Dissociation of the Dorsal–Cactus Complex and Phosphorylation of the Dorsal Protein Correlate with the Nuclear Localization of Dorsal

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Abstract. The formation of dorsal–ventral polarity in *Drosophila* requires the asymmetric nuclear localization of the dorsal protein along the D/V axis. This process is regulated by the action of the dorsal group genes and cactus. We show that dorsal and cactus are both phosphoproteins that form a stable cytoplasmic complex, and that the cactus protein is stabilized by its interaction with dorsal. The dorsal–cactus complex dissociates when dorsal is targeted to the nucleus. While the phosphorylation of cactus remains apparently unchanged during early embryogenesis, the phosphorylation state of dorsal correlates with its release from cactus and with its nuclear localization. This differential phosphorylation event is regulated by the dorsal group pathway.

THE activity of the rel family of proteins, which includes the lymphocyte transcription factor NF-κB, the avian oncogene v-rel, several vertebrate rel proteins, and the *Drosophila* morphogen dorsal (for review see Blank et al., 1992; Govind and Steward, 1991; also Steward, 1987; Ghosh et al., 1990; Kieran et al., 1990) is to a great extent controlled by their cytoplasmic-to-nuclear translocation. For example, NF-κB, a heterodimer consisting of two rel proteins, p65 and p50 (Baeuerle and Baltimore, 1989), is found in the cytoplasm of uninduced cells in a complex with its inhibitor, IκB (Baeuerle and Baltimore, 1988*a*). An as yet undefined signal transduction pathway initiated by such agents as IL-1 and the phorbol ester TPA results in the activation and nuclear targeting of NF-κB (Lenardo and Baltimore, 1989). It has been proposed, but not demonstrated, that the final step in the signal transduction pathway is the inactivation of IκB by phosphorylation, allowing NF-κB to localize to the nucleus (Kerr et al., 1991; Ghosh and Baltimore, 1990; Lenardo and Baltimore, 1989).

Different forms of IκB, IκBα, IκBβ, and IκBγ, as well as a less-conserved member, bcl3, are found in vertebrates (for review see Blank et al., 1992; Lienhard-Schmitz et al., 1991; Hatada et al., 1992; Inoue et al., 1992*a*; Kerr et al., 1992; Wulczyn et al., 1992; Haskill et al., 1991; Davis et al., 1991; Ohno et al., 1990; Baeuerle and Baltimore, 1988*b*). Recently, the *Drosophila cactus* gene has been cloned and sequenced and the amino acid sequence indicates that cactus represents the *Drosophila* homolog of the IκB proteins (Geisler et al., 1992; Kidd, 1992). These proteins share a motif, the cdc-10/SWI6 or ankyrin repeat (Breeden and Nasmyth, 1987), which is thought to function in protein–protein interactions, and possibly in anchoring proteins to the cytoskeleton (Blank et al., 1992). Although IκB-like proteins appear to function as cytoplasmic sequestering factors, recent data point to a function in the nucleus, possibly as a modifier of NF-κB transcriptional activity (Bours et al., 1993; Zabel et al., 1993).

Like NF-κB, dorsal is targeted to the nuclei as the last step of a multistep signal transduction pathway that has been well characterized. The selective relocalization of dorsal is positively regulated by the 11 dorsal group gene products in the pathway and negatively controlled by cactus protein (for review see Chasan and Anderson, 1993; Govind and Steward, 1991; Stein and Stevens, 1991). A direct interaction between the dorsal and cactus proteins was proposed based on genetic experiments and has recently been demonstrated biochemically (Govind et al., 1992; Geisler et al., 1992; Kidd, 1992; Roth et al., 1991). This dorsal–cactus complex is thought to be disrupted by a ventral signal generated by the transmembrane receptor Toll, and transmitted through the cytoplasmic components of the pathway, pelle and tube, resulting in the nuclear localization of dorsal. The nature of the signal and the exact molecular mechanism by which this signal acts is not known. Furthermore, it is not known whether the signal acts through dorsal or cactus, or through both proteins. It has been proposed that a kinase regulates the nuclear targeting of dorsal when it is transiently expressed in tissue culture cells (Norris and Manley, 1992).

The nuclear targeting of dorsal is selective. The protein is uniformly distributed in the cytoplasm of early embryos and is targeted to the nuclei at the blastoderm stage in a ventral-to-dorsal gradient, with the highest concentration of nuclear dorsal protein in the most ventral nuclei and decreasing levels towards the dorsal side. Nuclei on the dorsal side of the embryo remain devoid of dorsal protein (Roth et al., 1989; Rushlow et al.,1989; Steward, 1989; Steward et al., 1988). The

selective nuclear targeting of dorsal is thought to be the result of the asymmetric activation of the Toll receptor by a ligand that is matured in the extracellular space surrounding the embryo (Chasan and Anderson, 1993; Stein et al., 1991; Schneider et al., 1991; Hashimoto et al., 1991). The concentration of dorsal protein in each nucleus is ultimately responsible for the specific identity of each cell along the dorsal–ventral axis (Ray et al., 1991; Roth et al., 1991; Steward, 1989). Varying threshold levels of dorsal appear to control the activation or repression of specific zygotic genes (Ferguson and Anderson, 1991; Ip et al., 1992; Jiang et al., 1991; Rushlow et al., 1989; Thisse et al., 1987; Rushlow et al., 1987; St. Johnston and Gelbart, 1987).

In this paper we concentrate on the intracellular events that lead to the targeting of dorsal to the nucleus. We show that cactus is associated with dorsal in the cytoplasm of both ovaries and early embryos. Cactus appears to remain exclusively cytoplasmic as dorsal forms a nuclear gradient at the blastoderm stage, indicating that dorsal dissociates from cactus when it is targeted to the nucleus. Further we show that the maternal cactus protein is stabilized by its interaction with the dorsal protein. Finally, we demonstrate the dorsal group genes regulate the differential phosphorylation of dorsal. At least one of the dorsal isoforms, that is highly phosphorylated and does not bind to the cactus protein, represents a nuclear form of dorsal. We will discuss our results in the context of dorsal–cactus complex formation and the role of phosphorylation in the activation of dorsal by the signal generated by the dorsal group genes.

Materials and Methods

Fly Strains

Wild type flies and all mutants used were derived from Oregon R. The following genotypes were used for the crosses as described in the text.

dorsal stocks. dl^8 (a dorsal protein null) and *Df(2R)TW119* are described by Steward and Nusslein-Volhard (1986); dl^{011} is described by Isoda et al. (1992).

cactus stocks. $cact^{A2}$, $cact^{BQ}$ (obtained from S. Roth, Princeton University, Princeton, NJ), $cact^{E10}$ (obtained from Y. Hiromi, Princeton University), and *DF(2L)E10RN2/* are described by Roth et al. (1991).

dorsal-group stocks. $Toll^{10B}$ is described by Erdelyi and Szabad (1989), ea^4 and ea^{5027} are described by Chasan and Anderson (1989) and were obtained from K. Anderson (University of California, Berkeley, CA). $Toll^{r623}$ and $Toll^{rm9}$ are described by Gerttula et al. (1988); tub^{118}, tub^{238}, pll^{312}, and pll^{385} are described by Anderson and Nusslein-Volhard (1986) and were obtained from K. Anderson.

Generation of mAbs against the Dorsal Protein

The full-length dorsal cDNA was subcloned into a pET-3 vector at the NdeI site and a full-length dorsal protein was overexpressed in *Escherichia coli* BL21(DE3) (Steward, 1989). Induction and isolation of mAb lines were performed as described by Bopp et al. (1991).

Several different lines gave rise to mAbs that recognized both the full-length dorsal protein expressed in bacteria, dorsal protein from ovaries, and dorsal protein from 0–3-h embryos. These lines did not crossreact with any protein in dorsal null mutant embryo extracts. Of these, a subset of mAbs were determined to recognize epitopes in the COOH-terminal half of the bacterially expressed protein, as these mAbs did not recognize a bacterially expressed polypeptide encoded by a NdeI–SstI fragment (amino acid 1-403). Two monoclonals within this subset, 7A4-25 and 7A4-39 quantitatively immunoprecipitated the dorsal protein from native embryo and ovary extracts, and from in vitro translation reactions programmed with the *dorsal* mRNA.

For Western blot analysis, dorsal protein is detected using a sheep polyclonal antibody raised against the full-length dorsal protein and affinity-purified against the full-length dorsal protein.

Generation of mAbs against the Cactus Protein

The cloning and expression in bacteria of a cactus fusion protein have been described in detail (Kidd, 1992). A mouse immunized with a glutathione S-transferase–cactus fusion protein which produced serum specific to the cactus protein (Kidd, 1992) was sacrificed and monoclonal lines were generated as described (Bopp et al., 1991).

Hybridoma lines were initially screened based on reactivity to the cactus portion of a maltose binding protein (MBP)[1]–cactus fusion protein (MBP261; a gift from Simon Kidd, Laboratory of Genetics, Rockefeller University). Positive lines that recognized several polypeptides in the 68-kD range on Western blots of wild type ovary and embryo extracts were subcloned (these polypeptides were initially identified by reactivity to the mouse serum). Several lines, 2C2-50, 2C2-51, 3H12-4, 3H12-29, produced mAbs that were able to immunoprecipitate cactus protein from denatured embryo extracts. Monoclonals 2C2-50 and 2C2-51 were found to have an epitope(s) in the NH_2-terminal half of the cactus protein based on reactivity to a maltose binding protein-NH_2-terminal cactus fusion protein (MBP-261N; a gift from Simon Kidd). Monoclonals 3H12-4 and 3H12-29 did not crossreact with MBP-261N.

Whole Cell Extracts and Immunoprecipitations

Extracts from whole flies, ovaries and dechorionated embryos were all performed in the same way. Preparation of extracts and native immunoprecipitations are described in detail by Govind et al. (1992) with the addition of 10 mM sodium molybdate and 1 mM sodium vanadate.

SDS-PAGE and Western Blotting

Separation of polypeptides by minigel polyacrylamide gel electrophoresis (Bio Rad Laboratories, Cambridge, MA) was performed according to Laemmli et al. (1970). To facilitate separation of closely-migrating isoforms of cactus, a higher ratio of acrylamide to bis acrylamide (30%/0.2%) was used as the stock acrylamide solution. When used, these are labeled "low bis" SDS-PAGE in the text.

Protein samples separated by SDS-PAGE were transferred to Immobilon P blotting matrix (Millipore Corp., Milford, MA). The blots were stained for total protein by Ponceau S (Sigma Immunochemicals, St. Louis, MO), and blocked in 10 % nonfat dry milk (Carnation) in 0.5% Tween 20, 0.05% SDS in PBS (Block buffer). Blots were incubated for 4 h at room temperature (or overnight at 4°C) in primary antibodies diluted in Block buffer. The blots were washed for 3 × 10 min in Tween 20–PBS. The blots were then incubated for 2 h at room temperature with the appropriate biotinylated secondary antibody (Vector Labs., Burlingame, CA). After 3 × 30 min washes in Tween 20–PBS, the blots were then incubated with streptavidin-HRP (Vector Labs.) for an additional hour at room temperature. The blots were then washed for 3 × 30 mins in Tween 20–PBS and rinsed once in PBS. The protein bands were detected using HRP-enhanced chemoluminescence (ECL; Amersham Corp.).

Phosphatase Treatments

Whole embryo extracts were prepared from dechorionated 0–3-h embryos as described and denatured by boiling in a final concentration of 2% SDS. The samples were then dialyzed into either potato acid phosphatase buffer (1 mM Pipes, pH 6, 150 mM NaCl) or bacterial alkaline phosphatase buffer (50 mM Tris, pH 8.5, 300 mM NaCl) using a Centricon flowthrough filter (Amicon Corp., Danvers, MA) with a molecular cutoff of 10 kD. For potato acid phosphatase (PAP; Boehringer Mannheim Biochemicals, Indianapolis, IN), the samples were then treated for 2 h at 37°C with PAP or PAP plus 1 mM sodium molybdate (Sigma Immunochemicals). The same incubation conditions were duplicated for bacterial alkaline phosphatase (BAP; Promega Biotec, Madison, WI). The reactions were carried out at 37°C and stopped by the addition of 10% (vol/vol) cold TCA on ice. The acid precipitates were collected, neutralized and solubilized in SDS-loading buffer (SLB: 2% SDS, 65 mM Tris, pH 6.8, 10% glycerol, 20 mM DTT) before separation by SDS-PAGE. The reactions were terminated by boiling in an

1. *Abbreviations used in this paper*: BAP, bacterial alkaline phosphatase; ECL, enhanced chemoluminescence; MBP, maltose binding protein; PAP, potato acid phosphatase.

equal volume of SDS-loading buffer. The samples were then separated by SDS-PAGE and analyzed by Western blots.

Immunolocalization and Confocal Imaging

Embryo and ovary isolation and fixation were essentially as described in Steward (1989). Fixed embryos were blocked in 0.5% Tween 20–PBS with 10% BSA. The embryos were then incubated overnight at 4°C with primary antibodies diluted in the same buffer. They were then washed 3 × 15 min in Tween 20–PBS and incubated in the appropriate biotinylated secondary antibody for 2 h at room temperature. After washing in Tween 20–PBS for 3 × 30 mins, the embryos were further incubated with streptavidin–FITC or streptavidin–Cy3 (Jackson Immuno Research Labs., Inc., West Grove, PA) in the dark at room temperature for 1 h. Finally, the embryos were washed for 3 × 30 mins in Tween 20–PBS, washed once in PBS, and mounted on slides using Aquamount (Polysciences Inc., Warrington, PA). Optical sections were generated by confocal microscopy (MRC600; Bio Rad Laboratories).

Results

Distribution of the Cactus Protein

The *cactus* gene has been identified by its mutant maternal phenotype that shows ventralization of the embryo (Schupbach and Wieschaus, 1989). Its genetic interactions and molecular structure have been described in detail (Roth et al., 1991; Geisler et al., 1992; Kidd, 1992). The ventralized phenotype of the loss-of-function *cactus* mutants indicated that the dorsal group genes and cactus have opposing functions: cactus retains dorsal in the cytoplasm while the dorsal group genes promote nuclear targeting of dorsal. Injection rescue experiments suggested that cactus activity is uniformly distributed in the early embryo (Roth et al., 1991), and the cactus protein has been shown to bind to dorsal protein, blocking its DNA binding activity in vitro (Kidd, 1992; Geisler et al., 1992). Therefore it was of interest to determine if cactus becomes relocalized from the cytoplasm to the nucleus like the dorsal protein.

Based on the maternal function of *cactus* we expected to find that the gene is transcribed and possibly translated in the germline during oogenesis. In addition, since the majority of *cactus* alleles show zygotic lethality, it must also be expressed in other somatic tissues. In the ovary, the cytoplasm of the somatically derived follicle cells appear to stain with the antibody as soon as they are formed in posterior region 2b of the germarium (see *top arrow* in Fig. 1 *A*). This staining becomes more pronounced as the egg chamber matures (Fig. 1, *A–C*) and is maintained throughout oogenesis, as is evident in the optical section through the follicle cells of a stage 9–10 follicle (Fig. 1 *C*). Cactus protein is also abundant in the somatically derived sheaths (Fig. 1, *A* and *B*, **) covering the ovarioles and appears to be exclusively cytoplasmic. The zygotic function of cactus is unknown, although it results in lethality at several different developmental stages. Although cactus has no genetically defined function in the ovary, the abundance of cactus in the soma raises the possibility that it functions in ovarian development.

In the germline, staining of nurse cells is first visible at about stage 4 (*bottom arrow* in Fig. 1 *A*) and appears to increase as the follicle matures. Oocyte staining is visible at about stage 8 (Fig. 1 *B*) and increases in parallel to the staining observed in the nurse cells, demonstrating that *cactus* is transcribed and translated in the oocyte–nurse cell complex. Cactus protein appears to be cytoplasmically localized in both somatic and germline cells.

Embryos were double stained for both cactus and dorsal (Fig. 2). In early embryos cactus protein is uniformly distributed in the cytoplasm similar to the distribution of the dorsal protein (results not shown). At the blastoderm stage, cactus protein remains uniformly distributed in the cytoplasm around the nuclei (Fig. 2, *A* and *D*), while the dorsal protein becomes relocalized to the nuclei in a ventral-to-dorsal gradient (Fig. 2, *B* and *E*). The different localization patterns of cactus (Fig. 2, *D* and *G*) and dorsal (Fig. 2, *E* and *H*) are clearly visible in the magnified sections of the embryo. The cactus antibody is uniformly distributed in the cytoplasm on the ventral and dorsal side as well as in the pole cells (*arrow* in Fig. 2 *D*). In contrast, the dorsal protein is found in the nucleus on the ventral side and decreases in concentration in lateral nuclei (Fig. 2, *E* and *H*). It is also found in the pole cell nuclei (Fig. 2 *E*). On the dorsal side it is found in the cytoplasm with the cactus protein (Fig. 2 *E*).

The difference in the distribution of cactus and dorsal can be seen when anti–cactus and anti–dorsal antibody labeling are viewed simultaneously (Fig. 2, *C*, *F*, and *I*). While the red nuclear staining of dorsal remains virtually the same in the single and double exposure (Fig. 2, compare *I* to *H*), the cytoplasmic staining of cactus (*green*) and dorsal (*red*) results in the orange staining observed all along the dorsal–ventral axis. The lack of any nuclear staining shows that cactus is exclusively a cytoplasmic protein, even as dorsal becomes localized to the nuclei. Staining of postblastoderm stage embryos shows that cactus protein is localized exclusively to the cytoplasm throughout embryonic development (data not shown). In Schneider tissue culture cells, a cell line derived from late embryonic tissues, cactus staining is also cytoplasmic (Fig. 1 *E*).

Cactus Manifests Several Developmentally Regulated Forms

Genetic analysis of cactus indicates that it has distinct maternal and zygotic functions (Roth et al., 1991). Two cactus transcripts have been identified that encode essentially the same protein (Kidd, 1992). One of these transcripts is abundant in female ovaries and early embryonic stages, suggesting that it specifically encodes the maternal cactus protein. This maternal protein is predicted to be 18 amino acids longer at the COOH terminus than the zygotic protein (Kidd, 1992). Consistent with these observations, Western blot analysis of cactus protein expression reveals several polypeptides which are differentially expressed during development (Fig. 3). In whole male extracts (Fig. 3, lane *1*), two major polypeptides of 69 and 71 kD crossreact with the anti–cactus mAb. These two polypeptides are also detected in females (Fig. 3, lane *2*), ovaries from immature females (lane *3*), postblastoderm embryos (lanes *6* and *7*), and in Schneider tissue culture cells (lane *9*). In addition, a third prominent band of 72 kD is expressed in females (Fig. 3, lane *2*), in ovaries from mature females that contain late-stage oocytes and unfertilized eggs (lane *4*). In early embryos (0–3-h postfertilization) the 72-kD protein is the major form of cactus although a 71-kD protein is also detected (Fig. 3, lane *5*). The amount of the 72-kD form decreases after the cellular blastoderm stage, and the 69- and 71-kD forms appear. Except for minor differences, our results with a mAb against

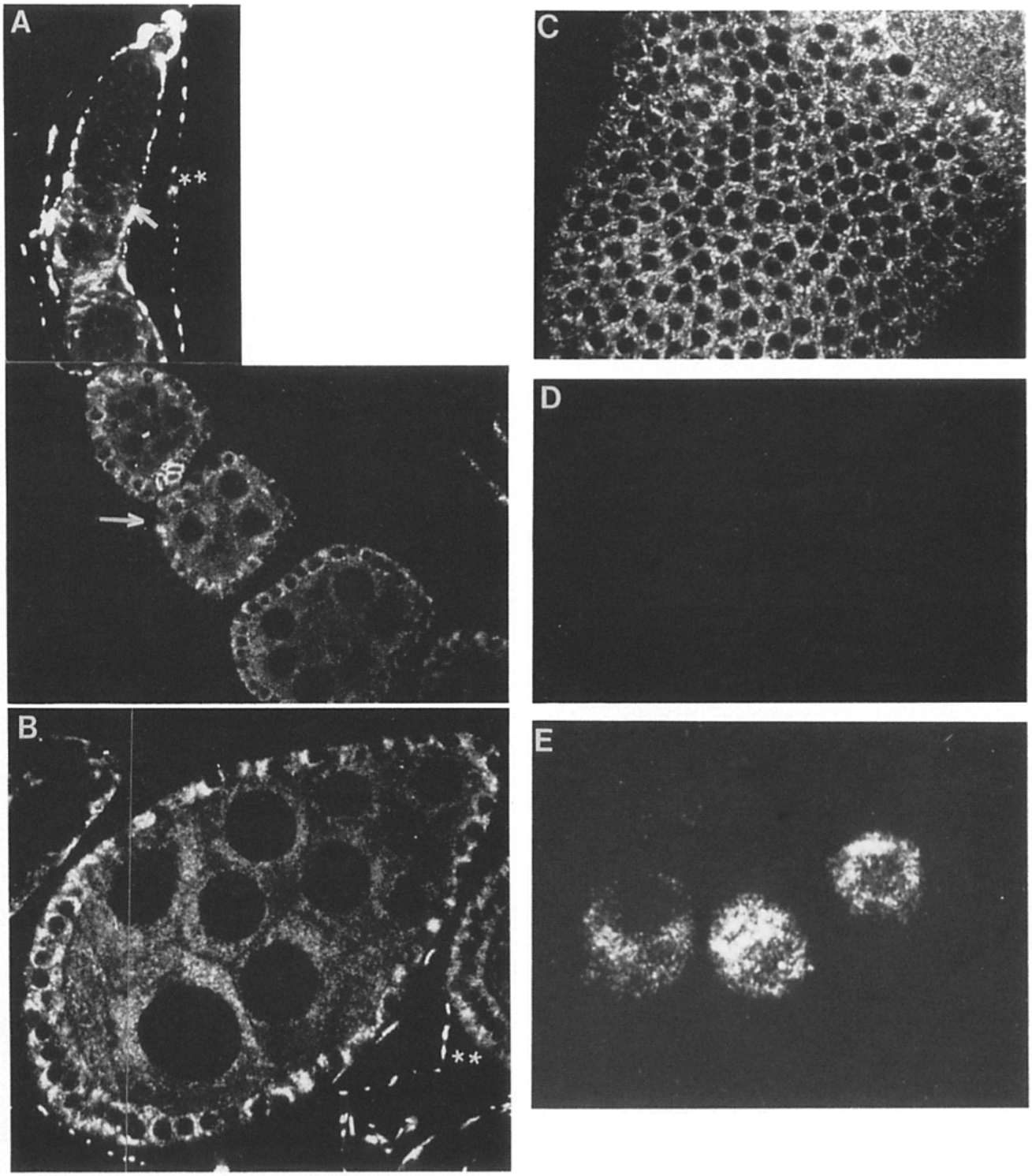

Figure 1. Localization of the cactus protein in the soma and germline of *Drosophila* ovaries. Ovaries from wild type Oregon R females were fixed and stained for cactus protein using anti-cactus monoclonal 3H12-29. Imaging was performed by Bio Rad confocal microscope. (*A*) Longitudinal section through an ovariole showing cactus protein expression limited to the somatically derived ovarian sheath (**), the follicle cells (*arrow*) surrounding the germline derived nurse cells. (*B*) Longitudial section through stage 8 egg chamber, showing expression of cactus in the cytoplasm of the nurse cells. Cactus protein is also seen to accumulate in the developing oocyte. (*C*) Surface view of about stage 10 egg chamber, showing cactus localization in the cytoplasm of the follicle cells. (*D*) Control ovariole treated with secondary antibody only. (*E*) Cytoplasmic localization of cactus in Scheider L2 *Drosophila* tissue culture cells.

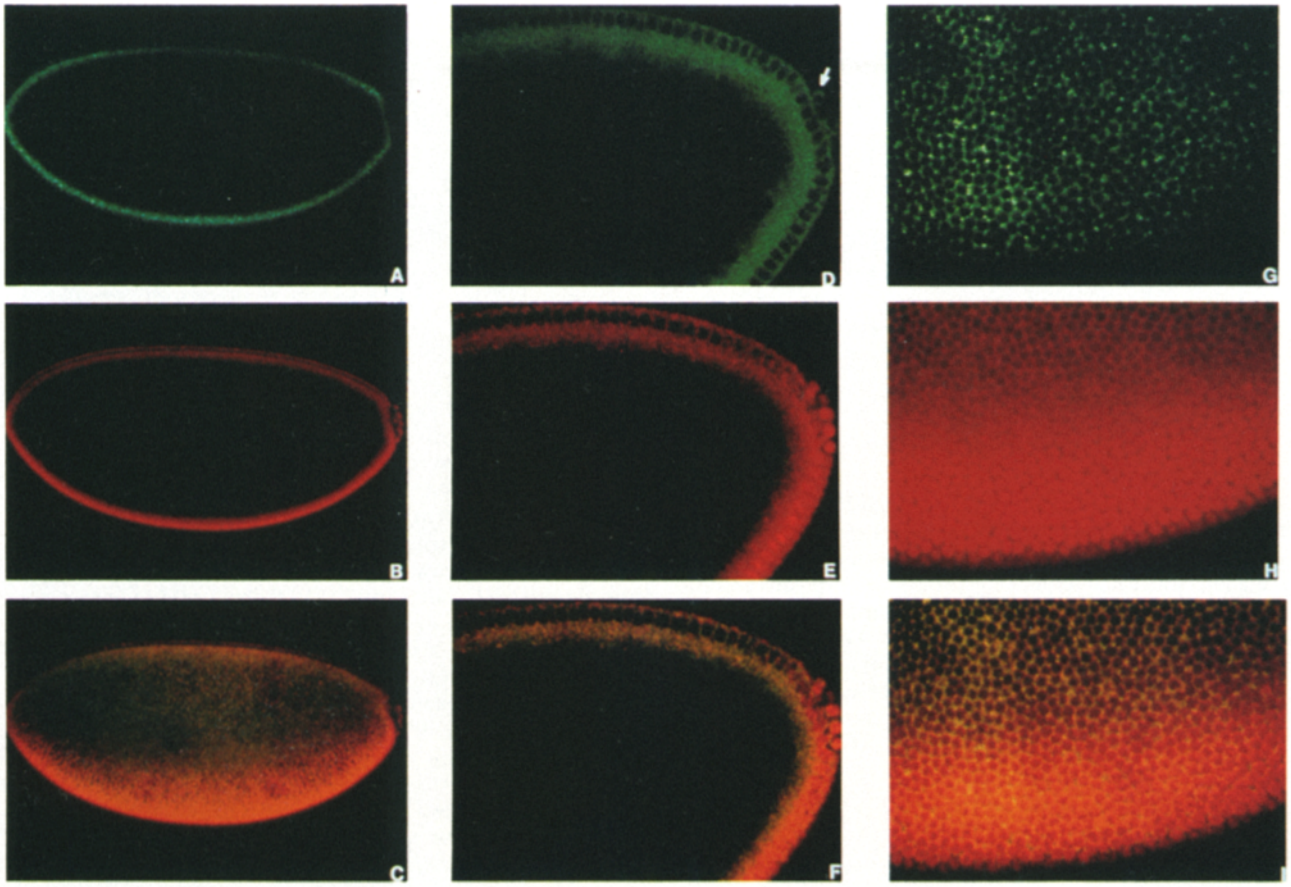

Figure 2. Double-labeling of cactus and dorsal in the early embryo. Embryos were fixed and stained for cactus using monoclonal 3H12-29 (*A*, *D*, and *G*; cactus staining has been imaged in *green*) and dorsal using an affinity-purified sheep anti–dorsal polyclonal antibody (*B*, *E*, and *H*; dorsal staining has been imaged in *red*). When viewed together, areas of overlapping cactus and dorsal staining appear as orange staining (*C*, *F*, and *I*). (*A–C*) Single optical longitudinal section of a syncytial blastoderm; *C* is a projection of several optical sections of the embryo shown in *A* and *B*. (*D–F*) Magnified view of the same embryo showing the posterior end with pole cells (*arrow*). (*G–I*) Magnified view of the ventral–lateral midsection of the same embryo.

cactus confirm those shown by Kidd (1992) using a polyclonal antibody.

The expression of the zygotic and the maternal forms of cactus observed on the Western blot correlates with the staining pattern in the soma and germline during oogenesis. The 69- and 71-kD proteins are detected in ovaries obtained from immature females (Fig. 3, lane *3*) but the 72-kD protein appears as the predominant form in mature ovaries (lane *4*). This suggests that the 69- and 71-kD polypeptides are expressed in the follicle cells, where cactus staining is first detected. The 72-kD band may represent a germline-specific form of cactus which is detected in the nurse cells and the egg chamber later in oogenesis. This protein is apparently deposited into the developing egg prior to fertilization since it is again detected in early embryos.

The results of the developmental Western blot indicate that different forms of the cactus protein actually perform the zygotic (somatic/zygotic) versus the maternal (germline/maternal) functions. The significance of two distinct isoforms of zygotic cactus is not clear. The faster migrating 69-kD form is the most abundant protein at each developmental stage but the 71-kD form is predominant in tissue culture cells, raising the possibility that there is a tissue-specific difference in the abundance of the two isoforms. If so, the 71-kD form may function in the subset of cells which are immortalized in the Schneider tissue culture line. A 71-kD cactus isoform is also detected in low levels in 0–3-h embryo extracts. This isoform may represent either a precursor to the maternal (72 kD) cactus protein, or the newly synthesized zygotic (71 kD) cactus which will function after the blastoderm stage.

Both Cactus and Dorsal Are Differentially Phosphorylated

It has been demonstrated that the vertebrate homologs of dorsal and cactus, the rel proteins and the IκB proteins, respectively, are phosphorylated (Kerr et al., 1991; Gilmore and Temin, 1988). Phosphorylation of IκB is thought to regulate its dissociation from NF-κB (Ghosh and Baltimore, 1990). Both cactus and dorsal have a larger molecular mass on Western blots than the predicted protein (Kidd, 1992; Steward, 1989; Roth et al., 1989). Like cactus, dorsal is detected on Western blots as several developmentally regulated isoforms. In the ovary a single dorsal protein is detected. The dorsal pattern becomes more complex after fertilization (Steward, 1989; Roth et al., 1989). The appearance of high molecular weight forms for both dorsal and cactus indicates that both proteins are modified.

To determine if dorsal and cactus are modified by phos-

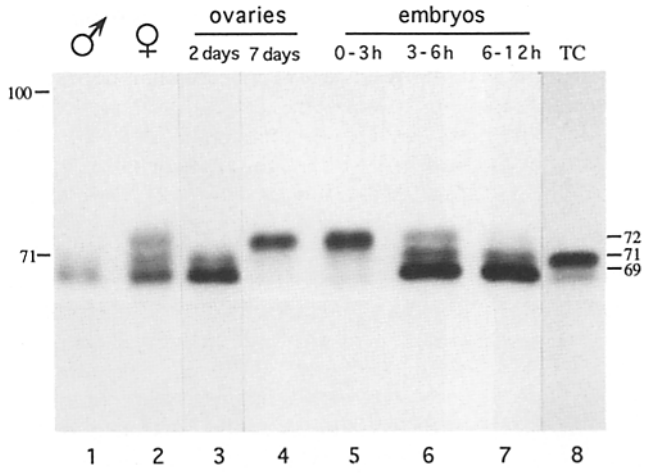

Figure 3. Developmental profile of cactus protein. Total protein extracts (40 μg of total protein/lane) were separated on 12% low bis SDS-PAGE (stock acrylamide = 30% acrylamide −0.2% bisacrylamide), blotted onto Immobilon (Millipore Corp.) and probed for cactus using monoclonal 3H12-29. Protein bands were detected using Enhanced Chemoluminescence (ECL). Oregon R 7-d-old males (lane *1*) and females (lane *2*); ovaries from 2-d-old females (lane *3*) or 7-d-old females (lane *4*); 0–3-h embryos (lane *5*); 3–6-h embryos (lane *6*); 6–12-h embryos (lane *7*). Schneider L2 *Drosophila* tissue culture cells (*TC*, lane *8*).

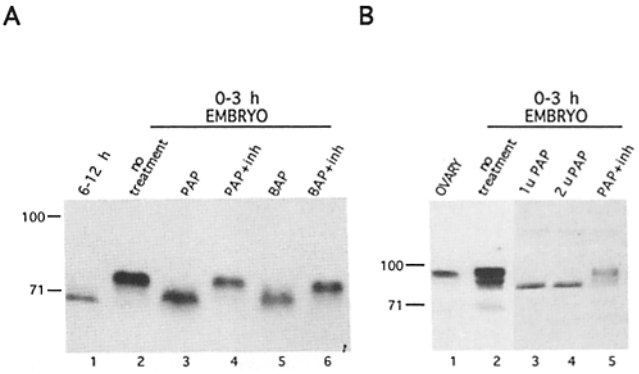

Figure 4. Maternal cactus and dorsal are phosphorylated. (*A*) Whole embryo extracts of 6–12-h embryos (lane *1*) or 0–3-h embryos (lanes *2–6*) were separated by 12% low bis SDS-PAGE, blotted on Immobilon, and probed for cactus using monoclonal 3H12-29. 0–3-h embryo extracts had been dialyzed into either acid phosphatase buffer (lanes *2* and *3*) or alkaline phosphatase buffer (lanes *4* and *5*). The extracts were treated with 2 U of potato acid phosphatase (*PAP*, lane *3*) or 2 U PAP plus 1 mM sodium molybdate (*PAP*+inh, lane *4*). Embryo extracts were treated with 150 U of bacterial alkaline phosphatase (*BAP*, lane *5*) or 150 U of BAP plus 1 mM sodium molybdate (*BAP*+inh, lane *6*). (*B*) Whole embryo extracts from mature ovaries (lane *1*) and 0–3-h embryos (lane *2*) were separated by 10% SDS-PAGE, blotted on Immobilon, and probed for dorsal using affinity-purified sheep anti–dorsal. Embryo extracts were prepared, dialyzed into acid phosphatase buffer, and treated with 1 U (lane *3*) or 2 U (lane *4*) of PAP. Incubations were also done in the presence of 1 mM sodium molybdate (lane *5*).

phorylation, extracts from ovaries and 0–3-h embryos were subjected to phosphatase treatment. The cactus protein present in 0–3-h embryo extracts is the 72-kD form (Fig. 4 *A*, lane *2*), clearly distinguishable from the zygotic 69- and 71-kD forms in 6–12-h embryos (lane *1*). Treatment with potato acid phosphatase (Fig. 4 *A*, *PAP*, lane *3*) or bacterial alkaline phosphatase (*BAP*, lane *5*) results in a shift of the 72-kD form to ~70 kD. The downward shift was specific to the phosphatase treatment, since treatment with phosphatase in the presence of an inhibitor results in no shift in apparent molecular weight (Fig. 4, lanes *4* and *6*). These results clearly demonstrate that the maternal form of cactus is a phosphorylated protein. The phosphatase-insensitive form has a slightly slower mobility than the predominant zygotic form, the 69-kD protein. The difference in their mobilities probably represents the difference in the amino acid sequences for the maternal versus the zygotic RNA (Kidd, 1992).

In parallel to cactus, dorsal was analyzed for possible phosphorylation forms (Fig. 4 *B*). In the ovary of mature females, a single dorsal isoform is detected (Fig. 4 *B*, lane *1*). Several different isoforms of dorsal ranging from 85 to 90 kD are detected in the 0–3-h embryo stage (Fig. 4 *B*, lane *2*). When these embryo extracts are treated with PAP (Fig. 4 *B*, lanes *3* and *4*), the multiple isoforms collapse to a single form of 85 kD. The downward shift is specific to phosphatase treatment, since treatment with PAP in the presence of a phosphatase inhibitor prevents any mobility shift (Fig. 4 *B*, lane *5*). The same 85-kD form is detected when ovary extracts are treated with PAP (data not shown). Since the mobility of this phosphatase-insensitive form of dorsal is similar to that of the bacterially expressed dorsal protein and the in vitro translation product, it most likely represents the unmodified form of the dorsal protein. These results show that both cactus and dorsal are phosphorylated in the ovary and the early embryo.

Analysis of the Cactus Protein in Various Mutant Backgrounds

To determine if the dorsal group genes have a detectable biochemical effect on maternal cactus, we analyzed the cactus protein product in loss-of-function and gain-of-function mutations of the dorsal group genes and compared these results to the cactus protein found in *cactus* mutants, as well as *dorsal* mutant backgrounds (Fig. 5).

The 72-kD maternal form of cactus is the major form seen in extracts from wild type embryos (Fig. 5, lane *1*; for comparison, lane *10* shows the zygotic forms present in Schneider tissue culture cells). Extracts from the loss-of-function *tube* (Fig. 5, lane *2*) or *pelle* (lane *3*) mutations show no differences in the levels or forms of cactus protein present. The same result is seen for a gain-of-function mutation $Toll^{10b}$, although the amount of protein detected is reduced (Fig. 5, lane *4*). Mutations in *tube* and *pelle* result in a strong dorsalized phenotype (D0) because dorsal protein fails to localize to the nuclei all along the dorsal–ventral axis. The $Toll^{10b}$ mutation has a strong ventralized phenotype (V1) because the dorsal protein accumulates in all the nuclei around the embryo circumference at a high level. In all these mutants, independent of the localization of dorsal, the forms of cactus protein detected are indistinguishable from wild type (Fig. 5, lane *1*).

In contrast to the dorsal group mutants, mutations in the *cactus* gene itself show pronounced differences in the mater-

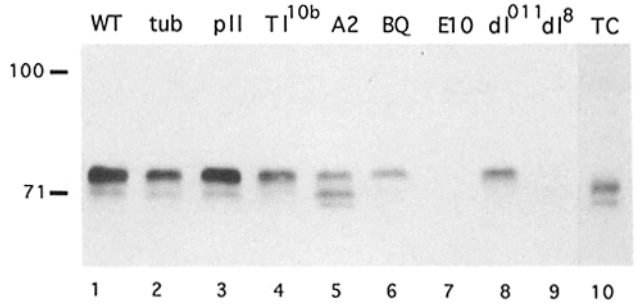

Figure 5. Cactus protein in wild type, dorsal group, *cactus*, and *dorsal* mutant embryos. Total embryo extracts were prepared for each of the following 0–3-h embryo collections. Lane *1*, wild type Oregon R (*WT*); lane *2–4* are mutants in the dorsal group genes; lane *2*, tub^{118}/tub^{238} (*tub*); lane *3*, pll^{312}/pll^{385} (*pll*); lane *4*, $Toll^{10b}/+$ (Tl^{10b}). Lanes *5–7* are mutants in *cactus*; lane *5*, $cactus^{A2}/cactus^{A2}$ (*A2*); lane *6*, $cact^{BQ}/Df(2L)EI0RN2$ (*BQ*); lane *7*, $cact^{E10}/cact^{E10}$ (*E10*); lanes *8–9* are mutants in *dorsal*; lane *8*, $dl^{011}/Df(2L)TW119$ (dl^{011}); and lane *9*, dl^{8}/Df(2L)TW119 (dl^{8}). For each sample, 40 μg of total protein were separated on a 12% "low bis" SDS-PAGE, blotted on Immobilon and probed for cactus using monoclonal 3H12-29.

nal cactus protein. The cactus proteins detected in embryonic extracts of $cactus^{A2}$, that shows a strong ventralizing phenotype (V2) but is zygotically viable, look different from wild type. There is a significant decrease in the 72-kD form and new bands of 69 and 70 kD appear (Fig. 5, lane *5*). It is unlikely that these new forms represent the zygotic component, since the zygotic 69-kD protein is not detected in early embryos (Fig. 5, lane *1*). It is more likely that all three polypeptides represent mutant forms of cactus which are reduced in their ability to regulate dorsal localization.

Two *cactus* gain-of-function mutations, $cact^{BQ}$ and $cact^{E10}$ were also analyzed. Embryos from mothers carrying these mutations show a dorsalized phenotype and dorsal protein remains cytoplasmic (Roth et al., 1991). Genetic analysis indicates that these mutants are not overproducers of cactus (Govind et al., 1993; Roth et al., 1991) but rather lead to products that inappropriately affect the localization of dorsal (Roth et al., 1991). In $cact^{BQ}/Df(2L)EI0RN2$, a form of cactus with the same apparent molecular weight as the maternal form of cactus is present, but at reduced levels (Fig. 5, lane *6*). In $cact^{E10}$, no protein is detectable (Fig. 5, lane *7*). It is possible that a mutant form of maternal cactus made in $cact^{E10}$ is not detected, possibly due to technical problems. In both these mutants, the levels of dorsal protein are similar to the wild type (data not shown), as has been previously suggested by embryo staining (Roth et al., 1991).

We also examined cactus in two dorsal mutant backgrounds. Based on genetic experiments that place dorsal function downstream of cactus, mutations in dorsal itself should not compromise the expression of the cactus maternal forms. Surprisingly, in embryos from dorsal protein null females ($dl^{8}/Df(2L)TW119$), no maternal cactus protein is detected even when twice the amount of protein was loaded relative to the other samples (Fig. 5, lane *9*). Loss of dorsal protein did not affect the expression of zygotic cactus, since in ovaries and older embryos of $dl^{8}/Df(2L)TW119$ the zygotic form is detected (data not shown). These results indicate that the stability of the maternal cactus gene product is linked during oogenesis and early embryogenesis to the presence of dorsal.

To determine if the presence of cactus protein is dependent on dorsal function or on the dorsal protein itself, we looked for cactus in a loss-of-function dorsal allele, dl^{011}. The dl^{011} mutation encodes a COOH-terminal truncation of the dorsal protein (amino acids 1-339; Isoda et al., 1992). Despite the fact that dl^{011} has an intact rel homology region, the protein retains only residual function. Embryos of $dl^{011}/Df(2L)TW119$ mothers display a strong dorsalized (D0-D1) phenotype (Isoda et al., 1992). The 72-kD form of the cactus protein is present in this mutant in reduced amounts (Fig. 5, lane *8*). The presence of the cactus protein in dl^{011} indicates that the stabilization of cactus depends on the presence of the dorsal protein itself, rather than its function. Furthermore, the NH_2-terminal region of dorsal encompassing the rel homology region is sufficient to stabilize the cactus protein.

Analysis of Dorsal in Dorsalized and Ventralized Mutants

The results of the analysis of cactus in various dorsal group mutant backgrounds indicate that the modification of cactus is not detectably affected by the Toll signal transduction pathway. We next addressed the possibility that the dorsal group gene products give rise to the modification of dorsal.

In wild type embryos, a complex pattern of isoforms is resolved from the single molecular weight form observed in late stage oocytes (Fig. 6, lane *1*). At least four bands ranging from 85–90 kD are resolved: a faint 90-kD band, two intermediate bands and an 85-kD band. Several other minor species can also be detected. As was demonstrated in the phosphatase experiment (Fig. 4), all of these isoforms cor-

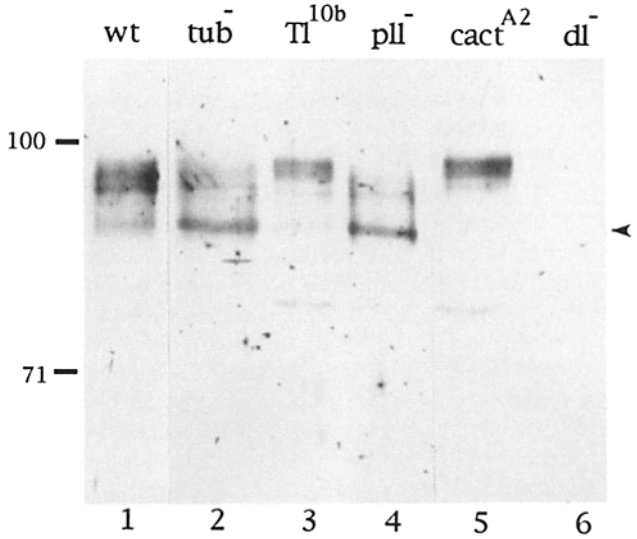

Figure 6. Dorsal protein in wild type, dorsal group, and *cactus* mutant embryos. Embryo extracts were prepared for each of the following 0–3-h embryo collections: lane *1*, wild type 0–3-h embryos; lane *2*, tub^{118}/tub^{238} (*tub-*); lane *3*, $Toll^{10b}/+$ (Tl^{10b}); lane *4*, pll^{312}/pll^{385} (*pll-*);lane *5*, $cact^{A2}/cact^{A2}$ ($cact^{A2}$), lane *6*, $dl^{8}/Df(2L)TW119$ (dl^{8}). For each sample, ~40 μg of total protein were separated on a 10% "low bis" SDS-PAGE, blotted on Immobilon and probed for dorsal using affinity-purified sheep anti-dorsal. This blot is representative of results obtained from several experiments.

respond to different phosphorylated forms of dorsal. A single band with an apparent molecular weight of 85-kD comigrates with dorsal purified from a bacterial expression system (Fig. 6, *arrow*), and most likely represents the unmodified isoform.

Strong loss of function mutations in the genes of the dorsal group pathway result in the absence of dorsal protein in nuclei and the retention of dorsal in the cytoplasm (Roth et al.,1989; Steward, 1989). In the dorsalizing *tube* (Fig. 6, lane *2*) and *pelle* (lane *4*) mutants, the higher molecular weight isoforms of dorsal are markedly reduced compared to wild type (lane *1*). A concomitant increase in the 85-kD isoform is observed. Similar differences are found in *easter* and *Toll* loss-of-function mutants (results not shown). These results clearly show that the phosphorylated state of dorsal in embryos is dependent on the "signal" transmitted from the Toll receptor to the cytoplasmic components, tube and pelle.

This conclusion is further strenghthened by the results obtained from ventralizing mutations, in which the nuclear localization of dorsal is no longer tightly regulated. In the $Toll^{10b}$, the gain-of-function allele in which the dorsal protein is found at high levels in all nuclei (Roth et al., 1989; Steward, 1989), two high molecular weight isoforms are observed and the lower molecular weight isoforms are barely detectable (Fig. 6, lane *5*). In particular, the 90-kD isoform which is faint in the wild type extracts is now readily detectable. In the loss of function cactus allele, $cactus^{A2}$, all nuclei contain dorsal protein but a shallow nuclear gradient is still detectable (Roth et al., 1989, 1991; Steward, 1989). The bands observed in extracts from $cact^{A2}$ are nearly identical to those of $Toll^{10b}$, except that a major intermediate isoform seen in the wild type embryo extract is more abundant in the extract from $cact^{A2}$ than in $Toll^{10b}$. This suggests that subtle biochemical differences in dorsal isoforms between $cact^{A2}$ and $Toll^{10b}$ accompany the differences in protein distribution. Together, the results from the dorsalizing and ventralizing mutations indicate that the high molecular weight isoforms of dorsal detected in embryos (particularly the 90-kD form) correspond to a nuclear form of the protein.

Characterization of the Dorsal–Cactus Complex

Genetic studies of the dorsal group genes and cactus (Govind et al., 1993; Roth et al., 1991) argue strongly for a direct interaction between the two proteins, and it has been shown that dorsal and cactus are found in a complex in ovaries and early embryos (Kidd, 1992). Based on the antibody staining results shown in Fig. 2, dorsal and cactus must dissociate when dorsal is relocalized to the nucleus. To characterize the nature of the dorsal–cactus complex, and to investigate possible changes in the proteins when they dissociate, immunoprecipitation studies on native extracts of wild type ovaries and embryos, as well as dorsalized and ventralized mutant embryos, were performed.

Total extracts from either ovaries or embryos were incubated with a mAb directed against the dorsal protein. The resulting immunoprecipitates were analyzed by Western blot for dorsal (Fig. 7 *A*), and then the same blot was reprobed for cactus (Fig. 7 *B*). No protein is immunoprecipitated from wild type embryo extracts using a pre-immune control (Fig. 7, *A* and *B*, lane *2*). When wild type ovary extracts are incubated with anti-dorsal antibody, a single high molecular weight form of dorsal (Fig. 7 *A*, lane *3*) and the 72-kD form

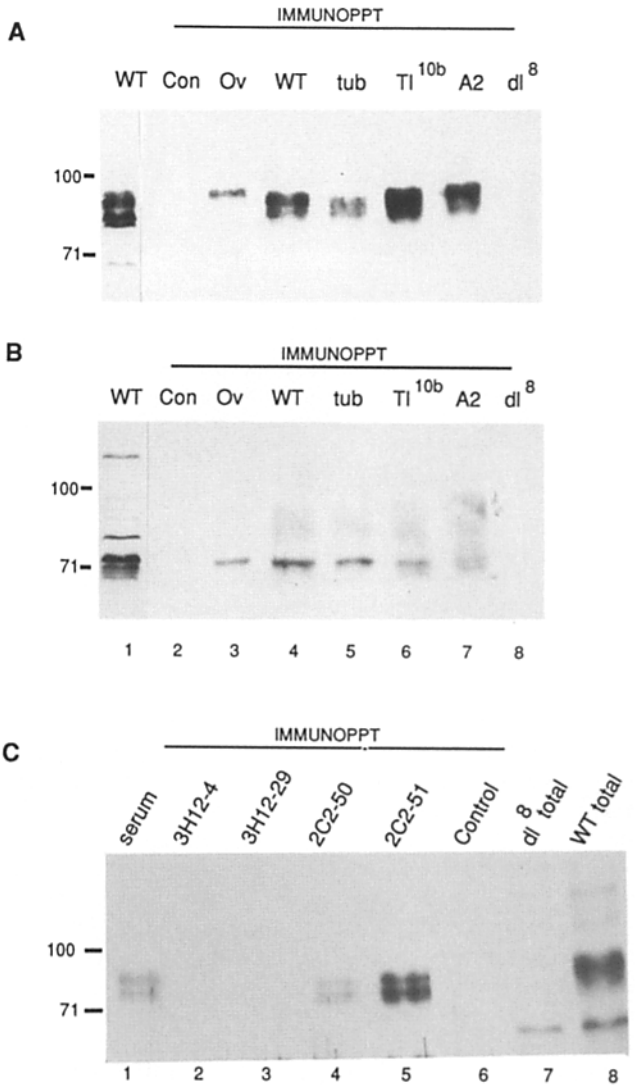

Figure 7. Immunoprecipitation of the dorsal–cactus complex. Total extracts from 0–3-h embryos were incubated with anti-dorsal monoclonal supernatants 4F7-25 and 4F7-39. Immunoprecipitates were separated by 10% SDS-PAGE, blotted to Immobilon, probed for dorsal protein using affinity-purified sheep anti-dorsal polyclonal antibodies (blot *A*). After developing, the Western blot was stripped and reprobed for cactus protein using monoclonal 3H12-29 (blot *B*). Lane *1*, 30 μg of total wild type embryo extract. The extracts for each immunoprecipitation are : lane *2*, Protein A–agarose alone; lane *3*, wild type ovaries; lane *4*, wild type embryos; lane *5*, tub^{118}/tub^{238}; lane *6*, $Toll^{10b}/+$; lane *7*, $cact^{A2}/cact^{A2}$; lane *8*, $dl^{8}/Df(2L)TW119$. Cactus associates with dorsal via the COOH-terminal region in vivo (blot *C*). Native extracts from 0–3-h wild type embryos were incubated with anti-cactus polyclonal serum or monclonal supernatants. The resulting immunoprecipitates were probed for the presence of dorsal protein. The anti-cactus antibodies used were: lane *1*, mouse anti-cactus polyclonal serum; lane *2*, monoclonal 3H12-4; lane *3*, monoclonal 3H12-29; lane *4*, monoclonal 2C2-50; lane *5*, monoclonal 2C2-51; lane *6*, protein A–agarose alone; lane *7*, total extract of 0–3-h $dl^{8}/Df(2L)TW119$ embryos; and lane *8*, total extracts of 0–3-h wild type embryos.

of cactus are coprecipitated (Fig. 7 *B*, lane *3*). In wild type embryo extracts, anti-dorsal coimmunoprecipitates multiple isoforms of dorsal (Fig. 7 *A*, lane *4*) but only the 72-kD form of cactus (Fig. 7 *B*, lane *4*). Immunoprecipitation using the

anti-dorsal antibody from extracts of dorsalized *tube* embryos (Fig. 7 *A*, lane *5*), or ventralized *Toll*^{10b} embryos (lane *6*) confirms that the pattern of dorsal isoforms is altered compared to the wild type (see Fig. 6 for comparison). However, the 72-kD cactus protein remains unchanged and is still associated with dorsal in these mutant backgrounds where the distribution of dorsal is disturbed. In the *cact*^{A2} mutant the two forms of cactus, observed in the total extracts (Fig. 5, lane *5*), associate with the dorsal protein, suggesting that these mutant forms of maternal cactus retain the ability to associate with dorsal.

While the ratio of cactus associated with dorsal is comparable in wild type and dorsalized mutant embryos, this ratio is altered in the ventralized mutants. In the *Toll*^{10b} extract less cactus protein is coprecipitated with the anti-dorsal antibody compared to the wild type. The decreased levels of cactus in complex with dorsal in the *Toll*^{10b} background would indicate that cactus does not interact, or interacts less effectively, with the highest isoforms of dorsal that predominate in this mutant background.

We repeated the immunoprecipitations on native wild type embryo extracts using anti-cactus antibody (Fig. 7 *C*). Fig. 7 *C* (lane *8*) shows total dorsal protein in 0–3-h wild type embryo extracts. The control (Fig. 7 *C*, lane *7*) shows that no dorsal protein is detected in an extract from an equivalent amount of 0–3-h *dl*^{8}*/Df(2L)TW119* embryos. Anti-cactus polyclonal antibodies raised in mouse can immunoprecipitate several dorsal isoforms from native extracts (Fig. 7 *C*, lane *1*). Comparison of the dorsal proteins present in an embryonic extract (Fig. 7 *C*, lane *8*) with the dorsal proteins immunoprecipitated with anti-cactus antibody (lanes *1*, *4*, and *5*) confirms that the lower dorsal bands preferentially associate with cactus while the 90-kD form of dorsal does not bind to cactus.

Two mAbs that recognize an epitope in the COOH-terminal half of cactus fail to immunoprecipitate dorsal from native extracts (Fig. 7 *C*, lanes *2* and *3*). Anti-cactus mAbs that recognize an epitope in the NH_2-terminal half of cactus are able to immunoprecipitate dorsal protein (Fig. 7 *C*, lanes *4* and *5*). These immunoprecipitations suggest that the COOH-terminal region of cactus may contact dorsal, rendering epitopes in this domain inaccessible to monospecific antibodies. This is particularly significant since the COOH-terminal half of cactus contains six conserved ankyrin repeats which are required for its interaction with dorsal in vitro (Kidd, 1992).

Discussion

Developmental Regulation of Cactus

Characterization of the cactus protein during early development in Drosophila reveals a complex pattern of isoforms. A single 72-kD phosphorylated isoform functions in the female germline, while the 69- and 71-kD forms carry out the zygotic function(s) of cactus (Fig. 3). In immature ovaries the maternal 72-kD form is barely detectable, while the two somatic forms are clearly visible. In mature ovaries only the maternal form is detected. These results agree well with our antibody staining experiments that show expression of presumably the zygotic form of cactus in somatic ovarian tissues like the follicle cells and ovarian sheath in previtellogenic stages. It is likely that the form detected in the nurse cells and the developing oocyte is maternal cactus.

While the function of the maternal form of cactus has been clearly defined genetically, little is known about the function of the zygotic cactus proteins (Roth, 1990). The presence of the zygotic isoforms in the follicle cells surrounding the developing egg chamber raises the possibility that cactus participates in some aspect of oogenesis, perhaps in the establishment of axis polarity of the egg shell and developing egg chamber (Schupbach et al., 1991). Genetic data indicates that cactus functions in larval and pupal development. The zygotic isoforms may function similarly to the maternal isoform: it may control the activity of another transcription factor, possibly a rel protein. Alternatively, the presence of ankyrin repeats in the cactus protein may indicate a structural role for the zygotic protein.

The Dorsal–Cactus Complex

Our experiments show that cactus and dorsal are complexed in the cytoplasm, and that when dorsal is targeted to the nucleus, cactus remains a cytoplasmic protein. This indicates that cactus performs its inhibitory function strictly as a cytoplasmic protein, sequestering dorsal in the cytoplasm rather than affecting its ability to bind DNA in the nucleus (Ballard et al., 1990; Blank et al., 1991; Kerr et al., 1991; Inoue et al., 1992*b*; Ruben et al., 1992; Bours et al., 1993). These results support the models of the formation of the dorsal protein nuclear gradient (Govind et al., 1993; Roth et al., 1989, 1991; Steward et al., 1989; Rushlow et al., 1989). The immunoprecipitation studies demonstrate that only the 72-kD phosphorylated maternal cactus forms a stable complex with the unique isoform of dorsal in late oogenesis, as well as associating with dorsal in early embryos.

Previous immunoprecipitation experiments have shown that several different isoforms of dorsal self-associate in vivo (Govind et al., 1992). Taken together, the immunoprecipitations suggest that the maternal cactus protein interacts with a dorsal oligomer. The molecular weight of the native dorsal complex found in early embryos is slightly larger than 210 kD (Govind et al., 1992), consistent with a single subunit of maternal cactus binding to a dorsal dimer. It is interesting to note that this complex is similar to the smallest native unit of the NF-κB/IκB complex which is thought to consist of a p50–p65 NF-κB heterodimer and one IκB monomer.

The interaction of dorsal protein with cactus is mediated by the rel homology region, since the NH_2-terminal 339 amino acids present in the dorsal mutant, *dl*^{011}, can stabilize cactus protein. This result is supported by the observation that truncations within the rel-homology region of dorsal fail to interact with cactus in vitro (Kidd, 1992). It is likely that the ankyrin repeats found in the COOH-terminal half of cactus mediate complex formation of cactus with the dorsal dimer in vivo. The results of the immunoprecipitation using COOH- or NH_2-terminal specific monoclonals suggest that the COOH-terminal epitopes of cactus are masked when cactus is complexed with dorsal. The ankryin repeats in cactus have been shown to be necessary for its interaction with dorsal in vitro (Kidd, 1992) and for the interaction of IκB-like proteins with NF-κB (Hatada et al., 1992; Inoue et al., 1992).

An assortment of unrelated proteins with functions rang-

ing from receptors to transcription factors contain varying numbers of these SWI6/ankyrin repeats. In proteins like ankyrin, SWI6/ankyrin repeats are thought to mediate the interaction with cytoskeletal proteins, such as spectrin. It is unlikely that the SWI6/ankyrin repeats of the maternal cactus isoform interact with some cytoskeletal feature in the embryo, since the dorsal–cactus complex is readily soluble under the mild extraction conditions used in the immunoprecipitation experiments. This motif probably provides the essential protein–protein interaction sites necessary to sequester dorsal in the cytoplasm.

Is Cactus Modified when the Dorsal–Cactus Complex Dissociates?

Based on the analysis of in vitro phosphorylation of the IκB proteins, it has been suggested that phosphorylation may regulate the dissociation of IκB-like proteins from the rel family proteins (Ghosh and Baltimore, 1990; Kerr et al., 1991). For example, phosphorylation in vitro prevents IκB from interacting with NF-κB (Ghosh and Baltimore, 1990). Maternal cactus is phosphorylated during oocyte maturation and we cannot detect a change in its phosphorylation state which corresponds to the formation of the dorsal nuclear gradient during embryogenesis. Therefore, apparently cactus is not the target of the dorsal group genes. However, it is possible that a transient modification occurs that fails to register as a detectable biochemical change in our experiments.

In either case, our results show that the stability of cactus is dependent on its interaction with dorsal. Cactus destabilization and degradation may account for the decreased levels of cactus in *Toll*^{10b} extracts, where the level of cytoplasmic dorsal is reduced and high levels of nuclear dorsal protein are observed in all nuclei. The stabilization of cactus protein by dorsal is specific to the maternal form, since in ovaries and embryos from *dorsal* null females, the zygotic cactus form is present (data not shown). The fact that cactus protein is unstable in the absence of dorsal shows that no other rel protein (dorsal-like) interacts with the maternal form of cactus.

Genetic experiments suggested that the relative concentration of dorsal and cactus are important for the formation of the nuclear gradient of dorsal. These experiments indicated that in a wild type embryo there is an excess of cactus. Increasing the genetic dose of cactus over that in wild type does not affect dorsal–ventral patterning (Govind et al., 1993). The observation that dorsal protein stabilizes cactus explains the genetic results. Regardless of the number of gene copies expressing cactus, the amount of cactus protein will be regulated posttranslationally by the concentration of cytoplasmic dorsal. Excess cactus protein is degraded in the absence of dorsal protein, presumably because it cannot form a complex and is free in the cytoplasm.

This observation may point to an important regulatory feature affecting nuclear targeting of the dorsal protein. The predicted amino acid sequence for cactus indicates the presence of a PEST sequence in the carboxy terminus, a motif associated with a high turnover rate for a protein (Kidd, 1992). Cactus protein could be rapidly degraded as the cactus–dorsal complex dissociates, preventing complex formation to reoccur and facilitating the rapid transport of dorsal to the nucleus. The rapid turnover of the cactus protein and the relative instability of the dorsal–cactus complex are also consistent with the observation that a strong cactus loss-of-function phenotype can be mimicked when antisense cactus mRNA is injected into early cleavage stage embryos (Geisler et al., 1992). This stabilization may be a regulatory feature of the IκB-like proteins since it has also been observed for IκB-α, which is stabilized by its interaction with the IκB-binding domain of the p65 subunit of cytoplasmic NF-κB (Sun et al., 1993).

Nuclear Dorsal Protein

We have shown that the dorsal protein exists as multiply phosphorylated isoforms that are developmentally regulated. Both dorsal and cactus are phosphorylated during oogenesis. The dorsal protein is initially phosphorylated in the mature ovary, resulting in a 90-kD protein. It is not clear why the ovarian form of dorsal is phosphorylated but it may serve as a stabilized storage form of the protein in unfertilized eggs.

After fertilization, an array of differently phosphorylated isoforms ranging from 85–90 kD are observed. When dorsal is strictly cytoplasmic (e.g., in *tube* or *pelle* embryos), the 90-kD form and the intermediate forms are greatly reduced, and the nonphosphorylated form of dorsal (85 kD) becomes prominent. When dorsal is predominantly nuclear (i.e., in *Toll*^{10b} embryos), the highest molecular weight forms become the predominant isoforms of dorsal. These results show that the phosphorylation of dorsal correlates with its nuclear localization. The close correlation between the phosphorylation state of dorsal and the signal transduction pathway that controls the nuclear targeting of dorsal argues for phosphorylation as a regulatory mechanism in dorsal nuclear targeting. However, based on our results we cannot exclude the possibility that dorsal is phosphorylated in the nucleus and that the dissociation from cactus and nuclear targeting are controlled by different mechanisms.

It is possible that several differentially phosphorylated dorsal isoforms actually enter the nuclei along the dorsal–ventral axis by virtue of their ability to form dimers with the highly phosphorylated forms. These different dimers could have varied DNA binding affinities or specificities for interaction with other transcription factors. Altered phosphorylation has been shown to be the critical component in defining the specific activity of several transcription factors (Hunter and Karin, 1992; Jackson, 1992). It is also possible that dimers containing different phosphorylated forms of dorsal might enter the nuclei at different rates, providing the mechanism for the formation of the dorsal nuclear gradient. Phosphorylation of a protein near a nuclear localization sequence has been linked to the rate of nuclear translocation (Rihs et al., 1991).

The Role of the Dorsal Group Genes in Regulating the Phosphorylation of Dorsal

When the dorsal isoforms observed in wild type are compared to those in *tube* and *pelle* mutants, it is apparent that in the wild type embryo most of the dorsal molecules become the target of a novel phosphorylation event which may be dependent on the activity of the dorsal group gene pathway.

There are several possibilities that may define the sequence of events leading to the phosphorylated forms of dorsal. (*a*) The dorsal group genes could activate a kinase which phosphorylates dorsal when complexed with cactus. This kinase might be the dorsal group gene *pelle,* which encodes a serine–threonine kinase homologous to *raf/mos* which functions as a kinase in D/V pattern formation (Shelton and Wasserman, 1993). (*b*) The phosphorylation of dorsal is indirectly dependent on the activity of the Toll receptor pathway. The dorsal group genes could function to signal the release of dorsal from cactus, resulting in free dorsal. Only free dorsal could be modified by a kinase(s) responsible for generating the high molecular weight isoforms of dorsal. (*c*) Once the nuclear gradient is formed, a nuclear protein kinase could phosphorylate dorsal to activate its transcriptional activity. It is unlikely that a single event results in the dissociation of the dorsal–cactus complex and the nuclear targeting of dorsal. Our results of the characterization of the dorsal and the cactus protein point to a complicated mechanism that may require several distinct phosphorylation events to establish the nuclear gradient and transcriptional activity of dorsal.

We thank Georgia Vasilakis and Leslie H. Huang for excellent technical assistance, Joe Goodhouse for his expertise with the confocal microscope, Kathryn Anderson and Siegfried Roth for sending us fly stocks, Marty Marlow for her help with mouse monoclonals, and Gordon Gray for fly food. Our thanks to Shubha Govind, Yasushi Hiromi, Siegfried Roth, Trudi Schupbach, and Jean Schwarzbauer for their comments on the manuscript. We also thank our other Princeton colleagues, especially Paul Schedl for stimulating discussions. Special thanks to Rita Bopp.

A. M. Whalen is a postdoctoral fellow of the New Jersey Commission on Cancer Research. This work was supported by grants from National Institutes of Health and National Science Foundation and the Horace W. Goldsmith Foundation.

Received for publication 1 June 1993 and in revised form 2 August 1993.

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