but does not dissociate from NF- κ B (Miyamoto et al. 1994). One interpretation of this result is that the degradation of I κ B in response to signal takes place when it is in the complex. However, in the absence of inhibitors, it has not been shown whether degradation of I κ B is a direct response to in vivo signaling or occurs as a secondary consequence of I κ B becoming separated from NF- κ B. To differentiate between these two possibilities for Cactus and Dorsal, we wanted to assay the response of Cactus to injection of activated *Spätzle* into embryos lacking Dorsal protein. If Cactus is a direct target for signaling, then activation of the signaling pathway should cause rapid degradation of Cactus even in the absence of Dorsal. If Cactus is degraded only as a consequence of being separated from Dorsal, then the level of Cactus protein in the absence of Dorsal should be unresponsive to signaling.

A complication of this experiment is that there is no detectable maternal Cactus protein in embryos or ovaries lacking Dorsal (Fig. 2, lane 1; Whalen and Steward 1993; Kidd 1992), suggesting that Cactus is unstable when not bound to Dorsal even prior to signaling. We found, however, that it is possible to produce Cactus protein in embryos lacking Dorsal protein, that is, embryos laid by *dorsal*[−] (*dl*[−]) females, by injecting in vitro-synthesized *cactus* RNA into the cytoplasm of these embryos and allowing translation in vivo (Fig. 2, lanes 2,4). To test the effect of signaling, activated *Spätzle* was injected into the perivitelline space of the embryos after translation of Cactus had begun (Fig. 2, lanes 3,5). We observed that Cactus was degraded in response to signaling at roughly the same rate in the embryos from *dl*[−] females as in embryos from wild-type females (cf. Fig. 2, lanes 2–5 with Fig. 1A), whereas it continued to accumulate in the absence of signaling (Fig. 2, lanes 2,4). We conclude that free Cactus is as effective a substrate for signal-dependent degradation as Cactus in the Dorsal–Cactus complex, indicating that Cactus degradation is a direct consequence of signaling.

This experiment also revealed that two kinds of degradation control the level of Cactus protein in the em-

Figure 2. Degradation of Cactus in response to signaling in the absence of Dorsal. To test the effect of signaling on Cactus protein in the absence of Dorsal, 1.5- to 2-hr-old embryos laid by *dl*[−] females were injected with *cactus* transcripts. (Lane 1) Uninjected *dl*[−] embryos contain no detectable Cactus protein. Following a 5-min incubation to allow translation of the injected RNA, activated *Spätzle* was injected into the perivitelline space of the experimental embryos (lanes 3,5) but not the controls (lanes 2,4). After an additional 5 or 15 min with or without injected *Spätzle*, cytoplasm was collected and assayed for Cactus protein as described in Fig. 1. In the absence of *Spätzle* (lanes 2,4) Cactus protein accumulated, whereas in embryos exposed to *Spätzle* for 5 or 15 min (lanes 3,5), the newly synthesized Cactus protein was degraded. The arrow points to the Cactus protein; the high molecular weight band was used as a loading control.

bryo: signal-dependent and signal-independent degradation. Signal-independent degradation eliminates free Cactus, so that essentially all of the Cactus in the embryo is complexed with Dorsal. Signal-dependent degradation destroys Cactus, whether or not it is complexed with Dorsal. Signal-dependent degradation of Cactus must be more rapid than signal-independent degradation, because we observed that the amount of Cactus in embryos lacking Dorsal was decreased dramatically by exposure to signal, even though both injected and control embryos were subject to signal-independent degradation (e.g., cf. Fig. 2, lanes 4 and 5).

A more stable mutant form of Cactus inhibits signaling

These experiments showed that Cactus protein stability is controlled by both signal-dependent and signal-independent degradation but did not test whether degradation of Cactus is necessary for signaling. We isolated an unusual allele of *cactus*, *cact*^{Su}, on the basis of its ability to block signaling. This mutant allowed us to investigate the importance of Cactus stability in proper signaling.

In a large-scale genetic screen for suppressors of a dominant, ventralizing allele of *easter*, *ea*⁸³¹, one suppressor mutation was isolated. *easter* acts genetically upstream of *Spätzle* and is homologous to serine proteases (Chasan and Anderson 1989; Morisato and Anderson 1994). All of the embryos (100%) laid by *ea*⁸³¹/+ females are moderately ventralized (Jin and Anderson 1990), whereas *Su*/+; *ea*⁸³¹/+ females produced 50% wild-type, hatching lar-

vae and 50% unhatched embryos with an apparently wild-type cuticular phenotype (Fig. 3A). The *Su* mutation also suppresses ventralizing mutations of several other dorsal group genes, including *Toll* (Fig. 3B), *spätzle*, and *cactus* (data not shown), demonstrating that the suppressor is not specific to *easter* but, instead, inhibits the activity of the pathway.

The *Su* mutation mapped by recombination to the second chromosome between *b* and *pr*, a region including the *dorsal* and *cactus* genes. However, the *Su* mutation complemented both *dorsal* and *cactus*, as well as all available deficiencies in this interval, suggesting that it could be a gain-of-function allele. To obtain loss-of-function alleles at this locus we carried out a genetic screen to revert the suppressing effect of the mutation (Materials and methods). We obtained four revertants that were no longer able to suppress *ea*^{83l} and mapped to the same site as *Su*. Three of these revertants failed to complement loss-of-function *cactus* alleles. We therefore concluded that the *Su* mutation was a gain-of-function allele of *cactus* and renamed it *cact*^{Su}.

Despite the ability of *cact*^{Su} to act as a strong dominant suppressor, it had no phenotype on its own: *cact*^{Su}/*cact*^{Su} flies are fully viable, and females produce wild-type embryos. The lack of an obvious effect on dorsal-ventral patterning by *cact*^{Su} was perplexing, given its strong suppression of ventralization. To look for more subtle effects of the mutation, we examined the Dorsal gradient in embryos from *cact*^{Su}/*cact*^{Su} females by examining sections stained with an anti-Dorsal antibody. Figure 4 shows that there is a reduction in the extent of the dorsal gradient in *cact*^{Su}/*cact*^{Su} embryos compared with wild type. In wild-type embryos, 27% of the nuclei

in the embryo circumference had high levels of nuclear Dorsal, whereas in *cact*^{Su}/*cact*^{Su} embryos this was reduced to 20%. The absence of a later embryonic phenotype indicates that there is enough regulative capacity in the patterning process to compensate for this reduction in the number of Dorsal-containing nuclei.

Cact^{Su} encodes a slightly truncated protein that appears to be more stable than wild-type Cactus

To determine the molecular basis of the *cact*^{Su} activity, we used reverse transcription-PCR to amplify the *cactus* mRNA from ovarian RNA of *cact*^{Su} females. The sequence revealed that the *cact*^{Su} allele has a single base pair change in the 5' splice donor of intron six, which should render this splice site defective. The predicted protein lacks the seventh exon of the maternal *cactus* mRNA, which encodes 23 amino acids, and in its place has 5 amino acids encoded by intron 6 (Fig. 5A). This mutant protein is almost identical to the zygotic form of Cactus, a naturally occurring splicing variant expressed later in development (Fig. 5A; Kidd 1992).

To characterize the Cact^{Su} protein, we performed Western blots of 0- to 2-hr embryo extracts from embryos laid by wild-type, *cact*^{Su}/+, and *cact*^{Su}/*cact*^{Su} females. As predicted from the mRNA sequence, the Cact^{Su} protein is smaller than the wild-type maternal protein (Fig. 5B). Unexpectedly, we found that in embryos from *cact*^{Su}/+ heterozygous females, there was 10 times as much Cact^{Su} protein as wild-type protein (Fig. 5B, lane 2), even though the amounts of wild-type and *cact*^{Su} RNAs in these embryos were equal (data not

A

$$\frac{ea^{83l}}{+}$$

$$\frac{Su}{+}; \frac{ea^{83l}}{+}$$

B

$$\frac{Tl^{84c}}{+}$$

$$\frac{Su}{+}; \frac{Tl^{84c}}{+}$$

Figure 3. The *Su* mutation suppresses ventralizing alleles of *easter* and *Toll*. *Su* was recovered in a screen for suppressors of a dominant ventralizing allele of *easter*, *ea*^{83l}. Embryos produced by *ea*^{83l}/+ females are moderately ventralized, whereas those produced by *Su*/+;*ea*^{83l}/+ females either hatched or appeared wild type (A). *Su* also suppressed the dominant phenotype of *Tl*^{84c} (B), as well as ventralizing alleles of *spätzle* and *cactus* (data not shown).

Figure 4. *cact^{Su}* narrows the Dorsal gradient. Embryos (2.5–3.5 hr) laid by wild-type or *cact^{Su}/cact^{Su}* females were stained with Dorsal antibody and then sectioned. The extent of the Dorsal gradient (marked by arrows) was calculated as the percentage of nuclei around the embryonic circumference that stained strongly for nuclear Dorsal. In the wild-type embryos, $27 \pm 2\%$ of the nuclei (24 of 91 in this section) contained Dorsal, whereas in embryos from *cact^{Su}/cact^{Su}* females only $20 \pm 2\%$ of the nuclei (17 of 94 in this section) contained Dorsal. Four to six sections of each embryo were counted. Eight wild-type embryos and five *cact^{Su}/cact^{Su}* embryos were scored, and the results of all sections were averaged for each genotype.

shown). Based on the observation that Cactus is unstable, the greater accumulation of *Cact^{Su}* protein could reflect its greater stability. Maternal Cactus is not detected in Western blots from *dl⁻* ovarian extracts (Kidd 1992; Whalen and Steward 1993; see also Fig. 2), presumably reflecting the instability of Cactus when not com-

plexed with Dorsal. If there is normally an equilibrium between the Dorsal/Cactus complex and free subunits, then any free Cactus protein would be a target for degradation and newly synthesized Cactus could take its place in the complex. If *Cact^{Su}* represents a more stable protein than wild type, it should eventually replace wild

Figure 5. Molecular characterization of *cact^{Su}* and *cact^{ΔPEST}*. (A) Schematic diagram of the wild-type maternal Cactus, zygotic Cactus, *Cact^{Su}*, and *Cact^{ΔPEST}* proteins (drawing is not to scale). The ANKYRIN domain is a set of five conserved ankyrin repeats. The PEST domain is a region rich in Pro, Glu, Asp, Ser and Thr. The first residue after the PEST sequence in *Cact^{Su}* is Glu rather than Val in zygotic Cactus because of the EMS-induced base change (T → A at the second nucleotide in intron 6) at the splice junction. *Cact^{ΔPEST}* lacks the entire PEST sequence, as well as the carboxy-terminal region deleted in *Cact^{Su}*. (B) Cactus Western blot of early embryo extracts from 0- to 2-hr embryos produced by wild-type, *cact^{Su} / +*, or *cact^{Su} / cact^{Su}* females. Arrows point to the wild-type Cactus (lane 1) and *Cact^{Su}* (lane 2) proteins. (Lane 3) In embryos from *cact^{Su} / +* heterozygotes, there was ~10 times more *Cact^{Su}* protein than wild-type Cactus protein. (C) Cactus Western blot of cytoplasm from wild-type embryos injected with identical concentrations of *cact⁺*, *cact^{Su}*, and *cact^{ΔPEST}* RNA (Materials and methods), followed by a 20-min incubation to allow translation of the transcripts. The

left-hand arrow points to the endogenous wild-type Cactus; the right-hand arrows point to the *Cact^{Su}* proteins in lane 2 (large arrow) and the *Cact^{ΔPEST}* proteins in lane 3. After 20 min of translation there is approximately three times more *Cact^{ΔPEST}* protein than wild-type Cactus present as calculated by densitometry. Note that both *Cact^{Su}* and *Cact^{ΔPEST}* accumulate phosphorylated and unphosphorylated forms, indicating that this phosphorylation site(s) is not located in the carboxyl terminus.

type Cactus, yielding a ratio of Cact^{Su} /wild-type Cactus similar to what we observed.

Deleting the PEST sequence stabilizes Cactus and strongly inhibits signaling

The amino acids deleted in Cact^{Su} lie just carboxy-terminal to the PEST region of the protein (Fig. 5A; Geisler et al. 1992; Kidd 1992). PEST sequences are rich in Pro, Glu, Asp, Ser, and Thr and are thought to be associated with rapid protein turnover (Rogers et al. 1986; Rechsteiner 1990). One possible explanation for the apparently increased stability of Cact^{Su} is that the lack of sequences immediately adjacent to the PEST region interferes with the destabilizing function of the PEST sequence. In this view, removing the PEST sequence entirely might result in an even more stable protein.

To test whether the PEST sequence does affect Cactus stability, we constructed a mutant form of *cactus*, $\text{cact}^{\Delta\text{PEST}}$, in which both the seventh exon and the entire PEST sequence were deleted. The ankyrin repeat domain remained intact in this mutant (Fig. 5A). To compare the stability of the different Cactus proteins, we injected in vitro-synthesized transcripts of cact^+ , cact^{Su} , and $\text{cact}^{\Delta\text{PEST}}$ into wild-type embryos and allowed them to be translated in vivo. We then collected the cytoplasm from these embryos and analyzed it by Western blot. After 20 min of translation, there was more $\text{Cact}^{\Delta\text{PEST}}$ and Cact^{Su} than wild-type Cactus (Fig. 5C), even though equivalent concentrations of RNA were injected (Materials and methods). This suggested that $\text{Cact}^{\Delta\text{PEST}}$ and Cact^{Su} are both more stable than wild-type Cactus.

To test whether $\text{cact}^{\Delta\text{PEST}}$ could block signaling like cact^{Su} , we injected $\text{cact}^{\Delta\text{PEST}}$ RNA transcripts into wild-type embryos. This resulted in weakly dorsalized embryos (Fig. 6), suggesting that the $\text{Cact}^{\Delta\text{PEST}}$ protein interfered with signaling. A stronger effect was seen when $\text{cact}^{\Delta\text{PEST}}$ RNA was injected into $\text{cact}^{\text{Su}}/\text{cact}^{\text{Su}}$ embryos, where 15% of the embryos were completely dorsalized (Fig. 6). This strong dorsalization was never observed with injection of cact^+ or cact^{Su} transcripts (data not shown). Therefore, $\text{Cact}^{\Delta\text{PEST}}$ is more effective at blocking signaling than Cact^{Su} and is capable of completely blocking all signaling in embryos.

Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ also degrade rapidly in response to signal

The above experiments showed that the more stable forms of Cactus, Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$, can block signaling. In principle, the ability to block signaling could reflect resistance to either signal-dependent or signal-independent degradation of Cactus. To determine whether Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ were more resistant to signal-dependent degradation than wild-type Cactus, we injected activated Spätzle into embryos laid by females carrying these mutants. Activated Spätzle elicited the rapid degradation of the Cact^{Su} protein at a similar rate as the wild-type protein (cf. Figs. 7A and 1A). There was more Cact^{Su} than wild-type Cactus per embryo at all time

$\text{cact}^{\Delta\text{PEST}}$ → Wild Type

$\text{cact}^{\Delta\text{PEST}}$ → $\frac{\text{cact}^{\text{Su}}}{\text{cact}^{\text{Su}}}$

Figure 6. $\text{cact}^{\Delta\text{PEST}}$ can block signaling. In vitro-synthesized $\text{cact}^{\Delta\text{PEST}}$ RNA was injected into embryos from wild-type or $\text{cact}^{\text{Su}}/\text{cact}^{\text{Su}}$ females. When injected into wild-type embryos, $\text{cact}^{\Delta\text{PEST}}$ RNA causes weak dorsalization (top). When $\text{cact}^{\Delta\text{PEST}}$ was injected into embryos produced by $\text{cact}^{\text{Su}}/\text{cact}^{\text{Su}}$ females, it caused complete dorsalization in 15% of the embryos (bottom). This strongly dorsalized embryo lacks all ventral and lateral structures and is composed of a hollow tube of dorsal cuticle. Weaker dorsalization was observed in the other 85% of injected embryos.

points, but the rate of degradation was approximately the same. Therefore, the Cact^{Su} protein, while encoding a more stable form of Cactus, is not resistant to signal-dependent degradation.

To test the response of $\text{Cact}^{\Delta\text{PEST}}$ to signaling, we made stable transformants of $\text{cact}^{\Delta\text{PEST}}$ (Materials and methods). When activated Spätzle was injected into embryos from transformant females carrying one copy of the $\text{cact}^{\Delta\text{PEST}}$ transgene and two wild-type copies of *cactus*, both $\text{Cact}^{\Delta\text{PEST}}$ and wild-type Cactus were degraded rapidly (Fig. 7B). Therefore, both Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ are degraded rapidly in response to signal.

Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ are resistant to signal-independent degradation

The previous experiments showed that Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ undergo normal rates of signal-dependent degradation. To test whether their greater stability might be caused by greater resistance to signal-independent degradation of free Cactus, we compared the level of protein present after injection of cact^+ , cact^{Su} , and $\text{cact}^{\Delta\text{PEST}}$ RNAs into embryos from *dl⁻* females prior to the normal time of signaling (Fig. 8). Because the mutant proteins were synthesized in the absence of Dorsal, and because no activated Spätzle was injected, the proteins were subject to signal-independent but not signal-dependent degradation. Thirty minutes after injection, there

Figure 7. Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ are degraded in response to signaling. Activated Spätzle was injected into embryos produced by $\text{cact}^{\text{Su}}/\text{cact}^{\text{Su}}$ females (A) or $P[w^+, \text{cact}^{\Delta\text{PEST}}]$ females (B). The arrow in A points to the Cact^{Su} protein. Cact^{Su} degrades rapidly in response to signaling: 15 min following injection there is still some detectable Cact^{Su} protein visible (lane 3), but its level is greatly reduced compared with uninjected embryos (lane 1). In B, embryos laid by transformant females carrying one copy of $\text{cact}^{\Delta\text{PEST}}$ and two copies of wild-type *cactus* were injected with activated Spätzle. This transformant line produces substantially less $\text{Cact}^{\Delta\text{PEST}}$ protein than wild-type Cactus. The arrow points to the wild-type Cactus protein; the open arrowhead points to the $\text{Cact}^{\Delta\text{PEST}}$ protein. Lane 1 contains cytoplasm from uninjected embryos. Five minutes after injection of activated Spätzle (lane 2), the levels of both wild-type Cactus and $\text{Cact}^{\Delta\text{PEST}}$ are greatly reduced.

was much more Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ protein present than wild-type Cactus (Fig. 8, lanes 3,6,9) even though equivalent amounts of RNA were injected (Materials and methods). Because cact^{Su} contains the entire wild-type 5'- and 3'-untranslated regions, the observed difference in protein levels is most likely the result of the greater stability of the Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ proteins rather than differential translation. This result suggests that the Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ proteins are relatively insensitive to signal-independent degradation and that it is the resultant increase in free Cactus in the embryo that inhibits signaling.

Discussion

Signal-dependent degradation of Cactus

We have shown that Cactus is rapidly degraded in response to stimulation of the *Drosophila* dorsal-ventral signaling pathway. The mammalian homolog of Cactus, I κ B, is degraded in response to signaling in tissue culture cells (Beg et al. 1993; Henkel et al. 1993; Palombella et al. 1994; Traenckner et al. 1994). Our experiments in whole embryos demonstrate that rapid signal-dependent degradation of Cactus takes place in the intact organism.

Two plausible models could explain how Cactus is degraded in response to the signal. One model is that signaling causes dissociation of the Dorsal/Cactus complex by modifying either Dorsal or Cactus, and the resulting free Cactus is degraded by signal-independent degradation (see below). The other model is that Cactus is degraded directly in the complex. Because Cactus is degraded in response to signal even in the absence of

Dorsal, we conclude that Cactus degradation is a direct target of signaling. We infer that in the wild-type embryo, Cactus in the complex is degraded and that this degradation releases Dorsal, allowing it to move to the nucleus.

All of the genetically defined components that act upstream of *cactus* in the pathway, *Toll*, *tube*, and *pelle*, are required for signal-dependent degradation of Cactus, confirming that Cactus degradation is an integral step in this signaling pathway. However, the nature of these components does not clarify how Cactus protein is degraded in response to the signal. Genetic analysis has shown that *tube* acts downstream of *Toll* (Hecht and Anderson 1993) and *pelle* acts downstream of *tube* (Grosshans et al. 1994). The *pelle* gene appears to encode a serine-threonine kinase (Shelton and Wasserman 1993), and because Cactus is a phosphoprotein, it could be a substrate for Pelle.

Rapid phosphorylation of I κ B in response to signaling and prior to its degradation has been observed in response to IL-1 and TNF- α (Beg et al. 1993; Miyamoto et al. 1994; Traenckner et al. 1994). Although we found that Pelle activity is required in vivo for Cactus degradation, we did not observe phosphorylation of Cactus in response to signaling. It is possible that such a phosphorylated form exists but was not detected by our methods because of its short half-life or because it did not cause a detectable mobility shift. Alternatively, Pelle might control Cactus degradation indirectly, phosphorylating an unknown intermediate in the pathway.

Our findings that the Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ mutants degrade rapidly in response to signaling demonstrate that the PEST sequence is not required for signal-dependent degradation of Cactus. This implies that there is another region of Cactus, located either in the amino terminus or

Figure 8. Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ are relatively resistant to signal-independent degradation of free Cactus in the absence of Dorsal. In vitro-synthesized transcripts of wild type *cactus*, cact^{Su} , and $\text{cact}^{\Delta\text{PEST}}$ were injected into embryos from *dl⁻* females. Cytoplasm was collected 10, 20, and 30 min after injection and analyzed by a Cactus Western blot. Identical concentrations of the three RNAs were injected (Materials and methods). The level of $\text{Cact}^{\Delta\text{PEST}}$ protein that accumulates after 30 min is greater than that of Cact^{Su} and much greater than that of wild-type Cactus (cf. lanes 3, 6, and 9). The arrows point to the Cactus proteins; the more slowly migrating band is a cross-reacting band.

in the ankyrin repeats, that is required for rapid degradation in response to signaling.

Two kinds of degradation control Cactus protein levels

We have shown that Cactus protein stability is regulated at two levels (Fig. 9). The first level of regulation is the signal-dependent degradation described above. The second is signal-independent degradation of the Cactus protein that is not complexed with Dorsal. We found that in the absence of Dorsal, the Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ proteins accumulate to a much higher level than wild-type Cactus protein after injection of equivalent amounts of RNA. Strong evidence that the greater accumulation of Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ reflects increased stability rather than increased translation comes from the comparison of Cactus protein levels in embryos from $\text{cact}^{\text{Su}}/\text{cact}^+$ heterozygous females. In these embryos, the level of Cact^{Su} is greater than the level of Cact^+ , and furthermore, the level of Cact^+ is 10-fold lower than in wild-type embryos. It is very unlikely that the decrease in Cact^+ is attributable to increased translation of Cact^{Su} . If, however, there is an equilibrium between free Cactus and Cactus in the Dorsal/Cactus complex, then because free Cact^{Su} is more stable than free Cact^+ , it will accumulate preferentially over time.

Our experiments indicate that in the absence of Dorsal, wild-type Cactus turns over much more rapidly than Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$. Because these mutants lack sequences in and near the PEST region, we infer that the PEST sequence is important for the degradation of free Cactus. The mechanism by which PEST sequences cause

protein degradation is unknown; however, our finding that Cact^{Su} , which retains the entire PEST sequence but lacks the adjacent amino acids, is more stable than wild-type Cactus suggests that the conformation of the PEST region is important for its function.

Increasing the amount of free Cactus blocks signaling

The normal consequence of signal-independent degradation is to remove nearly all Cactus protein that is not complexed with Dorsal from the embryo. The Cact^{Su} and $\text{Cact}^{\Delta\text{PEST}}$ proteins, which are resistant to signal-independent degradation, can effectively block signaling, revealing that excess free Cactus interferes with normal signaling. Free Cactus could block signaling in one of two ways. It could act as a buffer, binding to Dorsal after it is freed from the complex, decreasing its probability of translocating to the nucleus. Alternatively, free Cactus could compete with the Cactus in the Dorsal/Cactus complex for a limiting upstream component that targets Cactus for degradation, effectively titrating out the signal from upstream in the pathway.

A standing controversy in the field is whether the dorsal-ventral signaling pathway acts by modifying Dorsal, Cactus, or both. Our results show that signal-dependent degradation of Cactus does not require Dorsal, indicating that Cactus is a direct target of the pathway and suggesting that all signaling could be mediated via Cactus. However, even the strongest *cactus* mutants retain some residual dorsal-ventral polarity, with more Dorsal protein translocated into ventral than dorsal embryonic nuclei (Roth et al. 1991). Because we obtained completely dor-

Figure 9. Model of the two forms of regulation of Cactus protein degradation. (Amino) Amino-terminus of Cactus; (ANK) ankyrin repeat domain; (P) PEST region. Signal-dependent degradation of Cactus, which requires the action of *Toll*, *tube*, and *pelle*, causes degradation of Cactus in the Dorsal/Cactus complex, leading to the liberation of free Dorsal, which then migrates into ventral nuclei. This type of degradation is mediated by more amino-terminal regions, because the $\text{Cact}^{\Delta\text{PEST}}$ mutant is subject to signal-dependent degradation. (Right) Free Cactus that is not complexed with Dorsal is degraded by signal-independent degradation. The PEST sequence is important for this process, as the $\text{Cact}^{\Delta\text{PEST}}$ mutant that lacks a PEST sequence is resistant to signal-independent degradation.

salized embryos with the stabilized forms of Cactus protein, this demonstrates that excess Cactus can effectively block all signaling. We therefore conclude that degradation of Cactus is necessary for any nuclear translocation of Dorsal. Once Cactus is degraded, the additional modification of free Dorsal that has been observed (Gillespie and Wasserman 1994) may be required for Dorsal to achieve the highest nuclear concentrations.

The observation that Cactus, like I κ B, is rapidly degraded in response to signaling strengthens the parallels between the mammalian and *Drosophila* signaling pathways. Given the similarities that exist between the two systems, our findings on Cactus may also apply to I κ B. It should now be possible to test whether the level of I κ B protein, like Cactus, is controlled by both signal-dependent and signal-independent processes, whether normal signaling targets I κ B directly for degradation, and whether there is a specific kinase required for I κ B degradation. We plan to continue to exploit the genetic interactions in *Drosophila* to elucidate the mechanisms of signal-dependent protein degradation.

Materials and methods

Fly strains and mutagenesis

Oregon-R was used as the wild-type stock. The *easter* alleles are described in Chasan and Anderson (1989) and Jin and Anderson (1990). For *Tl*⁻, *tub*⁻, *p11*⁻, and *dl*⁻ females: *Tl*^{5BREQ}/*Tl*^{9QRE} (Anderson et al. 1985), *tub*^{R5.6}/*Df*(3R)XM3, *p11*⁷⁴/*Df*(3R)Ser^{+R82f} (Hecht and Anderson 1993), and *dl*⁸/*Df*(2L)119 (Roth et al. 1989) were used. Ethylmethane sulfonate (EMS) mutagenesis was carried out as described (Lewis and Bacher 1968). To isolate suppressors of *ea*^{83l}, mutagenized *ea*^{83l}, *knirps*/TM3, *tld*^{10E} males were crossed to *ea*¹, *tld*^{10E}/TM1, *knirps* females. Balancers carrying the zygotic lethal mutations had been isolated previously (Ferguson and Anderson 1992). The only viable progeny from this cross were *ea*¹, *tld*^{10E}/*ea*^{83l}, *knirps* males and females, which were placed in bottles and checked for fertility. The presence of *ea*¹ in these flies ensured that intragenic suppressors would not be recovered, as *ea*¹/*ea*⁻ females are sterile. Of 75,000 F₁ females, one was fertile, which proved to be *cact*^{Su}. In the screen to generate revertants of *cact*^{Su}, a transformant line was used that contained a dominant allele of *easter*, *ea*^{83l-125.3} inserted on the second chromosome (Jin 1991). *cact*^{Su}/CyO males were mutagenized with EMS and crossed to +/CyO females. Four thousand single F₁ females of the genotype *cact*^{Su}/CyO were crossed to *ea*^{83l-125.3}/CyO males. The embryos laid by *cact*^{Su}/*ea*^{83l-125.3} F₂ females were scored for ventralization. Four revertants were recovered; all four mapped to *cact*^{Su}, and three of these were ventralized over *cact*⁹⁹ (Roth et al. 1991).

Cuticle preparations

Embryos were collected for 24 hr and aged for another 24 hr to allow completion of embryonic development. The embryos were then dechorionated in bleach, dissected out of their vitelline membranes, fixed in 1:4 glycerol/acetic acid, and mounted in Hoyer's medium diluted 1:1 with lactic acid. Injected embryos were allowed to develop for 36 hr after injection before cuticle preparations were made.

Embryo injections

Synthetic SP6 RNA transcripts were generated by in vitro tran-

scription carried out in the presence of radioactively labeled ATP as described (Schneider et al. 1991) with the modification that two sequential 5 M ammonium acetate/isopropanol precipitations were carried out followed by Cerenkov counting. The transcripts were diluted as necessary to ensure equivalent concentrations. For RNA injections, transcripts were injected into 1- to 1.5-hr embryos (Chasan and Anderson 1989). Embryos were allowed to develop for cuticle preparations, or the cytoplasm was collected for Western analysis. Cytoplasm was collected by aspirating the contents of 15 embryos into the injection needle, avoiding any cytoplasm that had leaked out of the embryo after injection. The cytoplasm was deposited into the injection oil where it was recovered with a pipette and placed immediately in buffer on ice. The buffer was a 1:1 dilution of 20 mM HEPES (pH 7.5), 20 mM NaCl, 1 mg/ml of aprotinin, 1 mg/ml of leupeptin, 1 mg/ml of antipain, in 2× protein sample buffer (Ausubel et al. 1991) containing 10% 2-mercaptoethanol. A crude activated Spätzle preparation was made (Schneider et al. 1994), and a concentration equivalent to 150 U/ml, as described in Schneider et al. (1994) was used. The activated Spätzle injections were carried out in 2- to 2.5-hr embryos. For the sequential RNA and activated Spätzle injections, RNA was first injected into the cytoplasm as described above. The activated Spätzle was then injected into the perivitelline space on the dorsal side of the embryo as described (Schneider et al. 1994) using the same point of entry as for the RNA. Cytoplasm was collected for Western analysis as described above.

Antibody staining of embryos

Embryos (2.5- to 3.5-hr) were prepared and stained as described (Roth et al. 1989) using a 1:1000 dilution of rat anti-Dorsal antibody received from R. Steward (Rutgers University, Piscataway, NJ). A goat anti-rat-horseradish peroxidase (HRP) secondary antibody was used; staining was detected with DAB. Embryos were embedded in Durcupan (Fluka) resin and sectioned (10 μ m).

Cactus constructs and transformants

To sequence *cact*^{Su}, ovarian RNA was isolated (Ashburner et al. 1989a) from *cact*^{Su}/*cact*^{Su} females. An oligonucleotide (GAT CGA ATT CTT CTG CAT CCT TGT ATG CTT TA) directed against the 3' end of the *cactus* cDNA was used as a primer for reverse transcriptase, and RT-PCR was carried out (Ausubel et al. 1991) using this primer as the 3' primer and GAT CGG ATC CAT TCG CTA TCG AAA CGT G as the 5' primer. The 5' primer contained a *Bam*HI site, and the 3' primer contained an *Eco*RI site for cloning into pSP64Poly(A) (Promega). Sequencing was performed according to Ausubel et al. (1991.) *Cact*^{ΔPEST} was constructed by PCR mutagenesis using TTT GCC GTG CCA AAC GAA as the 5' primer and ACT GTC CGG ATC ATC ATA CGG TCTC as the 3' primer. This inserted two tandem stop codons just prior to the PEST sequence. *Age*I, a site just downstream of the stop codons, and *Bsp*EI, an upstream site, were used to clone the mutant fragment into the wild-type vector. *CaSpeR* and *pr25.7wc* were used to generate stable transformants (Ashburner 1989b).

Western blots

Samples were boiled for 5 min, spun at 13,000 rpm for 2 min, and run on a 7% polyacrylamide gel. The proteins were transferred onto nitrocellulose (Sigma) and probed with 1:1000 mouse anti-Cactus antibody from S. Kidd as described (Ausubel et al. 1991). A goat anti-mouse HRP-conjugated antibody (Bio-

Rad) was used as the secondary antibody, and ECL (Amersham) was used for detection. Scanning densitometry was used to measure band densities.

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