Development 128, 2963-2974 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV5918

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

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Accepted 9 May 2001

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/NFκB 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 proteosome 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 that was apparently insensitive to dorsal group-dependent degradation (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).

cact^{D13}, *cact*^{011}, *cact*^{BQ}, *cact* ^{E10} have been described (Roth et al., 1991). Transformants were generated in a *w*/*w*, *cact*^{D13}/CyO or *w*/*w*; *cact*^{011}/CyO background by conventional microinjection of P-element based constructs (Rubin and Spradling, 1982). *pipe*^{386} and *pipe*^{664} (Anderson and Nüsslein-Volhard, 1984) and *dl*^{1} (Nüsslein-Volhard et al., 1980) have been described previously.

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, Δ101, 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 *Bam*HI site of pCaMatβGalBam as *Bam*HI/*Bcl*I 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 CaMatbGal+, DF311 (a kind gift from Dominique Ferrandon; Matthews et al., 1989), which carries a single *Bam*HI 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 *Bam*HI/*Bcl*I and subcloned into *Bam*HI-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 production of IκBΔ144Cactus-*lacZ* and IκBmutantΔ144Cactus-*lacZ*, wild-type and mutant (Traenckner et al., 1995) derivatives of the 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 *Bam*HI/*Bcl*I and subcloned into *Bam*HI 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-Tube – Tube residues 1-462 (Grosshans

et al., 1994); and (6) JG-Pellino – Pellino residues 33-424 (Grosshans et al., 1999).

For interaction studies, yeast clones carrying plasmid combinations were streaked on galactose/raffinose plates that lacked Ura, His, Trp and Leu, and the ability to direct expression of the Leu2 reporter gene was assessed by observing growth.

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).

β-Galactosidase detection in embryos

Embryos were stained for β-galactosidase activity according to Klambt et al. (Klambt 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 (10mMP0_4 buffer, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM K_4[FeII$(CN)_6$], 3 mM K_3[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.

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 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 *pipe*/*pipe* 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 *cact*^{011} 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 *cact*^{011}/*cact*^{011} 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

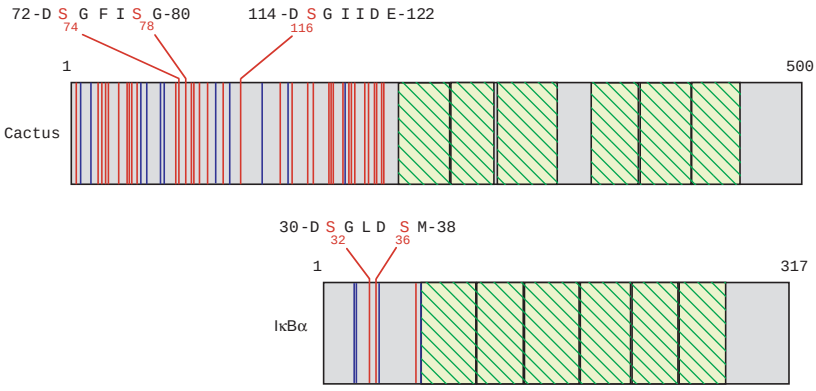

Fig. 1. Structural comparison between Cactus and IκBα. Schematic diagrams of Cactus and IκBα are shown with hatched yellow regions indicating the positions of the six complete ankyrin repeats in each protein. The position and sequence of the identified IKK target motif is shown above IκBα, as are the 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.

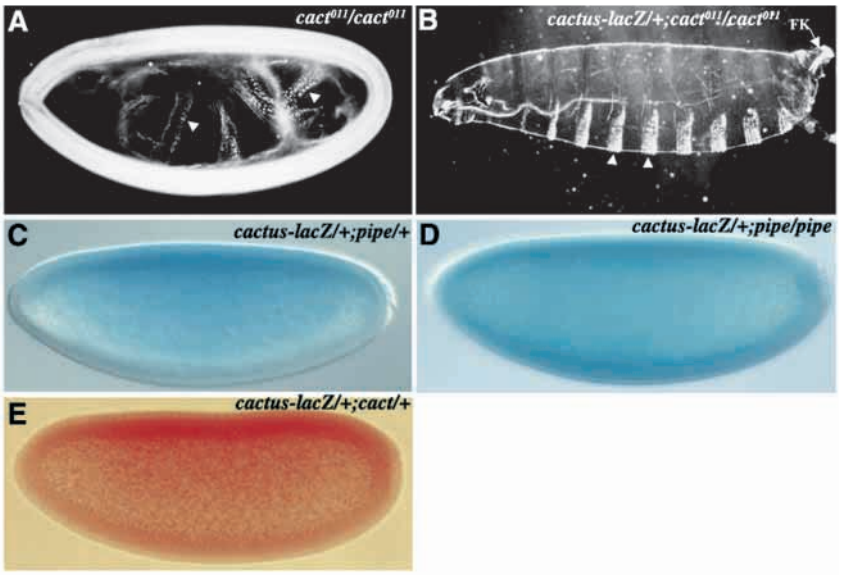

Fig. 2. Maternally expressed Cactus-*lacZ* fusion protein rescues Cactus function and exhibits dorsal group-dependent polar degradation. The Cactus-*lacZ* fusion protein was expressed in the female germline under the transcriptional control of the *alpha-tubulin 67C* promoter in *cact*^{011}/ *cact*^{011} females, resulting in hatching embryos with normal DV polarity (B). *cact*^{011}/ *cact*^{011} females normally produce ventralized embryos (A). Cactus-*lacZ* protein (visualized by enzymatic activity (C) or with anti-β-galactosidase antibody (E)) exhibited a dorsal enrichment in the progeny of wild-type females (*pipe*/+, C) similar to that seen for endogenous Cactus protein (Bergmann et al., 1996). Cactus-*lacZ* protein exhibited a uniform distribution in the progeny of dorsal group mutant females (*pipe*/*pipe*, D). In this and all other figures embryos are positioned with dorsal side upwards, anterior towards the left and maternal genotypes are shown at top right. Similarly, in all panels, arrowheads identify ventral denticle bands, while FK indicates the position of Filzkörper.

(*cact*^{BQ} and *cact*^{E10}) 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 ($DS_{32}G\Psi XS_{36}$ in IκBα versus $DS_{74}G\Psi XS_{78}$ 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 *cact*^{011}/*cact*^{011} 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 *cact*^{011} allele. Both *lacZ* activity (Fig. 3E) and antibody staining (not shown)

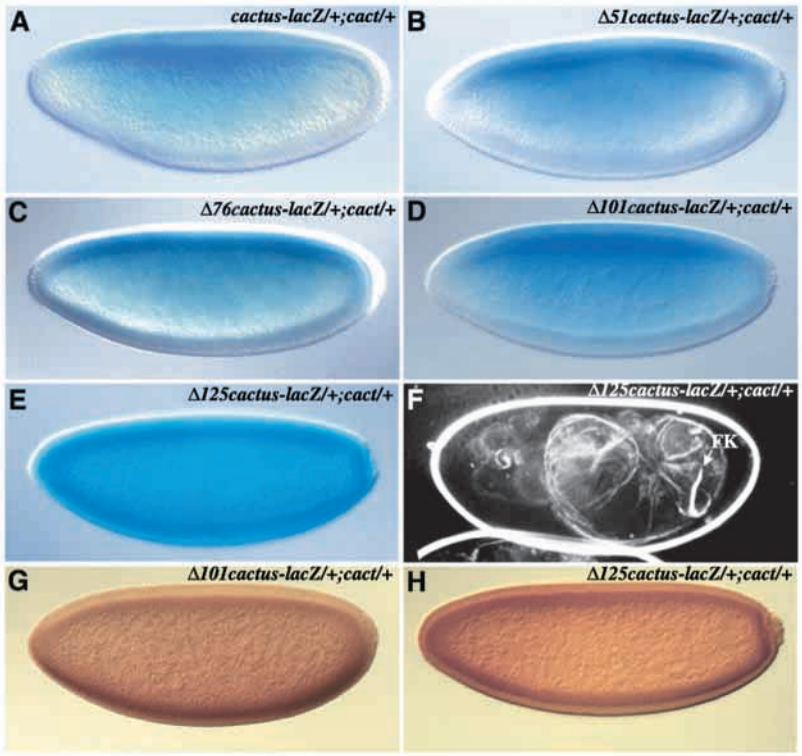

Fig. 3. Amino acid determinants present in the N-terminal 125 residues of Cactus are required for polarized degradation. The distribution of full-length Cactus-*lacZ* (A), or deleted derivatives lacking the first 51(B), 76(C), 101(D) or 125(E) amino acids, expressed under the control of the *alpha-tubulin 67C* promoter in *cact*^{D13}/+ females, visualized by β-galactosidase activity. Deletion of the first 125 amino acid residues resulted in loss of regulated degradation (E) and the production of dominantly dorsalized embryos, as assessed in the cuticular pattern (F). While Cactus-*lacZ* deletion derivatives that lacked as many as 101 amino acids gave rise to a normal Dorsal protein nuclear gradient (G), the deletion of 125 amino acids resulted in a Cactus-*lacZ* protein that retains Dorsal in the cytoplasm (H).

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 Δ*101cact-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 dl^1/dl^1 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 that 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 $cact^{011}$ 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.

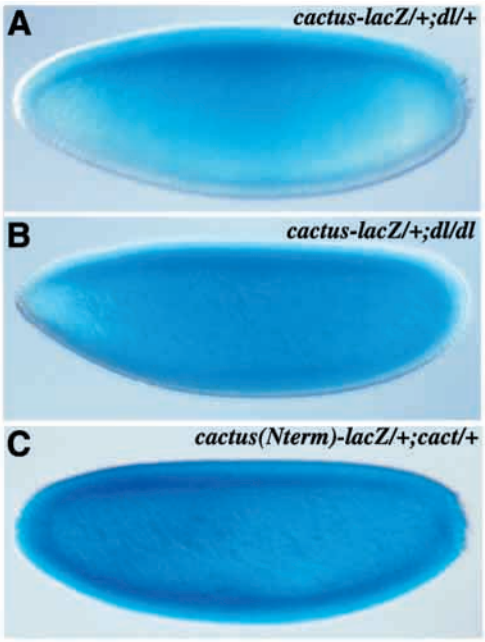

Fig. 4. Polarized degradation of the Cactus-*lacZ* protein requires Dorsal. Maternally expressed full-length Cactus-*lacZ* visualized by *lacZ* activity exhibits a normal polarized distribution in the progeny of females heterozygous for dl^1 (A), while ventral degradation is not observed the progeny of females homozygous for dl^1 (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 $cact^{BQ}/cact^{E10}$ maternal background. $cact^{BQ}/cact^{E10}$ mutant females produce completely dorsalized 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 Δ 76Cactus-*lacZ* (not shown) were capable of undergoing regulated degradation in a $cact^{BQ}/cact^{E10}$ mutant background. In addition, these constructs led to substantial rescue of the dorsalizing phenotype attributable to $cact^{BQ}/cact^{E10}$(see Fig. 5C,D). In contrast, Cactus-*lacZ* lacking the first 125 amino acids could neither form a DV gradient (Fig. 5B) nor rescue the $cact^{BQ}/cact^{E10}$ 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 $cact^{BQ}/cact^{E10}$ 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 Ser74 and Ser78 or all four of Ser74, Ser78, Ser82 and Ser83 had been converted to alanines had the ability to undergo ventral degradation in the embryos produced by $cact^{BQ}/cact^{E10}$ females (Fig. 6A,B). Moreover, both mutant constructs facilitated a significant level of rescue of the dorsalized phenotype associated with $cact^{BQ}/cact^{E10}$ maternal genotype (Fig. 6C,D), as well as rescuing the ventralized phenotype of embryos produced by $cact^{011}/cact^{011}$ mutant females (Fig. 6E,F).

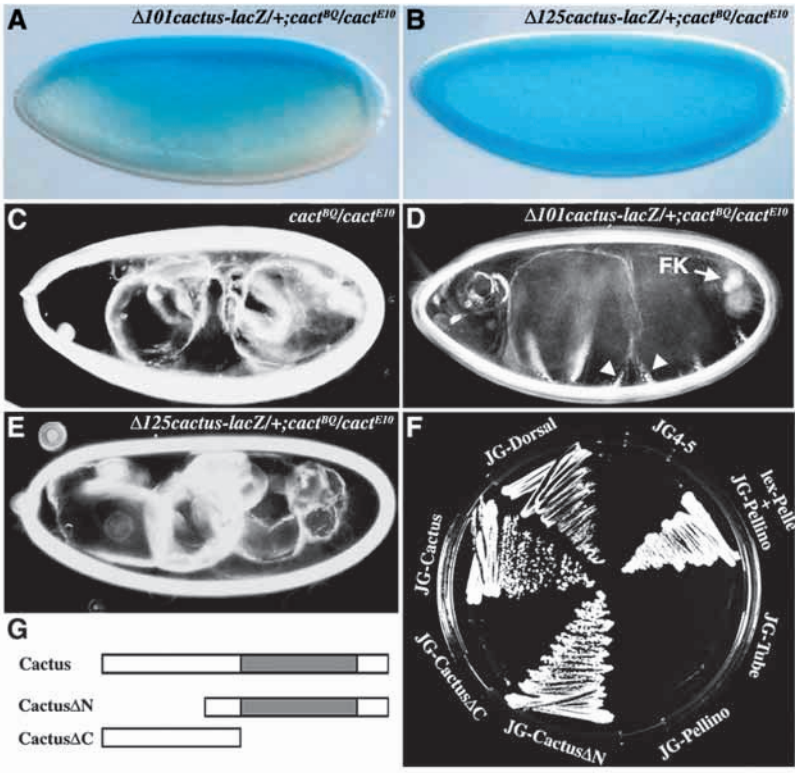

Fig. 5. Polarized degradation of Cactus-*lacZ* fusion proteins does not rely on endogenous Cactus. Enzymatic activity was used to determine Cactus-*lacZ* distributions in the progeny of $cact^{BQ}/cact^{E10}$ females, expressing endogenous mutant Cactus that does not undergo regulated degradation (Bergmann et al., 1996). While the Δ101Cactus-*lacZ* variant undergoes polarized degradation (A), the Δ125Cactus-*lacZ* variant does not (B). Similarly, Δ101Cactus-*lacZ* restored ventral and lateral elements (D) to the cuticles of embryonic progeny of $cact^{BQ}/cact^{E10}$ females (C), while Δ125Cactus-*lacZ* did not (E). Two-hybrid prey constructs containing full-length, N terminally deleted and C terminally deleted segments of Cactus fused to a bacterial activator segment in plasmid JG4-5 (Gyuris et al., 1993) were constructed (for extent of Cactus present in each construct, see G) and tested for their interaction with a lexA-Cactus bait (F). An interaction between Pelle and Pellino is shown as a positive control, while empty vector (JG4-5) and Tube and Pellino fusions to the activation domain fail to interact with lexA-Cactus and represent negative controls.

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)- (Fig. 7B) and the (S116A)Cactus-lacZ constructs (Fig. 7D) displayed a uniform distribution of Cactus-*lacZ* (Fig. 7B) and were both incapable of restoring ventral pattern elements when expressed in a $cact^{BQ}/cact^{E10}$ background (not shown). In contrast, the mutant derivative that carried (S104A) was capable of directing the formation of a Cactus-*lacZ* gradient (Fig. 7C) and of rescuing polarized Cactus function. 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 IκBα, 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 IκBα 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

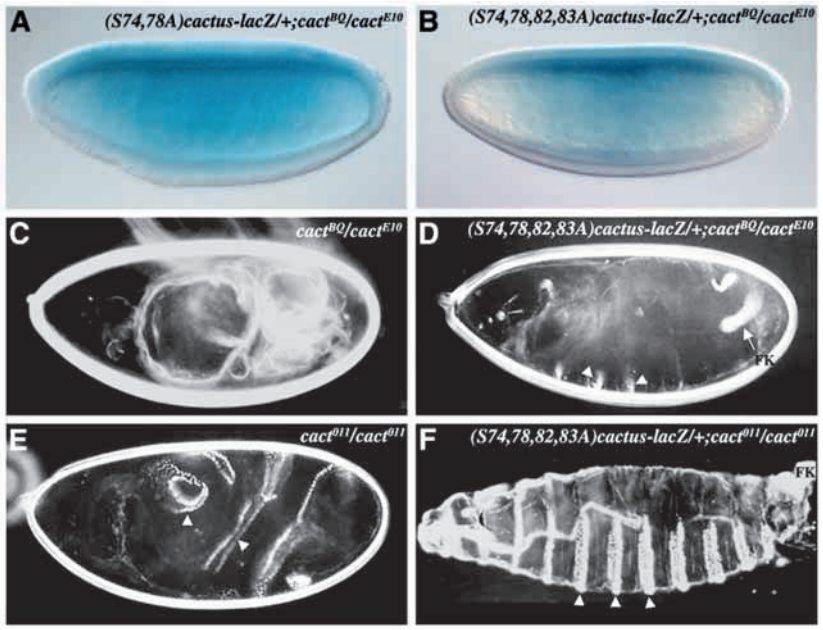

Fig. 6. The putative IκB Kinase (IKK) target motif is dispensable for the spatially regulated degradation of Cactus-*lacZ*. Mutated derivatives of Cactus-*lacZ* in which serines 74 and 78 (A) or serines 74, 78, 82 and 83 (B) had been converted to alanine residues were expressed under maternal transcriptional control and their distributions determined by *lacZ* activity. Both proteins exhibited normal polarized distributions and were capable of restoring ventral pattern elements (D) to the otherwise dorsalized progeny of $cact^{BQ}/cact^{E10}$ females (C). Similarly, the mutated constructs were capable of restoring lateral and dorsal pattern elements (F) and the ability to hatch to the otherwise ventralized progeny of $cact^{011}/cact^{011}$ females (E).

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

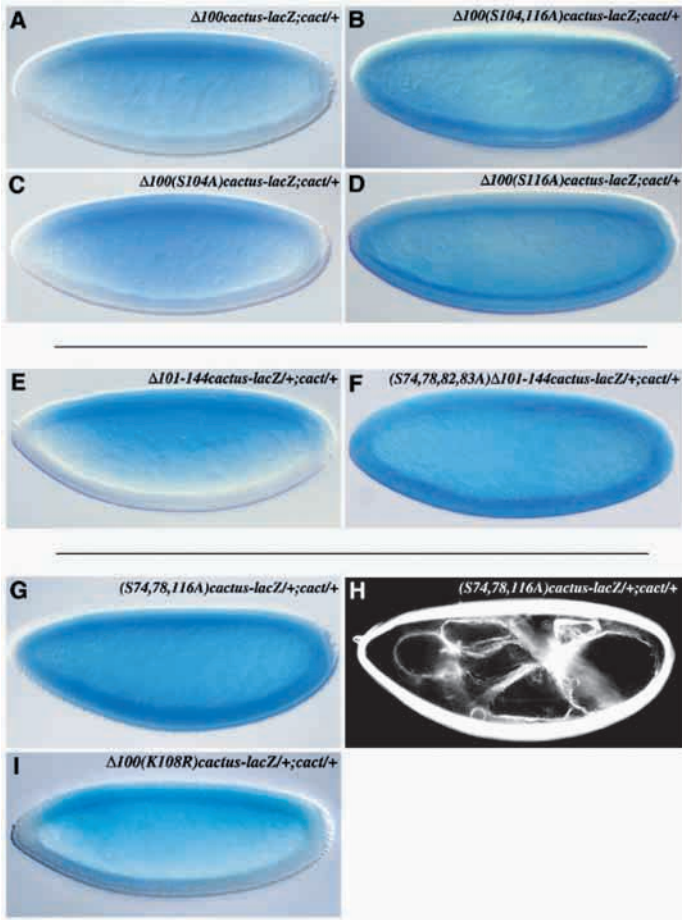

Fig. 7. Two separable regions in the Cactus N terminus direct polarized degradation. (A) Cactus-*lacZ* derivative lacking the N terminal 101 amino acid residues was subjected to site-directed mutagenesis to assess regulatory roles of serines 104 and 116 (A-D). The unmutated (A) and S104A (C) derivatives exhibited polarized degradation, as visualized by *lacZ* activity, while the S104,116A double mutant (B) or the S116A single mutant (D) led to a loss of degradation. Conversion of lysine108 to arginine did not perturb the Cactus-*lacZ* degradation gradient (I). To assess the regulatory roles of the putative IKK target motif in the absence of the influence of the S116 residue, the first 100 amino acids of Cactus were fused in frame to Δ144Cactus-*lacZ* and its distribution (E) compared with that of a mutant derivative in which serines 74, 78, 82 and 83 had been converted to alanine residues (F). Full-length Cactus-*lacZ* bearing the S74A, S78A and S116A mutants substitutions failed to undergo polarized degradation (G) and acted as a dominant dorsalizing mutant (H), demonstrating that the mutagenized residues are key regulatory determinants in the N terminus of Cactus.

able to direct the formation of a Cactus-*lacZ* degradation gradient (Fig. 8A) and, when expressed in the progeny of $cact^{011}/cact^{011}$ 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 $cact^{011}/+$ 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 $cact^{BQ}$ and $cact^{E10}$ 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 (DSGΨXS). 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

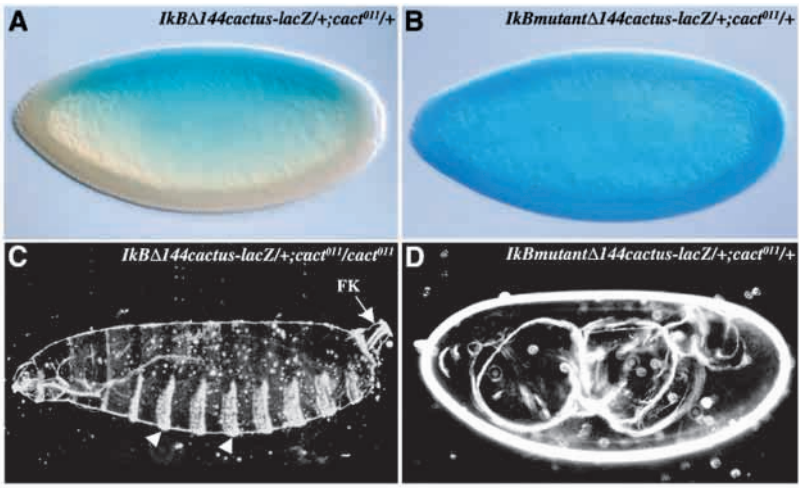

Fig. 8. The N terminus of IκBα can substitute for the N terminus of Cactus to direct regulated degradation. A Cactus-*lacZ* fusion derivative in which the first 71 amino acids of human IκBα were fused to the N-terminus of Δ144Cactus-*lacZ* was expressed under *alpha-tubulin 67C* transcriptional control 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 $cact^{011}$/ $cact^{011}$ 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 $cact^{011}$/+ 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 IKKε, 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 obtained 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.

We acknowledge M. Czaja, D. Ferradon, J. Friedman, R. Geisler, J. Jiang, S. Roth and N. Silverman for providing us with key reagents and advice on their use, and J. Lenz, H. Nguyen, G. Prelich and L. Stevens for suggestions, advice, encouragement and generously providing access to their equipment. N. F. thanks G. Prelich for graciously allowing her to work in his laboratory in the later stages of this work and C. Smith for his invaluable provision of materials for fly husbandry. J. G. thanks C. Nüsslein-Volhard, in whose laboratory some of these investigations were carried out. This work was supported by a grant from the American Cancer Society to D. S. N. F. was supported by a MARC predoctoral fellowship.

REFERENCES

Anderson, K. V. and Nüsslein-Volhard, C. (1984). Information for the dorsal–ventral pattern of the *Drosophila* embryo is stored as maternal mRNA. *Nature* **311**, 223-227.

Baldwin, A. S. (1996). The NF-κB and I κB proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**, 649-683.

Belvin, M. P. and Anderson, K. V. (1996). A conserved signaling pathway: the *Drosophila* Toll-Dorsal pathway. *Annu. Rev. Cell Dev. Biol.* **12**, 393-416.

Belvin, M. P., Jin, Y. and Anderson, K. V. (1995). Cactus protein degradation mediates *Drosophila* dorsal-ventral signaling. *Genes Dev.* **9**, 783-793.

Bergmann, A., Stein, D., Geisler, R., Hagenmaier, S., Schmid, B., Fernandez, N., Schnell, B. and Nüsslein-Volhard, C. (1996). A gradient of cytoplasmic Cactus degradation establishes the nuclear localization gradient of the Dorsal morphogen in *Drosophila*. *Mech. Dev.* **60**, 109-123.

Bhaskar, V., Valentine, S. A. and Courey, A. J. (2000). A functional interaction between Dorsal and components of the Smt3 conjugation machinery. *J. Biol. Chem.* **275**, 4033-4040.

Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y. and Ballard, D. W. (1995). Coupling of a signal response domain in I κBα to multiple pathways for NF-κB activation. *Mol. Cell Biol.* **15**, 2809-2818.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G. and Siebenlist, U. (1995). Control of I κBα proteolysis by site-specific, signal-induced phosphorylation. *Science* **267**, 1485-1488.

Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of* Drosophila melanogaster. Berlin: Springer.

Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D. and Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. *Genes Dev.* **9**, 1586-1597.

Chou, T. B. and Perrimon, N. (1992). Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. *Genetics* **131**, 643-653.

DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. and Karin, M. (1997). A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature* **388**, 548-554.

Drier, E. A., Huang, L. H. and Steward, R. (1999). Nuclear import of the *Drosophila* Rel protein Dorsal is regulated by phosphorylation. *Genes Dev.* **13**, 556-568.

Edwards, D. N., Towb, P. and Wasserman, S. A. (1997). An activity-dependent network of interactions links the Rel protein Dorsal with its cytoplasmic regulators. *Development* **124**, 3855-3864.

Gay, N. J. and Keith, F. J. (1991). *Drosophila* Toll and IL-1 receptor. *Nature* **351**, 355-356.

Geisler, R., Bergmann, A., Hiromi, Y. and Nüsslein-Volhard, C. (1992). cactus, a gene involved in dorsoventral pattern formation of *Drosophila*, is related to the IκB gene family of vertebrates. *Cell* **71**, 613-621.

Ghosh, S., May, M. J. and Kopp, E. B. (1998). NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**, 225-260.

Gillespie, S. K. and Wasserman, S. A. (1994). Dorsal, a *Drosophila* Rel-like

protein, is phosphorylated upon activation of the transmembrane protein Toll. *Mol. Cell. Biol.* **14**, 3559-68.

Govind, S. (1999). Control of development and immunity by rel transcription factors in Drosophila. *Oncogene* **18**, 6875-6887.

Grosshans, J., Bergmann, A., Haffter, P. and Nüsslein-Volhard, C. (1994). Activation of the kinase Pelle by Tube in the dorsoventral signal transduction pathway of *Drosophila* embryo. *Nature* **372**, 563-566.

Grosshans, J., Schnorrer, F. and Nüsslein-Volhard, C. (1999). Oligomerisation of Tube and Pelle leads to nuclear localisation of dorsal. *Mech. Dev.* **81**, 127-138.

Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**, 791-803.

Hashimoto, C., Hudson, K. L. and Anderson, K. V. (1988). The toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52**, 269-279.

Hashimoto, C., Gerttula, S. and Anderson, K. V. (1991). Plasma membrane localization of the Toll protein in the syncytial *Drosophila* embryo: importance of transmembrane signaling for dorsal-ventral pattern formation. *Development* **111**, 1021-1028.

Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P. and Baldwin, A. S. (1991). Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. *Cell* **65**, 1281-1289.

Hecht, P. M. and Anderson, K. V. (1993). Genetic characterization of *tube* and *pelle*, genes required for signaling between Toll and dorsal in the specification of the dorsal- ventral pattern of the *Drosophila* embryo. *Genetics* **135**, 405-417.

Hochstrasser, M. and Kornitzer, D. (1998). Ubiquitin-dependent degradation of transcription factors. In *Ubiquitin and the Biology of the Cell* (ed. J.-M. Peters, J. R. Harris and D. Finlay), pp. 279-302. New York: Plenum Press.

Ip, Y. T. (1995). Transcriptional regulation. Converting an activator into a repressor. *Curr. Biol.* **5**, 1-3.

Isoda, K. and Nüsslein-Volhard, C. (1994). Disulfide cross-linking in crude embryonic lysates reveals three complexes of the Drosophila morphogen dorsal and its inhibitor cactus. *Proc. Natl. Acad. Sci. USA* **91**, 5350-5354.

Jacobsen, R. H., Zhang, X. J., DuBose, R. F. and Matthews, B. W. (1994). Three-dimensional structure of beta-galactosidase from *E. coli*. *Nature* **369**, 761-766.

Jiang, J., Rushlow, C. A., Zhou, Q., Small, S. and Levine, M. (1992). Individual Dorsal morphogen binding sites mediate activation and repression in the *Drosophila* embryo. *EMBO J.* **11**, 3147-3154.

Jiang, J. and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F- box/WD40-repeat protein Slimb. *Nature* **391**, 493-496.

Karin, M. and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu. Rev. Immunol.* **18**, 621-663.

Kidd, S. (1992). Characterization of the *Drosophila* cactus locus and analysis of interactions between Cactus and Dorsal proteins. *Cell* **71**, 623-635.

Klambt, C., Jacobs, J. R. and Goodman, C. S. (1991). The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell* **64**, 801-815.

Liu, Z. P., Galindo, R. L. and Wasserman, S. A. (1997). A role for CKII phosphorylation of the Cactus PEST domain in dorsoventral patterning of the *Drosophila* embryo. *Genes Dev.* **11**, 3413-3422.

Lu, Y., Wu, L. P. and Anderson, K. V. (2001). The antibacterial arm of the *Drosophila* innate immune response requires an IκB kinase. *Genes Dev.* **15**, 104-110.

Maniatis, T. (1999). A ubiquitin ligase complex essential for the NF-κB, Wnt/Wingless, and Hedgehog signaling pathways. *Genes Dev.* **13**, 505-510.

Matthews, K. A., Miller, D. F. and Kaufman, T. C. (1989). Developmental distribution of RNA and protein products of the *Drosophila* α-tubulin gene family. *Dev. Biol.* **132**, 45-61.

Morisato, D. and Anderson, K. V. (1994). The spatzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the *Drosophila* embryo. *Cell* **76**, 677-688.

Morisato, D. and Anderson, K. V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. *Annu. Rev. Genet.* **29**, 371-399.

Nüsslein-Volhard, C., Lohs-Schardin, M., Sander, K. and Cremer, C. (1980). A dorso-ventral shift of embryonic primordia in a new maternal-effect mutant of *Drosophila*. *Nature* **283**, 474-476.

Pan, D. and Courey, A. J. (1992). The Same dorsal binding site mediates both activation and repression in a context-dependent manner. *EMBO J.* **11**, 1837-1842

Reach, M., Galindo, R. L., Towb, P., Allen, J. L., Karin, M. and Wasserman, S. A. (1996). A gradient of cactus protein degradation establishes dorsoventral polarity in the *Drosophila* embryo. *Dev. Biol.* **180**, 353-364.

Rechsteiner, M. (1990). PEST sequences are signals for rapid intracellular proteolysis. *Semin. Cell Biol.* **1**, 433-440.

Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z. and Rothe, M. (1997). Identification and characterization of an IκB kinase. *Cell* **90**, 373-383.

Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364-368.

Rose, M., Winston, F. and Hieter, P. (1988). *Laboratory Manual for Methods in Yeast Genetics*. New York: Coldspring Harbor Laboratory Press.

Roth, S. (1994). Axis determination. Proteolytic generation of a morphogen. *Curr. Biol.* **4**, 755-757.

Roth, S., Stein, D. and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59**, 1189-1202.

Roth, S., Hiromi, Y., Godt, D. and Nüsslein-Volhard, C. (1991). cactus, a maternal gene required for proper formation of the dorsoventral morphogen gradient in *Drosophila* embryos. *Development* **112**, 371-388.

Rubin, G. M and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable elements. *Science* **218** 348-353

Rusch, J. and Levine, M. (1996). Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. *Curr. Opin. Genet. Dev.* **6**, 416-423.

Rushlow, C. A., Han, K., Manley, J. L. and Levine, M. (1989). The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in *Drosophila*. *Cell* **59**, 1165-1177.

Rutschmann, S., Jung, A. C., Hetru, C., Reichhart, J. M., Hoffmann, J. A. and Ferrandon, D. (2000). The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*. *Immunity* **12**, 569-580.

Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T. and Ballard, D. W. (1995). Signal-induced degradation of IκB alpha requires site-specific ubiquitination. *Proc. Natl. Acad. Sci. USA* **92**, 11259-11263.

Schiestl, R. H. and Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**, 339-346.

Schneider, D. S., Hudson, K. L., Lin, T. Y. and Anderson, K. V. (1991). Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the *Drosophila* embryo. *Genes Dev.* **5**, 797-807.

Schneider, D. S., Jin, Y., Morisato, D. and Anderson, K. V. (1994). A processed form of the Spatzle protein defines dorsal-ventral polarity in the *Drosophila* embryo. *Development* **120**, 1243-1250.

Silverman, N., Zhou, R., Stoven, S., Pandey, N., Hultmark, D. and Maniatis, T. (2000). A *Drosophila* IκB kinase complex required for relish cleavage and antibacterial immunity. *Genes Dev.* **14**, 2461-2471.

Spencer, E., Jiang, J. and Chen, Z. J. (1999). Signal-induced ubiquitination of IκBα by the F-box protein Slimb/beta-TrCP. *Genes Dev.* **13**, 284-294.

Stein, D., Goltz, J. S., Jurcsak, J. and Stevens, L. (1998). The Dorsal-related immunity factor (Dif) can define the dorsal-ventral axis of polarity in the *Drosophila* embryo. *Development* **125**, 2159-2169.

Steward, R. (1989). Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. *Cell* **59**, 1179-1188.

Traenckner, E. B., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S. and Baeuerle, P. A. (1995). Phosphorylation of human I κBα on serines 32 and 36 controls I κBα proteolysis and NF-κB activation in response to diverse stimuli. *EMBO J.* **14**, 2876-2883.

Van der Meer, J. M. (1977). Optical clean and permanent whole mount preparations for phase-contrast microscopy of cuticular structures of insect larvae. *Dros. Info. Serv.* **52** 160.

Verma, I. M., Stevenson, J. K., Schwarz, E. M., van Antwerp, D. and Miyamoto, S. (1995). Rel/NFκB/I κB family: intimate tales of association and dissociation. *Genes Dev.* **9**, 2723-2735.

Wasserman, S. A. (1993). A conserved signal transduction pathway regulating the activity of the rel-like proteins dorsal and NFκB. *Mol. Biol. Cell* **4**, 767-771.

Wasserman, S. A. (2000). Toll signalling: the enigma variations. *Curr. Opin. Genet. Dev*. **10**, 497-502.

Whalen, A. M. and Steward, R. (1993). Dissociation of the Dorsal-Cactus complex and phosphorylation of the Dorsal protein correlate with the nuclear localization of dorsal. *J. Cell. Biol.* **123**, 523-534.

Whiteside, S. T., Ernst, M. K., LeBail, O., Laurent-Winter, C., Rice, N. and Israel, A. (1995). N- and C-terminal sequences control degradation of MAD3/IκB alpha in response to inducers of NF-κB activity. *Mol. Cell. Biol.* **15**, 5339-5345.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J. and Harper, J. W. (1999). The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and beta-catenin and stimulates IκBα ubiquitination in vitro. *Genes Dev.* **13**, 270-283.

Wojcik, E. J., Glover, D. M. and Hays, T. S. (2000). The SCF ubiquitin ligase protein slimg regulates centrosome duplication in *Drosophila*. *Curr. Biol.* **10**, 1131-1134.

Yang, J. and Steward, R. (1997). A multimeric complex and the nuclear targeting of the *Drosophila* Rel protein Dorsal. *Proc. Natl. Acad. Sci. USA* **94**, 14524-14529.