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RAPID COMMUNICATION

A Gradient of Cactus Protein Degradation Establishes Dorsoventral Polarity in the *Drosophila* Embryo

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Dorsoventral polarity in the *Drosophila* embryo is established by a signaling pathway active on the ventral and ventrolateral surfaces of the embryo. Signal transduction via the protein kinase Pelle frees the Rel-related protein Dorsal from its cytoplasmic inhibitor Cactus, allowing Dorsal to translocate into ventral and ventrolateral nuclei and direct gene expression. Here, we show by immunochemical analyses that Pelle-mediated signaling induces the spatially graded degradation of Cactus. Using a tissue culture system which reconstitutes Pelle-dependent Cactus degradation, we show that a motif in Cactus resembling the sites of signal-dependent phosphorylation in the vertebrate homologs IκB-α and IκB-β is essential for Pelle-induced Cactus degradation. Substitution of four serines within this motif with nonphosphorylatable alanine residues generated a mutant Cactus that still functions as a Dorsal inhibitor but is resistant to induced degradation. Injection of RNA encoding this altered form of Cactus has a dominant negative effect on establishment of dorsoventral polarity in the embryo. We conclude that dorsoventral signaling results in a Cactus concentration gradient and propose that signal-dependent phosphorylation directs the spatially regulated proteolysis of Cactus protein.

INTRODUCTION

Dorsoventral polarity of the *Drosophila* embryo is established by a nuclear concentration gradient of the transcription factor Dorsal (reviewed in St. Johnston and Nüsslein-Volhard, 1992; Morisato and Anderson, 1995). Prior to axis formation, Dorsal and its inhibitor, Cactus, are evenly distributed throughout the embryonic cytoplasm. Upon binding of the ligand Spätzle to the receptor Toll, a signal is transduced in the ventral and ventrolateral portions of the embryo that frees Dorsal from Cactus inhibition. Dorsal then translocates into nuclei, where it functions as a transcription factor, activating genes required for ventral and ventrolateral cell fates and repressing dorsal-specific genes. The nuclear concentration gradient of Dorsal thus defines the dorsoventral axis.

Dorsal is a member of the Rel family of transcription factors, which also includes the vertebrate NF-κB proteins (reviewed in Verma *et al.*, 1995). In addition to sequence similarity in their DNA binding domains, Rel family members share a common mode of activation. Inhibitory proteins, IκB-α and IκB-β, which are structurally and functionally related to Cactus, retain Rel-related proteins in the cytoplasm of unstimulated cells. Upon cell stimulation, NF-κB proteins, like Dorsal, are translocated to the nucleus, where they activate transcription of target genes containing κB recognition motifs.

Transduction of the gradient-determining signal to the Dorsal/Cactus complex requires the intracellular action of at least three gene products: Toll, Tube, and Pelle. Inactivation of Toll, Tube, or Pelle blocks Dorsal nuclear translocation, resulting in embryos that die as twisted tubes of dorsal

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epidermis (Anderson and Nüsslein-Volhard, 1984; Roth *et al.*, 1989). In contrast, the absence of Cactus function, or the presence of constitutively activated forms of Toll, Tube, or Pelle, promotes Dorsal nuclear translocation throughout the embryo, resulting in a ventralized phenotype (Schüpbach and Wieschaus, 1989; Roth *et al.*, 1989, 1991; Großhans *et al.*, 1994; Galindo *et al.*, 1995).

Pelle is a serine/threonine-specific protein kinase whose activity is required upstream of Dorsal and Cactus (Shelton and Wasserman, 1993; Hecht and Anderson, 1993). Inactivation of the Pelle catalytic domain blocks signal transduction to the Dorsal/Cactus complex. Furthermore, activated Pelle is sufficient to induce ectopic ventral-specific fates (Großhans *et al.*, 1994; Galindo *et al.*, 1995). Tube, which shares no obvious sequence similarity with any other previously described protein, interacts with Pelle in a yeast two-hybrid assay and may act to recruit Pelle to the plasma membrane (Letsou *et al.*, 1991; Großhans *et al.*, 1994; Galindo *et al.*, 1995).

The signal relay system controlling mammalian NF-κB activation in response to inflammatory stimuli is remarkably similar to the system responsible for Dorsal activation (Wasserman, 1993; Cao *et al.*, 1996). A potent activator of NF-κB is the proinflammatory cytokine interleukin 1 (IL-1), whose receptor displays considerable similarity to Toll (Schneider *et al.*, 1991; Gay and Keith, 1991). In addition, the mammalian protein most similar in sequence to Pelle is the interleukin-1 receptor-associated kinase (IRAK), which coimmunoprecipitates with the IL-1 receptor. The two proteins have substantial sequence identity in their carboxy-terminal catalytic domains (32%), as well as weaker similarity in their amino termini (Cao *et al.*, 1996).

Both protein phosphorylation and proteolysis have been demonstrated to play essential roles in the IL-1 pathway. Activation of the transmembrane IL-1 receptor in lymphocytes frees NF-κB by rapid signal-dependent degradation of IκB (reviewed in Verma *et al.*, 1995). Signal-dependent phosphorylation of IκB is essential for signal transduction; mutation of the serine residues in IκB that undergo signal-dependent phosphorylation blocks signal-dependent IκB degradation and NF-κB activation (Brown *et al.*, 1995; Brockman *et al.*, 1995; Traenckner *et al.*, 1995; DiDonato *et al.*, 1996).

Here, we explore signal-dependent degradation of Cactus and the potential role of phosphorylation in this process. We demonstrate that the amount of Cactus protein decreases in response to signaling in both wild-type embryos and cultured cells. We find that signaling in wild-type embryos results in a dorsoventral gradient of Cactus protein, with highest levels present on the dorsal side of the embryo and lowest levels in ventral regions. Last, we show that substitution of alanine for serine at four residues in Cactus, located in a stretch of sequence similar to the previously identified IκB phosphorylation motif, eliminates signal-dependent degradation of Cactus and blocks signal transduction. These results lead us to propose that signal-dependent phosphorylation of Cactus triggers its proteolysis and thereby establishes embryonic dorsoventral polarity.

MATERIALS AND METHODS

Drosophila Stocks

Drosophila melanogaster stocks were maintained on standard cornmeal–yeast–agar medium (Ashburner, 1989) at 18°, 22°, or 25°C. Oregon R was used as the wild-type stock. All other mutations and balancers are described in Lindsley and Zimm (1992).

Production of Cactus Antiserum

Plasmid pMALcact was constructed by cloning the 1.9-kb *Pvu*II fragment (encoding Cactus residues 22–500) of cDNA clone pcactNB27 (Geisler *et al.*, 1992) into the polylinker of pMAL-c (NEB Inc.) at the *Eco*RI site filled in with Klenow enzyme. The plasmid was transformed into *Escherichia coli* JM109(DE3), and the maltose binding–Cactus fusion protein (MBP-Cactus) expressed by isopropylthiogalactopyranoside (IPTG) induction of this plasmid. MBP-Cactus was purified to >90% homogeneity by passage of a bacterial extract over an amylose-resin column, extensive washing using both high-salt and low-salt buffers, and elution with buffer containing 10 m*M* maltose. Preparative SDS–PAGE, rabbit injection and processing of immune bleeds were performed as described in Gillespie and Wasserman (1994). To remove anti-MBP antibodies, crude antiserum was passed three times over an MBP-affinity column made by coupling maltose binding protein to Affi-Gel 10 resin (Bio-Rad). The flow-through was then passed three times over an MBP-Cactus-affinity column, and affinity-purified anti-Cactus antibodies were eluted with 100 m*M* glycine, pH 2.5, essentially as described in Harlow and Lane (1988).

Preparation of Crude Embryonic Lysates and Staged Embryonic Extracts

Crude embryonic lysates were prepared as described previously (Gillespie and Wasserman, 1994), except as follows: Lysis buffer for embryos (Lysis buffer I) consisted of: 62.5 m*M* Tris (pH 6.8), 10% glycerol, 2% SDS, 0.3 μ*M* aprotinin, 10 μ*M* leupeptin, 1 μ*M* pepstatin A, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF). Insoluble material was removed by centrifugation at 10,000*g* for 10 min 4°C. Protein concentration was determined using the Bicinchoninic acid (BCA) assay (Pierce). Bromophenol blue and 2-mercaptoethanol were then added to final concentrations of 0.004 and 5%, respectively. The samples were heated to 100°C for 1 min and stored at −20°C prior to electrophoresis. Staged embryonic extracts, obtained by microextraction of total embryonic contents of individually staged embryos with a large-bore microinjection needle, were the same as used previously (Gillespie and Wasserman, 1994) and had been stored at −70°C prior to use.

Immunoblot Analysis and Immunocytochemistry

Immunoblotting was done as described in Gillespie and Wasserman (1994), with the following modifications: protein samples were subjected to electrophoresis in 8% SDS–polyacrylamide gels and then transferred to PVDF membranes at 100 V for 1 hr. Cactus was detected using a 1/500 dilution of affinity-purified anti-Cactus antiserum in blocking buffer. The secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Calbi-

ochem), was used at a dilution of 1/30,000 in blocking buffer. For Fig. 6C, to control for loading, the PVDF membrane was reprobed after Cactus detection for biotinylated proteins with a 1:5000 dilution of streptavidin-conjugated alkaline phosphatase (TAGO Inc.). Quantitation was carried out with a Molecular Dynamics laser scanning densitometer.

Immunocytochemistry

Embryos were collected, fixed, devitellinized, and stained as described previously (Galindo *et al.*, 1995). Anti-Cactus antiserum was used at a dilution of 1:2000. For double-labeling experiments, embryos were processed identically through the incubation with secondary antibody. They were then incubated in blocking buffer containing a 1:10 dilution of normal rabbit sera for 30 min, followed by incubation for 2 hr in a 1:100 dilution of anti-Dorsal antibody conjugated to fluorescein (QuickTag FITC conjugation kit; Boehringer Mannheim Corp.). Following six 10-min washes with blocking buffer containing a 1:10 dilution of normal rabbit sera, embryos were mounted on slides in Fluoromount G.

Microscopy and Digital Image Processing

Image acquisition and analysis was done as described previously (Galindo *et al.*, 1995) using either an MRC-600 or an MRC-1024 (Bio-Rad) laser scanning confocal unit. Fluorescein and Cy3 images were collected separately, using the 488-nm and the 568-nm lines of the krypton–argon laser, respectively, to excite the fluorophores. The fluorescein signal was viewed through the 522DF32 filter, while the Cy3 signal was viewed through the 585EFLP filter. Image analysis was done using the Bio-Rad LaserSharp software.

Construct Formation

For tissue culture analysis, all mutagenized constructs were obtained using the Chameleon site-directed mutagenesis kit (Stratagene) and synthetic mutagenic primers. Plasmid DNA was purified using polyethylene glycol (Maniatis *et al.*, 1989). To construct the hemagglutinin (HA)-tagged $Cact^{WT}$ expression vector, an *Nde*I restriction site was introduced at the initiating methionine codon of the *cactus* cDNA (position 155). This modified *cactus* cDNA was then subcloned into the *Nde*I site at the 3′ terminus of two copies of the HA epitope sequence (ATGTACCCATACGATGTTCCAGATTACGCCATG) ligated to the *Xba*I site in Bluescript (KS^+) (pBS, Stratagene). The HA-tagged $Cact^{WT}$ expression construct was then obtained by cleavage of the above pBS-HA-$Cact^{WT}$ construct with *Kpn*I and *Ecl*136II, with the isolated *HA-*$Cact^{WT}$ cDNA placed via an *Eco*RV site into pAct5c (described in Norris and Manley, 1993). All *cactus* mutants were generated using pBS-HA-$Cact^{WT}$ as the template for mutagenesis; the altered cDNAs were then cloned as above into pAct5c.

For embryonic analysis, wild-type *cactus* cDNA (Geisler *et al.*, 1992; Kidd, 1992) was cloned via *Hin*dIII and *Eco*RI into the pSELECT vector (Promega), generating the S617 construct. Site-directed mutagenesis (Altered Sites System, Promega) of the *cactus* cDNA sequence was used to convert residues 459 and 460 (ACGCCT) to two tandem stop codons (TAGTAG), resulting in the *cact*Δ*PEST* construct. *Cact*Δ*PEST* cDNA was then cloned into a pSP64 (Promega) vector using the same *Hin*dIII and *Eco*RI restriction sites. To introduce the $cact^{SA4}$ mutation into *cact*Δ*PEST*, cDNA encoding the $cact^{SA4}$ mutation was obtained by cleavage of the above-mentioned $Cact^{SA4}$ construct with *Eag*I and *Sty*I and placed into *cact*Δ*PEST* similarly cleaved with *Eag*I and *Sty*I. The $cact^{SA4}$ construct was then generated by cleaving $cact^{SA4\Delta PEST}$ with *Sty*I and *Eco*RI and replacing the PEST deletion with wild-type sequences from construct S617.

Cell Culture

For tissue culture analysis, *Drosophila* Schneider (SL2) cells were grown at 25°C in Schneiders *Drosophila* media (Gibco) supplemented with 12% fetal bovine serum. DNA transfections were performed by the calcium-phosphate technique described in Han *et al.* (1989). One microgram of the Dorsal reporter was added to all transfections that included the Dorsal expression vector. All transfections included variable amounts of the parent pAct5c vector to bring the total amount of transfected DNA to 20 μg.

For luciferase assays, transfected cells were lysed for 20 min in buffer (100 m*M* Tris-acetate, 10 m*M* magnesium acetate, 1 m*M* EDTA) + 1% Triton X-100. Relative luciferase activity was determined by use of a luminometer. For immunoprecipitations from transfected SL2 cells, cells (2×10^6) were lysed in a 50 m*M* Hepes, pH 7.5, and 0.1% Triton X-100 buffer (lysis buffer II) that included 1 m*M* DTT and 10 μg/ml of the following protease inhibitors: leupeptin, pepstatin, bestatin, aprotinin, and PMSF. The lysates were incubated with 1 μl of the Dorsal antiserum and 60 μl of a 50% Protein A–Sepharose slurry for 3 hr with rotation at 4°C. Immunocomplexes were collected by centrifugation, washed five times with the above-mentioned lysis buffer II, boiled in SDS buffer, and electrophoresed on an 8% SDS–PAGE gel. HA-tagged Cactus protein was then detected by a monoclonal HA-specific antibody.

Embryo Injections and Cuticle Preparations

Embryo collection, RNA microinjection, and cuticle preparations were carried out as described previously (Driever *et al.*, 1990; Shelton and Wasserman, 1993), except that egg collection was performed at 25°C and embryos were dried for 5–7 min using a Zeiss air curtain incubator. For cuticle analysis, embryos were injected at stage 2 through the posterior end and RNA was deposited in the center of the embryo. For immunoblot analysis after RNA injection, stage 2 embryos were allowed to continue development until stage 4. Total embryonic contents were collected by aspirating embryonic contents into a large-bore injection needle. The microextract was then deposited into injection oil, mixed immediately with fresh, ice-cold lysis buffer I (described previously) at a concentration of 1 μl/embryo, boiled for 5 min, and stored at −20°C.

RESULTS

The Level of Cactus Correlates with the Level of Intracellular Signaling in Embryos

Anderson and colleagues have shown that exogenous Toll ligand triggers degradation of Cactus translated from microinjected RNA (Belvin *et al.*, 1995). However, the relationship between endogenous Cactus levels and signaling in embryos has not been examined. We investigated the effect

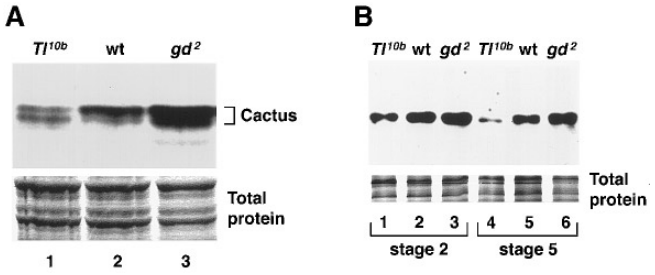

FIG. 1. Embryonic cactus protein levels correlate inversely with signaling. (A) Crude embryonic lysates from 0- to 3-hr Tl^{10b}, wild-type, and gd^2 embryos were resolved by SDS–PAGE on an 8% gel and analyzed by immunoblotting with affinity-purified anti-Cactus antiserum (top) or, as a loading control, by staining with Coomassie brilliant blue (bottom). (B) Staged embryonic extracts prepared from individually staged embryos (Gillespie and Wasserman, 1994) were subjected to electrophoresis and analyzed as described in (A). Whereas two maternal isoforms of Cactus are visible in the wild-type sample in A, only a single Cactus species is visible in B. Variability in the mobility and relative abundance of the two isoforms has been observed previously (Belvin *et al.,* 1995).

of signaling on maternal Cactus by immunoblot analysis of embryonic lysates from three different genetic backgrounds. In embryos from wild-type females the signal for Dorsal activation is transduced ventrally and absent dorsally; in embryos from females carrying a dominant *Toll* mutation (Tl^{10b}) signaling occurs around the entire embryonic circumference; and in embryos from *gastrulation defective* mutant (gd^2) females' signaling is eliminated, due to the failure to form active Toll ligand (Schneider *et al.,* 1991; Stein and Nüsslein-Volhard, 1992).

When lysates from 0- to 3-hr embryos were separated by gel electrophoresis and probed with anti-Cactus antisera, differences in the abundance of maternal Cactus were evident in a comparison of signal constitutive (Tl^{10b}), wild-type, and signal absent (gd^2) backgrounds (Fig. 1A). Immunoblotting of lysates from Tl^{10b} embryos revealed reduced amounts of maternal Cactus protein compared with wild-type lysates. In contrast, the amount of Cactus in embryos derived from gd^2 females was increased relative to wild-type. Thus the relative abundance of Cactus protein in the developing embryo correlates inversely with the level of intracellular dorsoventral signaling. In the absence of signal, the level of Cactus protein is elevated; with normal signaling, which is restricted to one half of the embryo, intermediate levels of protein are detected; and with global, constitutive signaling, the level of Cactus protein is very low. In contrast, the amount of Dorsal protein present in embryos is unaffected by the signaling state (Gillespie and Wasserman, 1994).

Next, we were interested in determining when the changes in Cactus levels occur relative to signal-dependent changes in Dorsal modification or localization. We have previously used immunoblots of microextracts of individually staged embryos to demonstrate that signal-dependent phosphorylation of Dorsal is readily detectable at stage 5, but not stage 2 (Gillespie and Wasserman, 1994). Using the same protein samples, we examined the levels of Cactus protein in embryos from Tl^{10b}, wild-type, and gd^2 females (Fig. 1B). At stage 2, Tl^{10b} embryos have less Cactus than wild-type embryos, which contain less Cactus than gd^2 embryos. Thus, a constitutively active Toll receptor induces degradation of Cactus prior to detectable signal-dependent Dorsal phosphorylation. Furthermore, Cactus degradation precedes the appearance of the Dorsal nuclear concentration gradient, which occurs at stage 3, 80 min postfertilization (Roth *et al.,* 1989; Rushlow *et al.,* 1989; Steward, 1989).

Comparison of Cactus levels in stage 2 and stage 5 embryos reveals two differences. First, for both genetic backgrounds in which signaling occurs (Tl^{10b} and wild-type), less Cactus is present at stage 5 than at stage 2. The decrease in both backgrounds was consistently 50% or greater. Second, the signal-induced differences in Cactus levels were more pronounced (by a factor of two) at stage 5 relative to stage 2. These differences correlate with a sharpening of the Dorsal nuclear concentration gradient that occurs between stages 2 and 5 (Roth *et al.,* 1989; Rushlow *et al.,* 1989; Steward, 1989).

A Dorsoventral Gradient of Cactus in Embryos

Degradation of Cactus as a direct consequence of dorsoventral signaling should be restricted to the ventral side of the embryo. We tested this hypothesis by investigating the distribution of Cactus in wild-type embryos using whole-mount immunofluorescent microscopy.

In wild-type embryos, during mid and late syncytial blastoderm stages, the distribution of Cactus is strikingly asymmetric (Fig. 2A). High levels of Cactus protein are present in the dorsal cytoplasm, where no intracellular signal transduction occurs. Little Cactus protein is present in the ventral cytoplasm of the embryo, where signaling is strongest. The transition from high to low levels is graded (Fig. 2C). The observed Cactus concentration gradient, therefore, inversely correlates with the state of intracellular signaling and is opposite in polarity to the Dorsal nuclear concentration gradient (Fig. 2B).

In gd^2 or Tl^{10b} embryos in which the signaling state is uniform across the dorsoventral axis, no Cactus gradient is observed. gd^2 embryos show uniform high levels of Cactus protein (Fig. 2D), whereas Tl^{10b} embryos contain a uniform low level of Cactus around the entire circumference of the embryo (Fig. 2E). Thus, the spatially regulated activation of intracellular signaling is responsible for the proper establishment of the Cactus concentration gradient.

Cactus Regulation Can Be Reconstituted in Tissue Culture

As a second route for exploring the control of Cactus abundance, we established a cultured cell system for Cac-

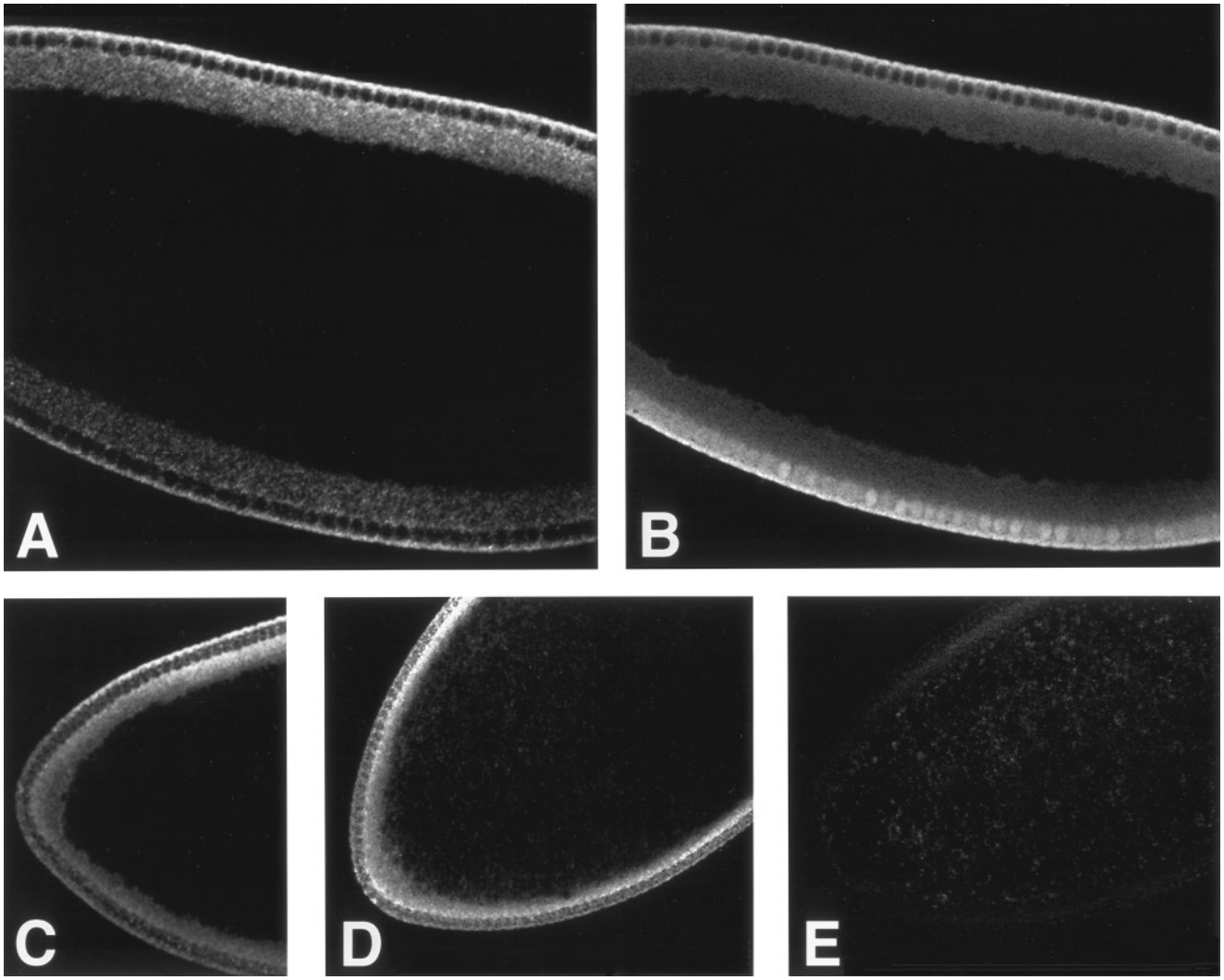

FIG. 2. Signal transduction generates a dorsoventral gradient of Cactus. (A, B) Wild-type embryos were labeled with anti-Cactus antisera detected with a Cy3-conjugated secondary antibody (A) and a fluorescein-conjugated anti-Dorsal antibody (B). (C, D, E) Embryos from wild-type (C), gd^2 (D), and Tl^{10b} (E) maternal genotypes were labeled with anti-Cactus antiserum and a Cy3-conjugated secondary antibody. All embryos were analyzed by confocal immunofluorescence microscopy and are shown oriented with the anterior surface to the left and the dorsal surface toward the top.

tus-dependent regulation of Dorsal activity, following the general approach taken previously (Norris and Manley, 1992; Kubota *et al.*, 1993). The reporter construct for our system consisted of a portion of the Zen promoter, which contains high-affinity Dorsal binding sites, placed 60 nucleotides upstream of the TATA box of a minimal collagenase promoter driving luciferase gene transcription. This construct was cotransfected into *Drosophila* Schneider (SL2) cells with expression vectors encoding Dorsal, Cactus, and various signaling molecules. Whole cell lysates were prepared and luciferase activity was assayed (Fig. 3).

Cotransfection of Dorsal stimulated luciferase expression fivefold. This activity was further enhanced as much as four- to fivefold by cotransfection of Twist, a Dorsal coactivator in embryos (Ip *et al.*, 1992). Cotransfection of Cactus with Dorsal and Twist reduced reporter expression to its basal level. Cactus inhibition of the Dorsal-dependent reporter activation was eliminated, however, by cotransfection of a Pelle expression vector in addition to Dorsal, Twist, and Cactus.

Having established a cultured cell system for exploring signaling to Cactus-inhibited Dorsal complexes, we wished to determine if the Pelle-dependent restoration of reporter gene expression reflected modification or degradation of Cactus. Since free Cactus is subject to rapid, signal-indepen-

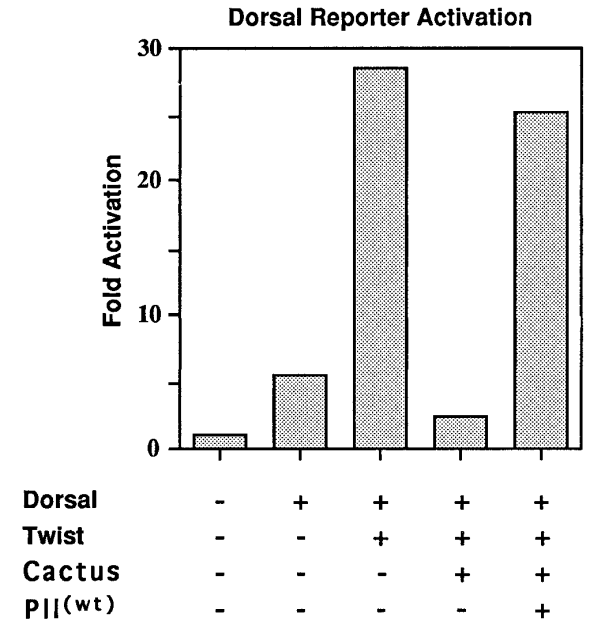

FIG. 3. Pelle-responsive gene activation in transfected tissue culture cells. A Dorsal-responsive reporter plasmid was cotransfected with the indicated expression vectors into *Drosophila* Schneider (SL2) cells. After 24 hr, cells were harvested and luciferase expression levels determined. Each bar indicates the relative luciferase activity for a representative experiment out of a minimum of three similar experiments done in duplicate.

dent degradation (Whalen and Steward, 1993; Belvin *et al.*, 1995), we examined the effect of Pelle on Dorsal-bound Cactus by precipitating Dorsal and any associated proteins with a Dorsal-specific antibody. To allow Cactus levels to be assayed by immunoblotting with an anti-HA monoclonal antibody, the amino terminus of Cactus was tagged with the influenza hemagglutinin (HA) epitope in these experiments.

The immunoprecipitation and immunoblot analysis indicated that the levels of Dorsal-bound Cactus are strictly correlated with Pelle protein kinase activity. In the absence of Pelle, cotransfection of Dorsal and Cactus resulted in the detection of substantial amounts of HA-Cactus coimmunoprecipitating with Dorsal (Fig. 4, lane 2). In the presence of Pelle, however, the amount of HA-Cactus found in association with Dorsal was greatly diminished (Fig. 4, lane 3). This reduction in the level of Dorsal-bound Cactus was strictly dependent on Pelle catalytic activity; cotransfection of catalytically inactive Pelle (K240R; Shelton and Wasserman, 1993) did not reduce the amount of coimmunoprecipitated Cactus (Fig. 4, lane 4).

Site-Directed Mutagenesis of an IκB-like Phosphorylation Motif in Cactus Blocks Pelle-Induced Degradation

Studies in vertebrate cell culture systems have shown that signal-dependent degradation of IκB-α and IκB-β requires phosphorylation at two serine residues, spaced four amino acids apart, in the amino-terminal portion of each protein (Brown *et al.*, 1995; Brockman *et al.*, 1995; Traenckner *et al.*, 1995; DiDonato *et al.*, 1996). Analysis of the Cactus protein sequence revealed four serine residues between amino acids 73 and 84 in a sequence context similar to the IκB-α and IκB-β phosphorylated serines (Fig. 5A). These Cactus residues are found in the region of Cactus postulated to be necessary for signal responsiveness, as a large amino-terminal deletion encompassing this portion of Cactus prevents Dorsal nuclear import (Roth *et al.*, 1991; Geisler *et al.*, 1992).

We used site-directed mutagenesis to investigate the role of the four serines in signal-dependent Cactus degradation. The *cactus* coding sequence was site-specifically altered so as to convert serines 74, 78, 82, and 83 to alanines, generating the Cact^{SA4} construct (Fig. 5A; Table 1). Upon transfection with 250 ng of the Pelle construct, the level of wild-type Cactus (Cact^{WT}) associated with Dorsal was reduced more than 50-fold (Fig. 5B, 5C), while the level of Dorsal-associated Cact^{SA4} was essentially unchanged (Fig. 5B). Cact^{SA4} was thus markedly more stable in Pelle-transfected SL2 cells than was wild-type Cactus.

To examine the function of individual serines in the IκB-like region, we mutated subsets of the four serine residues and assayed their activity in a dose–response study. The Cact^{SA2} construct contains alanines at positions 74 and 78, whereas Cact^{SA3} has alanines at positions 74, 78, and 83 (Table 1). These mutations were tested for their ability to stabilize Cactus in the presence of increasing amounts of Pelle (Fig. 5C). Upon transfection with 100 ng of the Pelle construct, the level of Cact^{WT} associated with Dorsal was reduced approximately 9-fold, while Cact^{SA2} and Cact^{SA3} levels were nearly unchanged (Fig. 5C). Upon cotransfection with 250 ng of the Pelle construct, Cact^{WT} levels were decreased a further 7-fold, whereas the overall decrease in Cact^{SA2} and Cact^{SA3} levels was only 3- and 2-fold, respec-

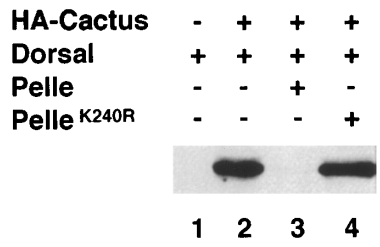

FIG. 4. Pelle-dependent degradation of Dorsal-bound Cactus in tissue culture cells. SL2 cells were cotransfected with the indicated expression constructs. After 24 hr, Dorsal-bound Cactus was immunoprecipitated with anti-Dorsal antibody. The immunocomplexes pellets were washed, fractionated by SDS–PAGE, and analyzed by immunoblotting with an anti-HA monoclonal antibody. Antigen–antibody complexes were then visualized by the Enhanced Chemiluminescence system (Amersham).

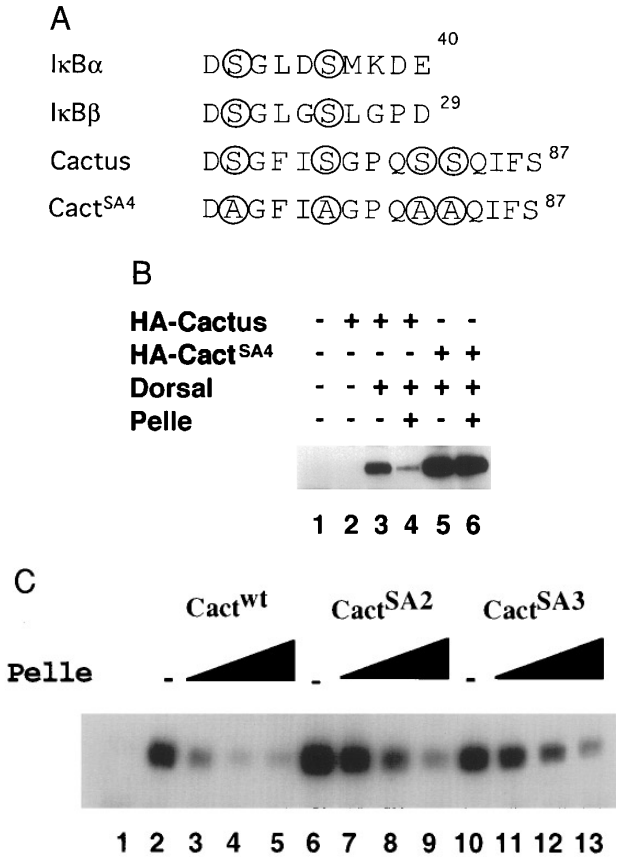

FIG. 5. Analysis of an IκB-like phosphorylation motif in Cactus. (A) Cactus shares sequence similarity with the IκB phosphorylation motif. The IκB phosphorylation motifs (DSGLXS) in IκB-α and -β are aligned with residues 73–78 in Cactus; numbers indicate position of the final residue shown in the amino acid sequence. Since Cactus residues 78–82 also show similarity to the DSGLXS motif, amino acids 73–82 of Cactus may contain two copies of the IκB phosphorylation motif. The four circles serines are those mutated to alanines in Cact^{SA4}. (B) Immunoprecipitates were prepared with anti-Dorsal antibody from SL2 cells transfected with the indicated expression constructs. After SDS–PAGE electrophoresis, coimmunoprecipitated HA-tagged Cact^{WT} and Cact^{SA4} were analyzed by immunoblotting using an anti-HA monoclonal antibody. (C) Wild-type or mutant Cactus bound to Dorsal was coimmunoprecipitated with anti-Dorsal antibody from untransfected SL2 cells (lane 1) or SL2 cells transfected with Cact^{WT} (lanes 2–5), Cact^{SA2} (lanes 6–9), or Cact^{SA3} (lanes 10–13) and increasing amounts of Pelle (0, 0.1, 0.25, and 1 μg). Coimmunoprecipitated HA-tagged Cact^{WT}, Cact^{SA2}, and Cact^{SA3} were analyzed as in (B).

tively. At this level of transfected Pelle, the abundance of Cact^{SA4} was unchanged (Fig. 5B, compare lanes 5 and 6).

These transfection experiments indicate that no single serine in the IκB-like motif is strictly required and the relative contributions of the four residues are unequal. The situation is thus more complex for Cactus than for IκB-α and IκB-β, in which both serines in the S-X-X-X-S motifs are required for inducible degradation (Brown *et al.*, 1995; Brockman *et al.*, 1995; Traenckner *et al.*, 1995; DiDonato *et al.*, 1996).

Mutations in the IκB-like Motif Render Cactus Resistant to Signal-Dependent Degradation in Embryos

To assay the importance of the IκB-like Cactus motif in dorsoventral patterning, RNAs corresponding to Cact^{WT} and Cact^{SA4} were generated and microinjected into wild-type embryos at a concentration of 1.5 μg/μl. We then measured the function of the dorsoventral signaling pathway by assaying cell fate markers in the cuticle secreted by the developing embryo (Anderson and Nüsslein-Volhard, 1984). Embryos injected with wild-type cactus RNA developed a wild-type cuticle pattern (Table 2). In contrast, injection of cact^{SA4} RNA resulted in a dorsalized cuticle pattern, characterized by the reduction in, or absence of, the ventral-specific denticles and the laterally derived filzkörper (Fig. 6A, Table 2). Cact^{SA4} RNA also dorsalized the cuticle, and hence blocked signaling, in signal constitutive *Tl^{10b}* embryos (Table 2). Thus, cact^{SA4} RNA has a dominant negative effect on the dorsoventral signaling pathway.

Based on the cultured cell experiments, we expected that the Cact^{SA4} protein would be resistant to signal-induced degradation. To test this prediction, we assayed protein levels in embryo injection experiments, using a modified Cactus construct which lacks the carboxy-terminal PEST sequence (Kidd, 1992; Geisler *et al.*, 1992). The CactΔPEST deletion removes 29 amino acids from the Cactus carboxy terminus and eliminates signal-independent but not signal-dependent degradation (Belvin *et al.*, 1995). Stage 2 wild-type embryos were injected with wild-type, cactΔPEST, or cact^{SA4}ΔPEST RNA and allowed to continue development until stage 4. Total embryonic contents were then extracted with a microinjection needle and analyzed by immunoblotting. Owing to the C-terminal truncation, the CactΔPEST and Cact^{SA4}-ΔPEST proteins were readily distinguishable from endogenous, full-length Cactus by SDS–PAGE (Fig. 6B).

Whereas CactΔPEST accumulated to significant levels in a wild-type background, this protein was barely detectable

TABLE 1
Cactus Mutant Constructs

Construct name	AA No. 74	AA No. 78	AA No. 82	AA No. 83
WT	Ser	Ser	Ser	Ser
SA2	Ala	Ala	Ser	Ser
SA3	Ala	Ala	Ser	Ala
SA4	Ala	Ala	Ala	Ala

TABLE 2
Injection of Cact^{SA4} *in Vitro* Synthesized RNA on Wild-Type and Tl^{10b} Maternal Phenotypes

Recipient genotype	Injected RNA	No. Scored	Cuticular phenotye			Extent of dorsalization	
			% Ventralized	% Wild-type	% Dorsalized	% VD and FK absent	% Reduced VD and/or FK
wt	None	20	—	100	—	—	—
	Cact^{wt}	20	—	100	—	—	—
	Cact^{SA4}	18	—	0	100	28	72
Tl^{10b}	None	20	100	—	—	—	—
	Cact^{SA4}	19	5	—	95	58	37

Note. RNA transcripts were produced *in vitro* and injected at a concentration of 1.5 μg/μl into stage 2 embryos of the indicated maternal phenotypes. Embryos from wild-type females were scored either as having a wild-type pattern (whether hatched or not) or as having a region of dorsalized cuticle [a reduction in or absence of ventral denticles (VD) or filzkörper (FK)]. Embryos from *Tl^{10b}* mutant females were scored as having either completely ventralized cuticle or dorsalized cuticle, as described above. Recipient maternal genotypes: wt, Oregon R; *Tl^{10b}*, *Tl^{10b}*/+.

in the signal constitutive *Tl^{10b}* genetic background (Fig. 6C, lane 2). In contrast, the Cact$^{SA4}\Delta$PEST protein was readily detectable in both backgrounds (Figs. 6B and 6C, lane 3). Dorsal protein levels in these extracts did not change in the presence of Cact$^{SA4}\Delta$PEST protein (data not shown).

These and the preceding experiments demonstrate that mutation of a putative phosphorylation site renders Cactus resistant to signal-dependent degradation in embryos and SL2 cells. The simplest interpretation of these results is that these serine residues, like those in the closely related IκB motif, are required as phosphoacceptor sites for Pelle or a Pelle-responsive kinase that targets Cactus for signal-dependent degradation.

DISCUSSION

We have used immunochemical techniques and molecular genetic analyses to explore the roles of phosphorylation and proteolysis in Cactus regulation. In both cultured cells and embryos we find that degradation of Cactus is dependent on a Pelle-mediated signal and that mutation of a putative phosphorylation motif blocks signal responsiveness. In embryos, we further find a gradient of Cactus protein. In ventral regions Cactus is degraded, allowing Dorsal to translocate into nuclei, whereas in dorsal regions Cactus persists, retaining Dorsal in the cytoplasm.

Although Dorsal appears to be the only partner for Cactus in the dorsoventral signaling pathway, Cactus may also regulate the Rel-related *Drosophila* immunity factor, or Dif, in the fly immune response (Ip *et al.*, 1993; Lemaitre *et al.*, 1995). Consistent with this hypothesis, we find that Cactus and Pelle regulate Dif-dependent reporter gene transcription in a manner comparable to that seen for Dorsal (M.R. and M.K., unpublished results).

The cell culture system we have used depends on Pelle catalytic function, but does not require the addition of the receptor or ligand that act upstream of Pelle in the embryonic signaling pathway. A likely explanation is that Pelle is activated by its high-level expression throughout the cytoplasm, obviating the need for pathway-dependent localization, modification, or protein–protein interaction.

Model for Dorsoventral Signal Transduction

Based on our findings, we propose a revised model for the establishment of the Dorsal nuclear concentration gradient (Fig. 7). For the purposes of discussion, we focus on two events in the signaling pathway:

Spatially graded activation of the protein kinase Pelle. We suggest that Pelle is recruited to the cell surface by Tube upon ventral and ventrolateral activation of Toll by processed forms of the ligand Spätzle (Morisato and Anderson, 1994; Schneider *et al.*, 1994). Although the role of Toll in this process remains unclear, four lines of evidence support this hypothesis: (1) Tube and Pelle are activated by localization to the plasma membrane (Großhans *et al.*, 1994; Galindo *et al.*, 1995); (2) Tube is required upstream of Pelle (Großhans *et al.*, 1994; Galindo *et al.*, 1995) and downstream of Toll (Hecht and Anderson, 1993), although evidence from cultured cell experiments suggests that Tube may also function downstream of Pelle (Norris and Manley, 1995; 1996); (3) Tube and Pelle interact through their amino-terminal domains (Galindo *et al.*, 1995); and (4) the amino-terminal domain of Tube is sufficient for signal transduction (Letsou *et al.*, 1993).

Spatially graded phosphorylation and degradation of Cactus. We propose that activated Pelle directs the phosphorylation of Cactus at serines located within the amino-terminal, IκB-like site. Signal-dependent phosphorylation would then target modified Cactus for degradation, thereby freeing Dorsal for translocation into ventral and ventrolateral nuclei. Although Pelle itself could be the kinase responsible for directly

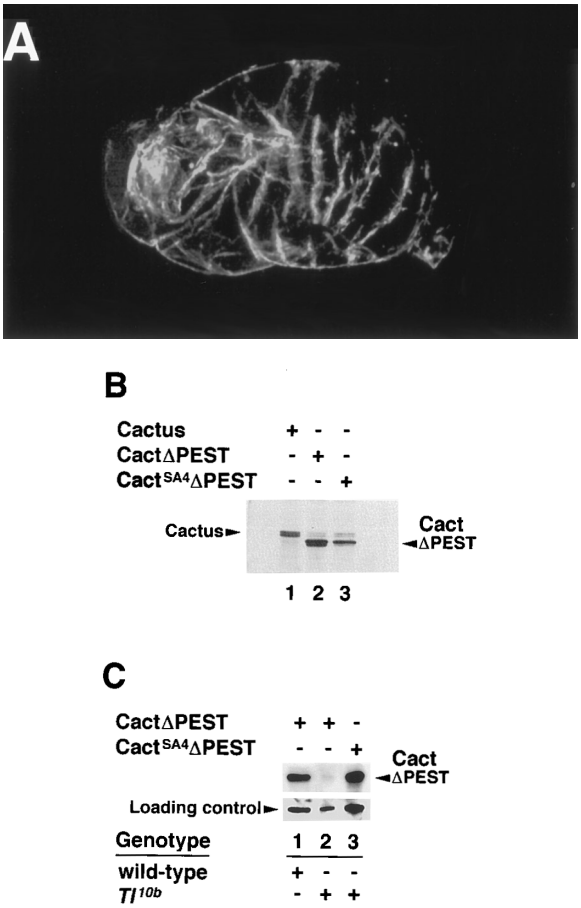

FIG. 6. Cact^{SA4} blocks signaling *in vivo*. (A) *In vitro* synthesized Cact^{SA4} RNA dorsalizes wild-type embryos. Cuticle obtained from a wild-type embryo injected at stage 2 through the posterior end with Cact^{SA4} RNA. The cuticle is dorsalized and lacks the ventral denticle belts and filzkörper indicative of ventral and lateral fates. (B) Wild-type stage 2 embryos were injected with the indicated RNA and allowed to develop to stage 4. Total embryonic contents were then collected by microextraction and examined by immunoblotting with anti-Cactus antibody. CactΔPEST migrates faster than wild-type Cactus protein on SDS–PAGE (lane 2), allowing the two species to be easily distinguished. (C) CactΔPEST and Cact^{SA4}ΔPEST RNA was injected as above into stage 2, *Tl*^{10b} embryos, with total cellular contents collected at stage 4. Immunoblot analysis with anti-Cactus antiserum shows the absence of any CactΔPEST protein (lane 2), whereas Cact^{SA4}ΔPEST protein is clearly present (lane 3). The membrane was then reprobed for biotinylated protein to control for loading.

modifying the target serines in Cactus, it exhibits only a weak ability to phosphorylate Cactus *in vitro* (C. Shelton and S.A.W., unpublished results; M.R. and M.K., unpublished results; Großhans *et al.*, 1994). Significantly, a high-molecular-weight, ubiquitin-dependent protein kinase activity has been recently identified in mammalian cells that can specifically phosphorylate the site of signal-dependent modification in IκB (Chen *et al.*, 1996). Modified Cactus, like IκB (Verma *et al.*, 1995; DiDonato *et al.*, 1996), is then likely degraded via the ubiquitin-mediated proteasome pathway, as degradation of Cactus is blocked in cell culture by calpain inhibitor I (M.R. and M.K., unpublished results).

Spatially graded phosphorylation and degradation of Cactus results in a gradient of Cactus protein in the embryo opposite in polarity to the Dorsal nuclear concentration gradient (Figs. 2A and 2B). Since modification and degradation of Cactus precedes detectable signal-dependent modification of Dorsal, it appears that formation of the Cactus gradient defines the Dorsal nuclear concentration gradient and, consequently, embryonic dorsoventral polarity.

Cactus as the Direct Target for Signal Transduction

Cactus is being continually synthesized as well as degraded in embryos, as evidenced by the ability of antisense cactus RNA to phenocopy a loss of *cactus* function (Geisler

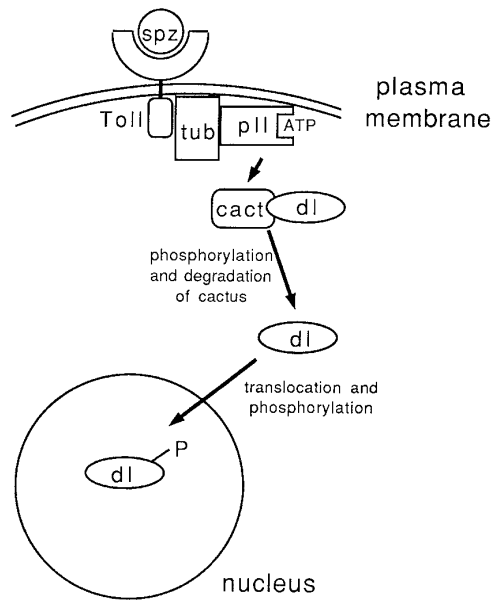

FIG. 7. Model of the signal transduction pathway for the regulated nuclear import of the Dorsal protein. Signal transduction is initiated on the ventral side of the *Drosophila* embryo by binding of a processed form of Spätzle (Spz) to the Toll receptor (Stein *et al.*, 1991; Morisato *et al.*, 1994; Schneider *et al.*, 1994). Activated Toll then transmits a signal via Tube and Pelle, leading to phosphorylation of Cactus at its IκB-like motif. Phosphorylation targets Cactus for signal-dependent degradation, freeing Dorsal for phosphorylation and translocation into nuclei. Signaling is strongest at the ventral midline, intermediate in lateral regions, and weakest at the dorsal surface. Therefore, the signal-dependent processes of Cactus phosphorylation, Cactus degradation, and Dorsal nuclear translocation are all graded along the dorsoventral axis.

et al., 1992). This synthesis and degradation of Cactus is apparently required to maintain a strict stoichiometry between Dorsal and Cactus in the cell (Govind *et al.,* 1993). Consequently, stabilization of Cactus by a carboxy-terminal truncation results in a narrowing of the Dorsal gradient (Belvin *et al.,* 1995). This diminution in signal responsiveness presumably reflects the accumulation of free Cactus, which diverts or absorbs a fraction of the signal intended for Dorsal-bound Cactus.

The degradation of free inhibitor and the stabilization of Dorsal-bound inhibitor is an important consideration in interpreting the phenotypic consequences of a loss of *cactus* function. Nüsslein-Volhard and colleagues have demonstrated that in the absence of Cactus protein, a slight gradient of Dorsal nuclear localization is still present (Roth *et al.,* 1991; Bergmann *et al.,* 1996). This observation could reflect transmission of signal directly to Dorsal. Alternatively, another Cactus-like inhibitor might complex with Dorsal and respond to the Pelle-mediated pathway. The inhibitor need not be present at substantial levels in wild-type embryos nor have a particularly high affinity for Dorsal. Rather, it need only bind Dorsal, and thereby be stabilized, in the absence of competition from Cactus. In this regard, it is worth noting that the transcript for Relish, a p105-like protein containing both a Rel domain and a Cactus/IκB-like domain, has been detected in 0- to 3-hr embryos (Dushay *et al.,* 1996).

Although both Cactus and Dorsal are modified in response to signaling, we believe that signal-dependent modification of Cactus precedes modification of Dorsal. Using extracts prepared from individually staged embryos, we find that signal transduction leads to Cactus degradation at earlier stages than Dorsal phosphorylation. Signal-dependent modification of Dorsal presumably occurs downstream of Cactus degradation, since loss of Cactus function induces Dorsal modification in the absence of signaling (Gillespie and Wasserman, 1994). Phosphorylation of Dorsal upon release from Cactus could reflect unmasking of a phosphorylation site or an encounter with a protein kinase upon translocation into nuclei (Gillespie and Wasserman, 1994; Belvin *et al.,* 1995).

It remains uncertain whether the normal target for signaling is free or Dorsal-bound Cactus. The fact that exogenous addition of ligand triggers Cactus degradation in the absence of Dorsal clearly demonstrates that signal can be transmitted to free Cactus (Belvin *et al.,* 1995). However, dissociation of Cactus from Dorsal may not be required for signaling, since modified IκB-α can be found associated with NF-κB (reviewed by Verma *et al.,* 1995). Indeed, it may be that signal-dependent phosphorylation and degradation of Cactus is unaffected by Cactus/Dorsal complex formation and thus that both free and bound Cactus are targets for Pelle-mediated signaling.

Parallels between the Dorsoventral and IL-1 Signaling Pathways

The findings presented here strengthen the parallels between the mammalian IL-1 and *Drosophila* dorsoventral intracellular signaling pathways. Cactus, like IκB-α and IκB-β, is degraded in response to signal. Furthermore, we find that the serine residues essential for Cactus proteolysis lie in a sequence motif with striking similarity to the signal-dependent phosphorylation motif of IκB-α and IκB-β. An additional parallel has recently been discovered through the identification of a protein kinase that associates with the IL-1 receptor upon ligand binding (Cao *et al.,* 1996). The catalytic domain of this IRAK and that of Pelle are more similar to one another than to the catalytic domain of any other known protein kinase (Cao *et al.,* 1996). Moreover, IRAK, Pelle, and Tube share identity in the amino-terminal region known to mediate interaction between Pelle and Tube (Feinstein *et al.,* 1995; Galindo *et al.,* 1995; Cao *et al.,* 1996). Although the direct substrates for IRAK and Pelle have not been defined, the similarity between the Cactus and IκB phosphorylation motifs, as well as between IRAK and Pelle, indicates conservation between the IL-1 and dorsoventral signaling pathways at the level of biochemical detail.

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