Separable and redundant regulatory determinants in Cactus mediate its dorsal group dependent degradation

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SUMMARY

Dorsal-ventral polarity within the Drosophila syncytial blastoderm embryo is determined by the maternally encoded dorsal group signal transduction pathway that regulates nuclear localization of the transcription factor Dorsal. Nuclear uptake of Dorsal, a Rel/NFkB homolog, is controlled by the interaction with its cognate IκB inhibitor protein Cactus, which is degraded on the ventral side of the embryo in response to dorsal group signaling. Previous studies have suggested that an N-terminally located kinase target motif similar to that found in IκB proteins is involved in the spatially controlled degradation of Cactus. We report studies of the in vivo function and distribution of fusion proteins comprising segments of Cactus attached to Escherichia coli β-galactosidase (lacZ). Full-length Cactus-lacZ expressed in vivo normalizes the ventralized phenotype of embryos that lack Cactus and faithfully reconstitutes dorsal group-regulated degradation, while fusion protein constructs that lack the first 125 amino acids of Cactus escape dorsal group-dependent degradation. Furthermore, Cactus-lacZ constructs that lack only the putative IκB-dependent kinase target-like motif can nevertheless undergo spatially regulated dorsal group-dependent degradation and we have identified the regulatory determinant responsible for dorsal group-dependent degradation of Cactus in the absence of this motif. Taken together, our studies indicate the presence of two distinct redundantly acting determinants in the N terminus of Cactus that direct dorsal group-dependent degradation. Strikingly, the regulatory domain of human IκBα can also direct polarized degradation of Cactus-lacZ fusion protein.

Key words: Drosophila, Dorsal-ventral polarity, Dorsal group, Dorsal protein, Cactus, IκB, IKK, NFκB, protein degradation

INTRODUCTION

In Drosophila, embryonic dorsal-ventral (DV) polarity is defined by a signal transduction pathway comprising the products of 12 known maternal effect genes (Morisato and Anderson, 1995), the dorsal group genes and cactus. The eleven dorsal group gene products act together to promote the activation and nuclear localization of Dorsal, whereas Cactus sequesters Dorsal and prevents its nuclear accumulation (Morisato and Anderson, 1995). A central event in the dorsal group signaling pathway is the ventral activation of the Toll receptor (Hashimoto et al., 1988; Hashimoto et al., 1991) by a processed form of its ligand, Spätzle (Morisato and Anderson, 1994; Roth, 1994; Schneider et al., 1994). Activated Toll then propagates a ventralizing signal into the cytoplasm of the syncytial blastoderm embryo and, acting through the products of the tube and pelle genes (Hecht and Anderson, 1993; Grosshans et al., 1994), induces the ventral degradation of Cactus (Belvin et al., 1995; Bergmann et al., 1996; Reach et al., 1996). In regions where Cactus is degraded, Dorsal is released, allowing its nuclear uptake (Bergmann et al., 1996; Reach et al., 1996). Depending on its nuclear concentration (Roth et al., 1989; Rusch and Levine, 1996) and promoter context (Jiang et al., 1992; Pan and Courrey, 1992; Ip, 1995), Dorsal then activates or represses specific zygotic genes required for DV patterning.

A key unresolved issue about Drosophila DV patterning concerns the nature of the molecular mechanism that spatially regulates Cactus degradation. Potential answers to this question may derive from studies of the regulation of the transcription factor Nuclear Factor kappa B (NFκB), which is homologous to Dorsal and controls aspects of the mammalian immune and inflammatory responses. (Wasserman, 1993; Belvin and Anderson, 1996).

NFκB is regulated by a diverse array of stimuli that lead to activation of the Interleukin 1 Receptor (IL-1R) whose intracellular domain is homologous to that of the Drosophila Toll receptor (Gay and Keith, 1991; Schneider et al., 1991). Upon binding of IL-1 to its receptor, an intracellular signal is transmitted that ultimately results in phosphorylation of IκBα...
at serines 32 and 36 (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995; Whiteside et al., 1995) by the IκB-
dependent kinase (IKK; DiDonato et al., 1997; Regnier et al., 1997; Karin and Ben-Neriah, 2000). Phosphorylation at these
sites is followed by polyubiquitination at a nearby lysine and
degradation through the ubiquitin proteasome pathway (Chen
et al., 1995; Scherer et al., 1995). Once freed from its inhibitor,
NFκB then enters the nucleus where it regulates transcription
(Verma et al., 1995; Ghosh et al., 1998).

cactus encodes a Drosophila homolog of IκBα. Like IκBα,
Cactus carries six complete copies of a 33 amino acid motif
termed the ankyrin repeat (Fig. 1). Cactus also contains a
region homologous to the IKK motif (Fig. 1; Whiteside et al.,
1995; Reach et al., 1996). The question of whether this motif
is involved in dorsal group-dependent degradation has been the
subject of some controversy. In one report, RNA encoding a
mutant form of Cactus, in which the critical serines (74 and
78) and two nearby serines (82 and 83) had been converted to
alanine, generated a dominant dorsalizing form of Cactus
(Reach et al., 1996). In a second report, RNA
injection was used to express N-terminally deleted forms of
Cactus that lacked the putative IKK target motif, which were
nevertheless capable of rescuing embryos from Cactus mutant
mothers to hatching, suggesting that the deleted form of Cactus
retained some aspect of normal regulation (Bergmann et al.,
1996).

In an effort to identify determinants of Cactus important for
its dorsal group-dependent degradation, and to address the
question of whether the IKK target-like motif in Cactus is
required, we have used a strategy in which transgenically
expressing forms of Cactus are expressed in vivo as in-frame
fusions to E. coli β-galactosidase (lacZ). We have found that
full length Cactus-lacZ expressed in the Drosophila germline is
capable of restoring normal pattern to the embryonic
progeny of cactus mutant females. Moreover, the Cactus-lacZ
fusion protein exhibits an asymmetric distribution along the
embryonic DV axis (hereafter referred to as the Cactus
degradation gradient) that is dependent on dorsal group
signaling. By constructing N-terminally deleted derivatives of
the Cactus-lacZ protein, we have been able to show that
determinants within the first 125 amino acids are required for
dorsal group-dependent degradation of Cactus-lacZ. Strikingly,
the identified IKK target-like motif is dispensable for regulated
degradation. Rather, our data show that in addition to the
previously identified IKK motif, a second distinct motif in the
N terminus of Cactus is capable of mediating signal-dependent
decay. Finally, we have been able to demonstrate that a
Cactus-lacZ derivative in which the endogenous regulatory
determinants have been substituted by those from IκBα can
nevertheless provide Cactus function and undergo polarized
degradation.

MATERIALS AND METHODS

Fly stocks and maintenance
All stocks were maintained employing standard conditions and
procedures. Larval cuticles were prepared according to Van der Meer
(Van der Meer, 1977). Embryos were staged according to Campos-
Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985).

Expression of Cactus-lacZ fusions in the female germline
Several strategies were used to generate the plasmids encoding the
Cactus-lacZ fusion proteins analyzed in this work. For plasmids
encoding full-length Cactus-lacZ, Δ51, Δ76, A101, Δ125 and Δ144
derivatives of Cactus-lacZ, and the Cactus(NTerm)-lacZ construct,
segments of the cactus-coding region were amplified by PCR, using
pNB3-cactus (Geisler et al., 1992) as a template. cactus-derived
PCR products were subcloned into the unique BamHI site of
pCaMatβGalBam as BamHI/BclI fragments, which placed the Cactus
protein determinants downstream of the Drosophila Tubulin alpha-4
(α67C) promoter and first seven codons of the β-galactosidase protein,
and upstream of the remainder of β-galactosidase. pCaMatβGalBam is a
derivative of CaMatGal(+), DF311 (a kind gift from Dominique Ferrandon; Matthews et al., 1989), which carries a
single BamHI cloning site. A similar strategy was used in the
preparation of the plasmid encoding Δ101(S104A)-Cactus-lacZ. However in this case, the N-terminal oligonucleotide used in the
amplification contained an altered sequence that introduced the
desired mutation into the Cactus part of the fusion protein.

In the case of the plasmids encoding the (S74,78A),
(S74,78,82,83A), Δ101(S116A), Δ101(S104,116A) and the
Δ101(K108R) derivatives of Cactus-lacZ, site-directed mutations
were initially introduced into pNB3-cactus using the Quik Change
Mutagenesis Kit (Strategene). In a second step, cactus
sequences were amplified using the mutant construct as a template and subcloned into pCaMatβGalBam as described above. The preparation of
(S74,78,116A) Cactus-lacZ required a second round of mutagenesis,
carried out on the S74,78A-containing Cactus construct, before the
amplification step for cloning into pCaMatβGalBam.

For the construction of Δ101-144Cactus-lacZ, PCR was used to
amplify the first 100 amino acids. The amplification product was cut
with BamHI/BclI and subcloned into BamHI cut Δ144Cactus-lacZ,
thus placing the first 100 amino acids (plus two additional codons encoding Asp and Pro) upstream of amino acid residue 145. For the preparation of (S74,78,82,83A)Δ101-144Cactus-lacZ, the quadruple
mutant derivative (S74,78,82,83A)Cactus-lacZ was used as the template for amplification, prior to subcloning into Δ144Cactus-lacZ.
Similarly, in the preparation of IκBαΔ144Cactus-lacZ and
IκBmutantΔ144Cactus-lacZ, wild-type and mutant (Traenckner et al.,
1995) derivatives of IκB cDNA were used as template for the
amplification of the region encoding the first 68 amino acids of IκB.
Amplification products were again cut with BamHI/BclI and
subcloned into BamHI cut Δ144Cactus-lacZ.

The sequences of oligonucleotides used in the generation of plasmid constructs described in this work are available on request.

Two-hybrid studies
Experiments with yeast were performed according to standard
protocols (Rose et al.,1988; Schiestl and Gietz, 1989; Gyuris et al.,
1993) using the yeast strain EGY48 carrying pSH18-34. The
two-hybrid bait plasmids used in this study contained the following
gene coding regions cloned downstream and in-frame with lexA in the
plasmid pEG202: (1) lex-cactus – Cactus residues 2-510; and (2) lex-
pelle – Pelle residues 1-511 (Grosshans et al., 1994).

The two-hybrid prey plasmids used in this study contained the
following gene-coding regions, cloned downstream and in frame with
the bacteria-derived activator sequence carried in pJG4-5: (1) JG-
Cactus – Cactus residues 2-510; (2) JG-CactusΔN – Cactus residues
146-510; (3) JG-CactusΔC – Cactus residues 2-219; (4) JG-Dorsal –
Dorsal residues 2-678; (5) JG-Joke – Joke residues 1-462 (Grosshans

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cleavage-stage embryos (data not shown), but at syncytial blastoderm stage, this distribution became enriched dorsally, consistent with the view that Cactus present on the ventral side of the embryo is degraded via the action of dorsal group signaling. This dependence on dorsal group signaling was demonstrated by the observation that Cactus-lacZ expressed in the progeny of pipelpipe dorsal group mutant females never exhibited a ventral loss of Cactus-lacZ, remaining uniformly expressed throughout cleavage and blastoderm stages of embryogenesis (Fig. 2D).

Females homozygous for the hypomorphic cac011 allele produce ventralized (V3, Roth et al., 1991) embryos (Fig. 2A), consistent with a partial loss of Cactus function (Roth et al., 1991; Bergmann et al., 1996). Expression of Cactus-lacZ in cac011/cac011 homozygote females resulted in the production of hatching progeny (Fig. 2B), indicating that the Cactus-lacZ fusion protein is capable of providing Cactus function. A PEST domain (Rogers et al., 1986; Rechsteiner, 1990) in the C-terminal region of Cactus has been implicated in the overall lability of Cactus, although this motif is not believed to play a role in dorsal group-dependent Cactus degradation (Belvin et al., 1995; Bergmann et al., 1996; Liu et al., 1997). Consistent with this notion, the protein distributions and rescuing abilities of transgenic inserts that expressed full-length versus PEST-deleted fusions to lacZ were indistinguishable (data not shown).

**Determinants in the first 125 amino acids of Cactus are required for dorsal group-dependent degradation**

In order to define elements within Cactus required for dorsal group-dependent degradation, we constructed derivatives of Cactus-lacZ with N-terminal deletions of various sizes. Cactus-lacZ that lacked the PEST domain was selected as the starting point for this and all other analyses to be described. Several previously reported observations have suggested that determinants in the N terminus of Cactus are involved in its regulated degradation. First, two P-element-derived alleles

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**RESULTS**

**A Cactus-lacZ fusion protein provides Cactus function and exhibits dorsal group-dependent spatial regulation**

Previous attempts to investigate the distribution of maternally expressed Cactus protein have been complicated by the zygotic expression of the gene early during embryogenesis (Bergmann et al., 1996). In order to focus exclusively on the function and dorsal group regulation of Cactus expressed maternally, we generated transgenic females that expressed Cactus as an in-frame N-terminal fusion to *E. coli lacZ and expressed it in the germline under the control of the α-tubulin 67C promoter, which is expressed exclusively maternally in the nurse cells (Matthews et al., 1989). The embryonic progeny of females that expressed the Cactus-lacZ fusion exhibited a distribution of both β-galactosidase protein (Fig. 2E) and lacZ enzymatic activity (Fig. 2C) identical to that published previously for endogenous Cactus protein (Bergmann et al., 1996). Cactus-lacZ expressed in the progeny of wild-type females was distributed uniformly in

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**Embryo antibody staining**

Antibody staining of embryos was carried out as described by Roth et al. (Roth et al., 1989). For detection of Cactus-lacZ, a monoclonal antibody against β-galactosidase (40-1a, Developmental Studies Hybridoma Bank, University of Iowa) was used in conjunction with an HRP-conjugated secondary anti-mouse antibody. For detection of Dorsal protein, a rabbit polyclonal antibody was used (Roth et al., 1989) in combination with biotinylated anti-rabbit secondary antibody and visualized with avidin/HRP complex (Vector Laboratories).

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**β-Galactosidase detection in embryos**

Embryos were stained for β-galactosidase activity according to Kamb et al. (Kamb et al., 1991) with the following modifications. Embryos were dechorionated in heptane saturated with 2.5% glutaraldehyde, then resuspended in 1-2 ml of heptane. After addition of an equivalent volume of methanol, the embryos were shaken vigorously for 30 seconds and allowed to settle. The heptane and most of the methanol were then aspirated. Embryos were then briefly rinsed once with methanol and once with 80% ethanol, followed by aspiration of the residual alcohol. The embryos were washed extensively (three times for five minutes each, four times for 30 minutes each) in 10 ml of PBT(PBS + 0.5% Triton X-100), then transferred to 1.5 ml Eppendorf tubes and stained in 1 ml of staining solution (10mMPO4 buffer, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 3 mM K2[FeII(CN)6], 3 mM K3[FeIII(CN)6], 0.3% Triton X-100 and 20 μl of 10%X-Gal solution in DMSO) until color was detected (20 minutes to overnight). Embryos were washed thoroughly with PBT and then incubated overnight at 65°C. Finally, the embryos were mounted in PBS/glycerol (1:1) and photographed using Nomarski optics.

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**Fig. 1.** Structural comparison between Cactus and 1xBtz. Schematic diagrams of Cactus and 1xBtz are shown with hatched yellow regions indicating the positions of the six complete ankyrin repeats in each protein. The position and sequence of the identified IKK target motif is shown above 1xBtz, as are the position of the two Cactus regulatory elements discussed in this study. The position of serine (red) and lysine (blue) residues located N-terminal to the first ankyrin repeat are shown. Lysine and serine residues present in other parts of the proteins are not shown.
(cactBQ and cactE10) that result in the expression of a Cactus protein lacking the N-terminal 144 amino acids lead to a gain-of-function dorsalizing phenotype, owing to its inability to undergo dorsal group-dependent degradation (Bergmann et al., 1996; Roth et al., 1991), thus leading to cytoplasmic retention of Dorsal. Second, Cactus carries a stretch of amino acids similar to the IKK target motif in the N terminus of Cactus (DS32 GYXS36 in IκBα versus DS74 GYXS78 in Cactus; Fig. 1; Reach et al., 1996). Although our previous work suggests that this portion of Cactus is not required for dorsal group-dependent degradation (Bergmann et al., 1996), another group has reported evidence to suggest that this motif does play a role in Cactus degradation (Reach et al., 1996). However, neither publication directly assessed the subcellular distribution of Cactus that lacked this motif. Rather, both studied the ability of Cactus derivatives that lacked the putative IKK region to provide polarized Cactus function. Our observations of Cactus-lacZ indicated that it would be possible to assess the distribution and function of modified derivatives of Cactus directly, including ones with alterations in the putative IKK target motif.

Cactus-lacZ derivatives lacking the first 51, 76, 101, 125 or 144 amino acids of Cactus were expressed transgenically in the female germline. Deletions of up to 101 amino acids resulted in a fusion protein that rescued the ventralized phenotype of cactD13/+ females (not shown) and exhibited ventral degradation of Cactus-lacZ protein (Fig. 3A-D). When Cactus-lacZ fusions lacking the first 125 (Fig. 3F) or 144 (not shown) amino acids of Cactus were similarly expressed in the female germline, these fusion proteins led to the formation of embryos that were strongly dorsalized, regardless of whether the expressing females were wild type or homozygous for the cactD11 allele. Both lacZ activity (Fig. 3E) and antibody staining (not shown)
revealed that these proteins were expressed uniformly along the DV circumference of blastoderm embryos and had lost their ability to form a gradient. Staining with anti-Dorsal confirmed that the dorsalizing phenotype attributed to these transgenes resulted from constitutive cytoplasmic retention of Dorsal (Fig. 3H). By contrast, Dorsal protein expressed in wild-type embryos (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989) or in ΔI01cact-lacZ females (Fig. 3G) exhibited a ventral-to-dorsal nuclear gradient with highest nuclear levels ventrally. Taken together, these results indicate that regulatory determinants capable of mediating polarized degradation of Cactus are present even when the first 101 amino acids, which includes the putative IKK target motif, are deleted. These determinants, however, are lost with the deletion of an additional 24 amino acids, suggesting the existence of critical residues in interval between amino acid residues 101 and 125 of Cactus.

**Spatially-specific degradation of Cactus-lacZ requires Dorsal**

Cactus-lacZ transgenes that undergo ventral degradation in the embryos produced by otherwise wild-type females failed to exhibit spatially asymmetric distribution in the embryos of dl¹/dl¹ females (Fig. 4B). These observations indicate that Dorsal protein is required for the observed dorsal group-dependent degradation of the Cactus-lacZ constructs and suggest that, in vivo, the species of Cactus-lacZ undergoing ventral degradation exists in a nascent complex with Dorsal protein. This idea is supported by the observation that a fusion protein comprising the first 160 amino acids of Cactus (but lacking the ankyrin repeat domain, which interacts with Dorsal) fused in-frame to lacZ was incapable of directing spatially regulated degradation (Fig. 4C), in spite of the fact that our other observations indicate that key regulatory elements are present in this segment of Cactus. Taken together, these findings suggest that an interaction with Dorsal is necessary for graded degradation.

**Dimerization with endogenous Cactus is not responsible for regulated degradation of Cactus-lacZ**

As cactus is a zygotically required gene, it is not possible to generate adult females that completely lack Cactus function, although it is possible to specifically eliminate Cactus function in the germline by generating clones (Roth et al., 1991; Bergmann et al., 1996). In the experiments described above, the function and distribution of Cactus-lacZ was assessed after expression in females that also express either wild-type Cactus or the hypomorphic cact⁶⁶ allele. Although the predominant form of Cactus detected in vivo is a heterotrimeric complex that includes one molecule of Cactus and two molecules of Dorsal (Isoda and Nüsslein-Volhard, 1994), Cactus dimers have also been detected (Isoda and Nüsslein-Volhard, 1994; Edwards et al., 1997). The existence of a dimerized form of Cactus raises the possibility that a degradation gradient of Cactus-lacZ exists that originates through an interaction with endogenous Cactus protein that is itself undergoing regulated degradation. In such a situation, the introduced Cactus-lacZ might not itself be providing determinants necessary for regulated degradation. The ability to associate with endogenous Cactus might be sufficient to bring it under regulatory control.

![Image](351x476 to 536x721)

**Fig. 4.** Polarized degradation of the Cactus-lacZ protein requires Dorsal. Maternally expressed full-length Cactus-lacZ visualized by lacZ activity exhibits a normal polarized distribution in the progeny of females heterozygous for dl¹ (A), while ventral degradation is not observed in the progeny of females homozygous for dl¹ (B). Similarly, a deletion derivative Cactus(Nterm)lacZ in which the N-terminal 161 amino acids of Cactus are fused to lacZ, and which lacks the Dorsal-binding ankyrin repeat region, did not exhibit spatially regulated degradation (C).

In order to test this idea directly, we expressed the N terminally deleted Cactus-lacZ derivatives described above in a cacBQ/cacE¹₀ maternal background. cacBQ/cacE¹₀ mutant females produce completely dorsIALIZED progeny in which all Dorsal protein was constitutively retained in the cytoplasm (Roth et al., 1991). As mutant Cactus protein produced by these two alleles fails to undergo dorsal group-dependent degradation (Bergmann et al., 1996), any spatially asymmetric degradation of the Cactus-lacZ fusion protein produced in this mutant background must result from the intrinsic ability of the fusion protein to respond to regulatory inputs. We observed that Cactus lacking the N-terminal 101 amino acids (Fig. 5A), as well as full-length, Δ51 and Δ76Cactus-lacZ (not shown) were capable of undergoing regulated degradation in a cacBQ/cacE¹₀ mutant background. In addition, these constructs led to substantial rescue of the dorsalizing phenotype attributable to cacBQ/cacE¹₀ (Fig. 5C,D). In contrast, Cactus-lacZ lacking the first 125 amino acids could neither form a DV gradient (Fig. 5B) nor rescue the cacBQ/cacE¹₀ phenotype (Fig. 5E).

The possibility of a Cactus protein that lacks cis regulatory elements undergoing regulated degradation by virtue of interacting with regulated protein is also contradicted by the finding that the Δ125Cactus-lacZ fusion protein does not undergo polarized degradation in embryos that also contain wild-type Cactus (Fig. 3E), in spite of the fact that two-hybrid analysis indicates that Cactus lacking the first 125 amino acids can dimerize with full-length Cactus (Fig. 5F), at least in *Saccharomyces cerevisiae*. This indicates that the regulated degradation of Cactus constructs lacking the putative IKK
target motif cannot be explained by a mechanism in which the mutant Cactus interacts with the endogenous Cactus, thereby bringing the introduced protein under its regulatory influence. Taken together, these observations argue that Cactus-lacZ undergoes regulated degradation under the influence of regulatory determinants intrinsic to the fusion protein and focus on the interval between amino acids 101 and 125 as containing a crucial regulatory determinant.

Expression of Cactus-lacZ constructs in the cact<sup>BQ</sup>cact<sup>E10</sup> background also provided the most compelling demonstration of the dispensable nature of the putative IKK motif for regulated Cactus degradation. Cactus-lacZ constructs in which both Ser<sup>74</sup> and Ser<sup>78</sup> or all four of Ser<sup>74</sup>, Ser<sup>78</sup>, Ser<sup>82</sup> and Ser<sup>83</sup> had been converted to alanines had the ability to undergo ventral degradation in the embryos produced by cact<sup>BQ</sup>cact<sup>E10</sup> females (Fig. 6A,B). Moreover, both mutant constructs facilitated a significant level of rescue of the dorsalized phenotype associated with cact<sup>BQ</sup>cact<sup>E10</sup> maternal genotype (Fig. 6C,D), as well as rescuing the ventralized phenotype of embryos produced by cact<sup>011</sup>cact<sup>011</sup> mutant females (Fig. 6E,F).

**Redundant determinants in the N terminus of Cactus can direct regulated degradation**

The experiments described above focus attention on the interval between amino acids 101 and 125 as containing key regulatory determinants of dorsal group-dependent Cactus degradation. In order to determine their roles, the two serine residues located in this interval (104, 116) were converted to alanine both individually and together, in the context of a Cactus-lacZ construct lacking the first 101 amino acids. Embryos that expressed the (S104,116A)-lacZ construct (Fig. 7B) and the (S116A)Cactus-lacZ constructs (Fig. 7D) displayed a uniform distribution of Cactus-lacZ and were both incapable of restoring ventral pattern elements when expressed in a cact<sup>BQ</sup>cact<sup>E10</sup> background (not shown). In contrast, the mutant derivative that carried (S104A)Cactus-lacZ (Fig. 7A) was capable of directing the formation of a Cactus-lacZ gradient (Fig. 7C) and of providing polarized Cactus function (not shown). These observations illustrate a key role for serine residue 116 in the regulation of Cactus distribution, at least in the absence of the first 101 amino acids of Cactus.

In the case of IkBα, a pair of lysine residues located about 10 residues to the N-terminal side of the phosphorylated serines at residues 32 and 36 provide the basis for regulated ubiquitination (Scherer et al., 1995). In the case of Cactus, a lysine residue located at residue 108 could provide a similar function in ubiquitin addition if serine 116 is indeed a target for phosphorylation. To test a requirement for lysine 108 in the context of S116-dependent regulation of Cactus, K108 was converted to arginine in a Cactus-lacZ derivative that lacked the first 101 amino acids. Interestingly, this construct was capable of forming a DV gradient of Cactus degradation (Fig. 7I), and of providing polarized Cactus function (not shown), demonstrating that a lysine residue need not be located N-terminal of S116 to facilitate its regulatory function. While studies of IkBα regulation indicate a crucial reliance on lysine residue positioning (Scherer et al., 1995), other proteins regulated by ubiquitination (e.g. C-Jun, Gcn4) do not rely on specific lysine residues (Hochstrasser and Kornitzer, 1998). Therefore, Cactus, while different from IkBα, may not be unusual in this respect.

Although the results above indicate that S116 is capable of imparting dorsal group-dependent regulation to Cactus, they do not necessarily indicate that this is the only serine residue in the N terminus of Cactus capable of providing this function. To determine whether other regulatory motifs are contained within the N-terminal 101 amino acids, residues 1 to 100 were fused directly to amino acid 145 of Cactus, in the context of a lacZ fusion protein. In this way, residues extending from amino acids 101 to 144 were deleted, removing the influence of S116. Interestingly, this construct was still capable of forming a
The Cactus IKK target motif is redundant

Cactus-lacZ degradation gradient, as assessed by lacZ staining (Fig. 7E), and was capable of restoring polarized cactus function to the progeny of cact^BQ/cact^E10 females (not shown). When serines 74, 78, 82 and 83 were converted to alanine in the context of the construct that lacked amino acids 101-144, the resultant construct was no longer capable of undergoing polarized degradation (Fig. 7F). Taken together, these results indicate that two redundant and separable determinants, at least one contained in the first 101 amino acids of Cactus, and a second located between residues 101 and 125, are capable of directing Cactus to undergo dorsal group-dependent degradation. Final confirmation of this notion was provided by the observation that full-length Cactus-lacZ in which just serines 74, 78 and 116 had been converted to alanine residues was incapable of undergoing polarized degradation (Fig. 7G) and was dominantly dorsalizing (Fig. 7H).

IκB regulatory determinants can substitute for Cactus sequences that direct polarized degradation

To test for functional similarity in the mechanisms directing regulated degradation of IκB versus Cactus, we created a Cactus-lacZ fusion construct that lacked the N-terminal regulatory determinants described above and instead carried a 68 amino acid stretch of human IκBα, which includes the characterized IKK target site. Strikingly, this construct was...
able to direct the formation of a Cactus-lacZ degradation gradient (Fig. 8A) and, when expressed in the progeny of cact<sup>β11/β11</sup> females, was able to rescue the ventralized phenotype to hatching (Fig. 8C). In contrast, when serine residues 32 and 36 of IκB were converted to alanine in the context of the same construct, the resultant protein did not undergo polarized degradation (Fig. 8B) and dominantly dorsalized progeny of cact<sup>β11/+</sup> females that would have otherwise produced hatchers (Fig. 8D). These observations suggest that the IκBα regulatory determinants can recruit the degradation machinery that normally mediates dorsal group-dependent degradation of Cactus and that the endogenous regulatory determinants of Cactus are likely to act in an analogous manner to those in IκBα.

**DISCUSSION**

Considerable study of the regulation of NFκB following activation of the IL-1R indicates that a specific kinase complex mediates the phosphorylation of IκBα at serines 32 and 36 (DiDonato et al., 1997; Regnier et al., 1997; Karin and Ben-Neriah, 2000), followed by recruitment of SCF β-TrCP complex (Spencer et al., 1999; Winston et al., 1999), ubiquitination of IκBα at lysines 21 and 22 (Scherer et al., 1995) and degradation. Several experiments have demonstrated the key importance of the identified serines and lysines in the degradation of IκBα. Conversion of serines 32 and 36 to alanine (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995; Whiteside et al., 1995) or of lysines 21 and 22 (Scherer et al., 1995) to arginine results in a dominant gain-of-function effect, and in an inability to activate NFκB activity bound to the mutant IκBα. In contrast, conversion of serines 32 and 36 to glutamate results in constitutive degradation of IκBα and of constitutive NFκB activity (Brockman et al., 1995).

The regulation of Dorsal nuclear localization by Cactus has remained less well understood. To some extent, this knowledge gap stems from a more complicated structure of Cactus, in comparison with other IκB proteins. Cactus, like IκBα and the other IκB proteins, carries six copies of the ankyrin repeat motif (Geisler et al., 1992; Kidd, 1992), which in all characterized IκB activities are involved in the physical interaction with their cognate Rel homologous proteins (Verma et al., 1995; Baldwin, 1996; Belvin and Anderson, 1996; Ghosh et al., 1998). The identification of the gain-of-function alleles cact<sup>βQ</sup> and cact<sup>β10</sup> has directed attention at the region of Cactus N-terminal to the ankyrin repeat regions, as potentially containing determinants of dorsal group-mediated regulation (Roth et al., 1991; Bergmann et al., 1996). In IκBα, the 72 amino acids to the N-terminal side of the ankyrin repeat region contain three serine residues and four lysine residues (Fig. 1), including the critical ones described above (Haskill et al., 1991). In the case of Cactus, the 228 amino acids to the N terminus of the first ankyrin repeat contain 36 serine residues and 11 lysines (Geisler et al., 1992; Kidd, 1992; Fig. 1), providing the possibility for much greater level of complexity in the control of Cactus regulation.

In the N-terminal 100 amino acids of Cactus, a segment with strong homology to the putative IKK target motif has been identified (Fig. 1) and investigated via injection of RNA carrying mutations and deletions in this region. However no consensus was obtained as to whether the IKK target-like motif is essential for the regulation of Cactus. In the studies of both Reach et al. (Reach et al., 1996), and Bergmann et al. (Bergmann et al., 1996), the phenotypes observed following expression of the mutant Cactus derivatives was a composite of the effects of the mutant together with the endogenous (wild-type or mutant) Cactus present in the embryos. Additional variability in the phenotypes is also likely to have arisen as a consequence of differences in the amount of RNA injected from embryo to embryo. In neither case was the distribution or function of the introduced Cactus assessed directly.

In order to address these technical issues and as a way of assessing the intrinsic ability of mutant Cactus derivatives to undergo spatially regulated degradation, we turned to the strategy of assaying directly the distribution and functionality of introduced Cactus in the context of Cactus-lacZ fusions. Full-length Cactus-lacZ was observed to reconstitute function and binding to Dorsal (data not shown). Strikingly, this protein also exhibited dorsal group-dependent regulation of subcellular distribution, as assessed by lacZ enzymatic activity, in spite of the fact that active β-galactosidase acts as a tetramer (Jacobsen et al., 1994). This suggests that tetramerization did not inhibit the ability of the protein to interact with Dorsal or the other cellular machinery mediating spatially regulated degradation.

By constructing specific mutant derivatives of Cactus in the context of a lacZ fusion protein, we have been able to demonstrate the redundant nature of the previously hypothesized IKK motif in Cactus. Deletions mutants that lack this region and site-directed mutants in which the critical serines have been converted to alanine residues have illuminated a second putative regulatory target in the N terminus of Cactus, located at S116. Taken together, these results indicate that redundant determinants present in the N terminus have the potential to confer upon the protein the ability to be degraded in a graded fashion. Interestingly, serine 116 is contained within the context DSGIID. Substitution of the second aspartic acid residue to a serine would convert this to the IKK consensus target motif (DSGPPXS). In view of the finding that conversion of the critical serines 32 and 36 in IκBα to glutamic acid results in a constitutively ubiquitinated protein, serine 116 may be present in the context of a second IKK-like target motif, one that is in fact predisposed towards an interaction with the degradation machinery, after a single phosphorylation event.

What is the basis for the presence of redundant regulatory determinants in the N terminus of Cactus? The formation of a gradient of nuclear Dorsal where high, intermediate, low and undetectable levels are required (Roth et al., 1989; Rusch and Levine, 1996) might necessitate a level of regulatory control in Cactus degradation that is not required of IκB in vertebrate cells where NFκB is simply active or inactive. On the ventral side of the *Drosophila* embryo, one might suppose that all regulatory serines in Cactus are phosphorylated, leading to complete degradation with partial phosphorylation in lateral regions, which leads to intermediate levels of degradation. While our studies of the distribution of Cactus-lacZ fusion that lacks either putative regulatory motif shows that both sites can act alone in the formation of a Cactus gradient, we cannot rule out the possibility of subtle changes in degradation gradients formed by these mutant proteins, in comparison with that...
Fig. 8. The N terminus of IκBα can substitute for the N terminus of Cactus to direct regulated degradation. A Cactus-lacZ fusion derivative in which the first 71 amino acids of human IκBα were fused to the N-terminus of Δ144Cactus-lacZ was expressed under α-tubulin 67C transcriptional control and its distribution determined by lacZ activity. This construct exhibited polarized degradation (A) and the ability to rescue DV polarity in the otherwise ventralized progeny of cac011/cac011 mutant females (C). In contrast, a derivative in which the IKK target site serines 32 and 36 had been converted to alanine exhibited a uniform distribution (B) and led to the dominant dorsalization of what would have otherwise been normal hatching progeny from cac011/+ females (D).

exhibited by wild-type Cactus. Interestingly, in support of the idea that multiple regulatory determinants are required for the formation of a normal Dorsal gradient, our previous observations (Bergmann et al., 1996) indicated that Cactus constructs lacking the previously identified putative IKK motif did not behave in an entirely wild-type manner, in comparison with introduced wild-type Cactus. Rather, this construct was weakly dorsalizing, leading to our suggestion that multiple determinants in Cactus were required for the formation of normal Cactus and Dorsal gradients.

Requirement for Cactus function in multiple processes in Drosophila provides a second possible reason for the presence of multiple regulatory determinants that mediate degradation. In addition to DV pattern formation, Cactus-mediated regulation of Dorsal/Rel-homologues plays a part in immune function in the fly as well as other processes (Wasserman, 1993; Wasserman, 2000; Govind, 1999). The presence of two identified regulatory domains may reflect a situation in which the N-terminal region of Cactus acts as an antenna to integrate several regulatory inputs, leading ultimately to the destabilization of Cactus. In this regard, it is interesting to speculate that additional members of the 36 serines present in the N-terminal domain of Cactus not identified in this study remain candidates for putative target sites for Cactus regulation in other processes. Whether either or both of the regulatory determinants identified in this study influence Cactus function in the Drosophila immune response remains an interesting topic for speculation. Along these lines, we have previously shown that expression of the Rel homologous Drosophila protein Dif can confer a significant restoration of DV pattern elements to embryos that lack Dorsal (Stein et al., 1998). This rescue and the polarity of the rescued embryos depends upon the state of the dorsal group signaling pathway and upon Cactus, suggesting that Cactus can bring Rel homologous proteins under the control of signal transduction pathways in a way that is specific to the cell type. Thus, while there is no evidence to suggest that Dif activity in the Drosophila immune system is graded, Dif activity in rescuing Dorsal minus embryos exhibits a Cactus-dependent graded response, presumably owing to the ability of Cactus to undergo graded degradation. Ultimately, an answer to the question of why Cactus carries more than one regulatory determinant capable of inducing degradation awaits the identification of the specific components of the phosphorylation and degradation machinery that targets serines 74, 78 and 116 (and potentially others) in the regulation of Cactus in DV patterning, immune function and in the other processes to which Cactus contributes in the life of the fly.

In spite of the presence of IKK target-like motifs in the Cactus N terminus, there remains uncertainty about the identity of the kinase(s) that modifies these targets. Lu et al. (Lu et al., 2001) and Rutschmann et al. (Rutschmann et al., 2000) have identified mutations that affect the Drosophila homologs of IKKβ and IKKγ, respectively, and while these mutations have striking effects on the bacterial arm of the Drosophila immune system, they have modest if any effects on the pathway affecting embryonic DV polarity (Rutschmann et al., 2000; Lu et al., 2001). Furthermore, Silverman et al. (Silverman et al., 2000) have shown that the Toll signaling pathway is not inhibited by RNAi-mediated inhibition of either DmIKKβ or DmIKKγ. It remains to be determined whether a Drosophila homolog of IκKε, discussed by Silverman et al. (Silverman et al., 2000), constitutes the Cactus kinase that acts in DV pattern formation.

How similar are the events of dorsal group dependent degradation of Cactus to the mechanism accomplishing degradation of IκBα? Previous studies have identified a role for the F-box protein B-TrCP, in the regulated degradation of phosphorylated IκBα (Spencer et al., 1999; Winston et al., 1999). B-TrCP, a component of the IκB Ubiquitin ligase (SCF), targets IκB for degradation in response to signals that induce NFκB activity, binding directly to phosphorylated IκBα and thereby providing specificity to the degradation process (Maniatis, 1999). By analogy to the situation for IκBα it is possible that phosphorylation of serines 74 and 78, or of serine 116 results in recruitment of an SCF complex containing an F-box specificity-conferring protein, followed by ubiquitination and degradation of Cactus. Spencer et al. have demonstrated that Drosophila embryos deficient for Slimb, a fly homolog of B-TrCP, show an impaired ability to activate the expression of Dorsal target genes (Spencer et al., 1999). While inferring a role for Slimb in targeting the degradation of Cactus during embryogenesis, they did not test directly for an effect on the distribution of Cactus. We have observed that Cactus-lacZ
expressed in *slimb*/*slimb* germline clones (Chou and Perrimon, 1992) exhibits a weak though reproducibly asymmetric distribution (data not shown), and the embryos produced develop polarized pattern elements along the DV axis (data not shown). In addition to a requirement of Slimb in WG and HH signaling (Jiang and Struhl, 1998) Slimb plays a role in centrosome duplication (Wojcik et al., 2000), and we have observed that *slimb* mutant germline clone-derived embryos exhibit aberrant early events in embryogenesis (data not shown), suggesting that *slimb* function is required prior to the events establishing DV polarity. This early embryonic phenotype may complicate the accurate assessment of Slimb function in the regulation of Cactus.

Belvin et al. (Belvin et al., 1995) were the first to demonstrate that degradation of the Cactus protein mediates the formation of the Dorsal nuclear gradient (Belvin et al., 1995). They demonstrated that upon perivitelline injection of activated Spätzle into early eggs, endogenous Cactus protein could be observed to rapidly decay. They found a similar injection-dependent decay of Cactus protein upon injection into the embryonic progeny of dorsal mutant females, demonstrating that dorsal group-dependent degradation of Cactus did not depend on the presence of Dorsal. Paradoxically, we have found that spatially asymmetric degradation of Cactus-lacZ protein does require the presence of Dorsal protein. A number of possibilities could explain the discrepancy. One possible mechanism might arise from the existence of both signal-dependent and -independent modes of Cactus degradation. Cactus that is not complexed with Dorsal has been observed to be extremely labile. Part of the rationale of the experiments in which Belvin et al. (Belvin et al., 1995) demonstrated that Dorsal was not required for regulated Cactus degradation was to demonstrate that the dorsal group-dependent degradation did not arise simply from the signal-dependent degradation that occurs in a situation in which the Dorsal/Cactus complex breaks down. However, several studies have suggested dorsal group-dependent regulatory inputs that act directly upon Dorsal in mediating its nuclear input (Whalen and Steward, 1993; Gillespie and Wasserman, 1994; Drier et al., 1999; Bhaskar et al., 2000). Regulatory inputs that lead to a spatially regulated dissociation of the Dorsal/Cactus complex, or directly to Dorsal nuclear uptake might be expected to lead to the graded degradation of Cactus-lacZ via the signal-independent degradation mechanism. Conceivably, the Cactus-lacZ constructs do not support the regulated ubiquitination of the fusion protein, while retaining the ability to undergo a dorsal group-dependent dissociation from Dorsal. Such a situation could account for the dependence upon Dorsal protein. Arguing against this notion is the fact that while the Cactus C-terminal PEST domain has been implicated in the effectiveness of signal-independent degradation of Cactus, deletion of the PEST domain does not seem to markedly influence the formation of the Cactus-lacZ degradation gradient. It is also interesting to note that several investigators have suggested evidence that in vivo, Cactus exists in a multiprotein complex with Dorsal, Pelle and Tube (Yang and Steward, 1997; Edwards et al., 1997) leading to speculation that this multiprotein complex is an essential element of dorsal group signaling and supporting the notion that the presence in this complex and an interaction with Dorsal is a prerequisite dorsal group-dependent phosphorylation and/or ubiquitination of Cactus. Ultimately, an understanding of the discrepancy between our data and that of Belvin et al. (Belvin et al., 1995) requires a more complete understanding of the molecular machinery that mediates the graded degradation of Cactus, a focal point of future studies.

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REFERENCES


