The dorsal protein enhances the biosynthesis and stability of the *Drosophila* I κ B homologue cactus

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ABSTRACT

The cactus and dorsal proteins are *Drosophila* homologues of mammalian I κ B cytoplasmic anchor proteins and rel/NF κ B transcription factors respectively. They are required for the generation of embryonic dorso-ventral polarity and probably at later developmental stages for an innate immune response. In this paper we report on the properties of SLDL, a derivative of the SL2 cell line in which dorsal is expressed constitutively. In SLDL cells biosynthesis of cactus protein is stimulated by ~4-fold when compared with SL2 cells. Enhanced biosynthesis of cactus protein cannot be explained solely on the basis of increased expression of the cactus gene as the level of the corresponding mRNA is only 2-fold higher than in SL2 cells. On the basis of these findings we propose that free cytoplasmic dorsal protein is able, directly or indirectly to stimulate translation of the cactus mRNA. Such an arrangement would enable the dorsal protein to be buffered in the cytoplasm of the resting cell over a wide range of concentrations. We also show here that subsequent to biosynthesis the cactus protein is either rapidly degraded or incorporated into complexes with dorsal. Protein that does not associate with dorsal has a half-life of ~40 min whereas that which is incorporated into complexes is very stable, having a half life in excess of 24 h. The complexed cactus protein is acted on by protein kinases which generate distinct phosphorylated isoforms.

INTRODUCTION

The cactus protein is a *Drosophila* homologue of the mammalian I κ B family of cytoplasmic anchor proteins (1,2). It functions to regulate the nuclear localization of the rel/NF κ B transcription factor dorsal during the generation of embryonic dorso-ventral polarity and may also be required in larvae for an innate immune response activated by exposure to bacterial lipopolysaccharides (3,4). The overall primary structure of cactus is strikingly similar to mammalian I κ B proteins and especially Mad3 and pp40. Like cactus, these proteins function to retain rel/NF κ B transcription factors in the cytoplasm and are dissociated in response to a number of extra cellular stimuli including that mediated by the

type 1 interleukin 1 receptor (5). They have in their C-termini 6 tandem copies of the 33 amino acid ankyrin repeat motif, sequences that are absolutely required for association with cognate rel/NF κ B transcription factors. Pairwise comparisons show that each repeat is more highly related to the corresponding repeat in other I κ B proteins than to other repeats in the same molecule and it is likely that the repeats form into α -helical bundles which comprise a basic binding domain (6). The ankyrin repeat domains are flanked at the C-terminal by proline rich sequences implicated in protein stability (7) and overlapping these PEST sequences, by several consensus phosphorylation sites for casein kinase 11.

Several studies have demonstrated the importance of protein stability in the regulation of rel/NF κ B complexes (8-11). For example, stimulation of the pre B cell line 70Z/3 by several agents known to act through NF κ B results in the rapid degradation of I κ B α followed by a slow resynthesis. Treatment of cells with antioxidants and protease inhibitors prevents degradation of I κ B and activation of NF κ B complexes. There is strong evidence that I κ B α is phosphorylated constitutively and in response to stimulation by tumour necrosis factor but the evidence for signal dependent modification mediated by interleukin 1 is not conclusive (8,9,12).

Dorsal and cactus associate together in the embryo as a heterotrimer consisting of two molecules of dorsal and one of cactus (13). In the development of embryonic dorso/ventral polarity, complexes dissociate in response to a ventrally restricted signal transduced from the peri-vitelline space of the embryo by a *Drosophila* homologue of the interleukin 1 receptor, Toll (14,15). Genetic studies have identified a mutant form of Toll, Toll10B, which is constitutively activated (16) and this receptor can be used to reconstitute the signal transduction pathway leading to nuclear localization of dorsal in the *Drosophila* tissue culture cell, SL2 (17). SL2 cells do not express dorsal to detectable levels and thus can provide a background lacking in rel/NF κ B transcription factors which is difficult to obtain in mammalian cells.

The *cactus* gene is expressed throughout the life cycle of *Drosophila* and due to alternative splicing, produces two isoforms that differ marginally at the C-terminal. The larger form termed 'maternal/zygotic' is produced during oogenesis and throughout development whereas the smaller form termed 'zygotic' is not found in the developing ovary but is expressed at all other stages. The maternal/zygotic form of cactus is present during early

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Figure 1. Immunofluorescent confocal microscopy of SL2 cells stained with dorsal and cactus antibodies. SL2 cells were transfected with pACTdl (2 μ g), fixed and stained as described in Materials and Methods. (A) Immunofluorescent image of dorsal protein. (B) Immunofluorescent image of cactus protein.

embryogenesis as a phosphoprotein (1,2). However the nature of the protein kinases that modify cactus is unknown and as yet there is no evidence published of signal dependent phosphorylation.

In this paper we show that in a cell line constitutively expressing dorsal the biosynthesis of cactus is enhanced by a factor of 5-fold. We also show that cactus which is incorporated into complexes with dorsal is at least 30-fold more stable than the unbound form.

MATERIALS AND METHODS

Cell lines and cell culture

The culture and manipulation of SL2 cells has been described previously. SLDL cells were constructed by co-transfecting SL2 cells with pACTdl (18), (0.5 μ g) and F9 (10 μ g) (19). pACTdl does not have a selectable marker but the carrier plasmid encodes resistance G418. It seems that prior to integration the transfecting DNAs concatenate and therefore pACTdl and F9 almost invariably co-integrate.

Immunofluorescent microscopy

Dorsal protein was detected in SL2 cells using a rat anti-dorsal polyclonal antibody and fluorescein conjugated anti-rat secondary antibody as described previously (17). Antibodies specific for cactus were raised in rabbit using CACT-Bgl protein (6) and the anti-serum was purified as previously described (19). Cactus protein was detected using primary antibody at a dilution of 1/200 and a goat anti-rabbit whole antibody conjugated to Texas red.

The cactus and dorsal proteins in the cells was viewed with an MRC 600 confocal microscope.

Western blot

Proteins were separated by SDS-PAGE. In order to separate closely migrating isoforms of cactus protein a higher than normal ratio of acrylamide to bis-acrylamide was used (final concentrations 7 and 0.04% respectively). Gels were transferred to nitrocellulose membranes (Hybond C+, Amersham International) in 48 mM Tris, 39 mM glycine, 0.04% SDS and 20% methanol using a semi-dry electroblotter. The blots were blocked with 10% milk powder, 0.1% Tween-20 in phosphate buffered saline (PBS) and then incubated with a 1/50 000 dilution of rabbit anti-cactus antibody in the same solution (1% milk powder). The membrane was then reacted with anti-rabbit antibody conjugated with horse radish peroxidase (Vector Labs, 1:1000 dilution), washed and visualised using enhanced chemiluminescence (Amersham International). Phosphatase treatment was performed as described by Whalen and Steward (20).

Metabolic labelling of SL2 and SLDL cells and immunoprecipitation of cell extracts

Trans ^{35}S label (1000 Ci/mmol) was obtained from ICN. For labelling of cactus, cells were seeded at 1×10^6 cells/ml in 60 mm plates. The media was aspirated, replaced with M3 lacking methionine (M3-Met) and the cells were incubated for 30 min. The media was then aspirated and replaced with 1 ml of M3-Met containing 200 $\mu\text{Ci/ml}$ 'Trans ^{35}S ' label. Cells were labelled for

Figure 2. Dorsal protein is constitutively expressed in SLDL cells and is localised in the cytoplasm. (A) Structure of pACTdl plasmid. (B) Cell extracts (5×10^5 cells/lane) were fractionated by 7% low-SDS-PAGE and analysed by Western blot using rat anti-dorsal antibody. Cell extracts were prepared from SL2 cells (lane 1), mock transfected cells (lane 2), SLDL cells (lane 3) and DL5E (a cloned derivative of SLDL) (lane 4). (C) SLDL cells were stained for immunofluorescence microscopy with rat anti-dorsal antibody and a rhodamine conjugated anti-rat secondary antibody. Nuclei were visualised by staining with DAPI (D).

10 min in the case of protein biosynthesis assays. For pulse chase experiments, the cells were labelled for 1 h, washed $3\times$ in PBS and incubated for the indicated times in M3 media. To prepare cells lysates, the media was aspirated and the cells solubilised in 'I' lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with PiC (1 mM phenyl methyl sulphonyl fluoride, 0.014 mg/ml pepstatin and 0.01 mg/ml leupeptin). Insoluble material was removed by centrifugation (14 000 g, 10 min). Antibody was added (1/40th dilution) and the lysate incubated at 4°C overnight. Then 30 μl of a 50% slurry of either Protein-A Sepharose (for rabbit anti-cactus) or Protein-G Fastflow (for rat anti-dorsal) was added and the solution was incubated at 4°C for a further 90 min. Finally, the immunoprecipitates were washed $3\times$ in I lysis buffer and then solubilised in SDS-PAGE sample buffer. To determine the overall level of protein biosynthesis, the incorporation of label into TCA insoluble counts was measured (21). Radiolabelled bands in both pulse chase and Northern blot experiments were quantified using the ImageQuant software of a Molecular Dynamics Model 425 PhosphorImager.

Northern blot analysis

Whole RNA was prepared from 5 ml of mid-log phase SL2 and SLDL cells. The cells were collected by centrifugation (180 g) and dissolved in guanidinium thiocyanate solution. The RNA was then pelleted through 5.7 M CsCl at 150 000 g for 16 h. RNA samples (15 μg) were separated in a 1.1% agarose gel in the presence of 6% formaldehyde. The blots were annealed with random-prime labelled probes (Boehringer-Mannheim) generated either from a cDNA of *Drosophila* ribosomal protein L18a (22) or with a probe derived from an internal *Bg/II* fragment of cactus (6).

RESULTS

Transient expression of dorsal enhances cactus protein in *Drosophila Schneider* (SL2) cells

SL2 cells were transiently transfected with the dorsal expression plasmid pACTdl (18) and stained for dorsal and cactus using specific antibodies raised in rat and rabbit respectively [(17) and

Materials and Methods]. The cells were examined for the presence of dorsal and cactus by immunofluorescent confocal microscopy (see Materials and Methods). As can be seen in Figure 1 dorsal is only detected in those cells that have taken up the pACTdl plasmid. By contrast, cactus protein is present at low levels in all cells but at a much higher level in those cells transfected with the pACTdl (Fig. 1B). Control experiments show that the observed enhancement of cactus staining is not caused by cross-reaction of the primary or secondary antibodies (result not shown).

Cell line SLDL expresses the dorsal protein constitutively

A cell line that expresses dorsal constitutively (SLDL) was prepared as described in Materials and Methods. The expression of dorsal in SL2 and SLDL cells was investigated by Western blot analysis (Fig. 2B) and by immunofluorescent microscopy using a specific rat anti-dorsal antibody (17). As shown in Figure 2C, most of the cells in this polyclonal line express dorsal and the protein localises to the cytoplasm (Fig. 2C, cf. Fig. 2D). SLDL cells can be propagated indefinitely without losing the ability to express dorsal.

Dorsal induces a phosphorylated isoform of cactus protein

We have analysed the nature of cactus protein in SL2 and SLDL cells using the Western blot method (Fig. 3A). In SLDL cells cactus protein is expressed to a much higher level than in SL2 cells. In addition while only two forms of cactus can be detected in SL2 cells a third species of slower mobility is present in SLDL cells (Fig. 3A). A similar distribution of cactus isoforms is seen in uninduced ST10BDL, a cell line that expresses dorsal constitutively and the Toll 10B receptor under heat shock regulation. Densitometry indicates that this species constitutes ~25% of the total cactus protein (Fig. 3B). The cactus protein in SLDL cells can be converted into two isoforms corresponding to the zygotic and maternal/zygotic protein by treatment with phosphatase (Fig. 3C). Taken together, these results show that the dorsal protein can induce the formation of a distinct phosphorylated isoform of cactus.

Cactus is associated with dorsal in SLDL cells

To determine whether dorsal and cactus are physically associated in SLDL cells, cell extracts were immunoprecipitated with a rat anti-dorsal antibody. The immunoprecipitates were then separated by SDS-PAGE and probed by Western blot for the presence of cactus (Fig. 4A). This experiment shows that cactus protein is present in immunoprecipitates of SLDL cells but not of SL2 cells. It is also apparent that dorsal is associated with all three isoforms of cactus in a similar ratio to that found in whole protein extracts of SLDL cells. In the reciprocal experiment, all phosphorylated isoforms of the dorsal protein can be detected in cactus immunoprecipitates of SLDL but not SL2 cells (Fig. 4B). These results show that in SLDL cells cactus and dorsal are associated with each other and that phosphorylation does not affect their ability to interact.

Enhanced biosynthesis of cactus protein in SLDL cells

We have compared the biosynthesis of cactus protein in SL2 and SLDL cells. Cells were labelled for 10 min with ^{35}S methionine and cactus protein was purified by immunoprecipitation. As can

Figure 3. Isoforms of Cact in SLDL cells. (A) Western blotting of Cact in SLDL cells. Extracts were prepared from SL2 (SL), mock transfected (F9), SLDL (lane 3) and ST10BDL cells (STDL). Proteins were fractionated by 7% Low-SDS-PAGE (30 $\mu\text{g}/\text{lane}$) and analysed by Western blotting using anti-cactus antibody. Isoforms of cactus are indicated on the right: Phosphorylated form (P-cact), maternal/zygotic form (M-Cact) and zygotic form (Z-Cact). Apparent molecular weights of marker proteins in kDa are indicated on the left. (B) Densitometric measurement of A. Western blot of cactus was scanned with a Leica Quantimet 500 densitometer. Values at the bottom show relative distance of peaks. Abbreviations for cells are as in panel A. (C) Phosphatase treatment of cactus from SLDL cells. Extract was prepared from SLDL cells and dialysed into phosphatase buffer by dilution and concentration in a Centricon-10. The sample was divided into four and incubated, as indicated, with phosphatase inhibitors and bacterial alkaline phosphatase (BAP) at 37°C overnight. Isoforms of Cact and apparent molecular weights are indicated as in Figure 3.

Figure 4. Association of dorsal and cactus in SLDL cells. (A) Immunoprecipitation using anti-DL antibody. Cell extracts were prepared from SL2 (SL) or SLDL cells (SLDL) and immunoprecipitated using anti-dorsal antibody (α -DL-IP) and then analysed by Western blotting using anti-cactus antibody. Cell extract from SLDL cells was also run along side the immunoprecipitated samples as a standard for cactus isoforms (C). Apparent molecular weights of marker proteins in kDa are indicated on the left. (B) Immunoprecipitation using anti-cactus antibody. Cell extracts were prepared from SL2 (SL) or SLDL cells (SLDL) and immunoprecipitated using anti-cactus cells (α -Cact-IP) and then analysed by Western blotting using anti-DL antibody alongside unpurified cell lysate from SLDL cells.

be seen in Figure 5, considerably more label is incorporated into cactus in SLDL cells than in SL2 cells. This difference has been quantified with a phosphor-imager and compared to the general rate of protein biosynthesis. As summarised in Table 1, there is a 4-fold enhancement of cactus biosynthesis in SLDL cells compared to SL2 cells and this enhancement is more marked in the case of the maternal/zygotic than the zygotic isoform. It is possible that the observed enhancement reflects a substantially higher rate of turnover of cactus in SL2. However the observed half-life of cactus in SL2 cells (see below and Fig. 7) suggests that this is not a significant factor.

A 2-fold increase in the level of mRNA in SLDL cells

To determine whether the increased biosynthesis of cactus can be accounted for by an increased concentration of the corresponding mRNAs, we have probed RNA prepared from SLDL and SL2 cells by Northern blot (Fig. 6). Two identical blots were hybridised with probes specific for cactus and ribosomal protein L18a respectively (see Materials and Methods). The amount of hybridisation of the L18a probe to the two RNA preparations was

closely similar but by contrast hybridisation of the cactus probe to the SLDL RNA was significantly greater than to the SL2 preparation. The amount of hybridisation of the cactus probe to the two preparations was quantified using the phosphor-imager and found to be 1.9-fold higher in SLDL cells. This analysis also indicates that L18a is expressed ~100- and 200-fold higher than cactus in SLDL and SL2 cells respectively, assuming that the probes were labelled to an equivalent specific activity.

Table 1. The relative metabolic rate in SL2 and SLDL cells

Source of lysate	Protein concentration (mg/ml)		Incorporation of radioactivity Synthesis of Cact		
	(10 ⁴ c.p.m./μg protein)		Maternal /zygotic	Zygotic	Total
SL2	6.46	2.14	1.024	1.0	1.0
SLDL	6.33	2.03	4.6	2.9	3.7

The cell lysates used in Figure 5 were measured for protein concentration and radioactivity.

Differential stability of bound and unassociated cactus protein

To investigate the relative stability of cactus isoforms in SLDL and SL2 cells we have carried out pulse chase experiments. Cells were labelled with ³⁵S methionine for 1 h and then the turnover of the cactus protein was monitored over a 24 h period. As shown in Figure 7, incorporation of label into cactus was much greater in SLDL cells than in SL2. Analysis with the phosphor-imager indicates that ~8-fold more label was incorporated into cactus in the SLDL cells. Furthermore a second set of proteins of *M_r* 84–90 kDa corresponding in mass to the multiple phosphorylated isoforms of cytoplasmic dorsal protein were purified by immunoprecipitation of the SLDL protein extracts with anti-cactus antibodies. These proteins were confirmed to be dorsal isoforms by probing of the immunoprecipitate with rat anti-dorsal antibody in a Western blot experiment (see Fig. 4B). The dorsal protein purified by immunoprecipitation must be in a complex with cactus and thus the cactus:dorsal ratio gives a measure of the relative amounts of bound and unassociated cactus protein. We have used the phosphor-imager to quantify dorsal and cactus protein in the immunoprecipitates and, as shown in Figure 7, the cactus:dorsal ratio drops quickly during the first hour from a value of 0.9 and then remains unchanged at ~0.45 during the rest of the period of the chase. As the dorsal and cactus mRNAs are being translated in the same cytoplasm, they will incorporate stoichiometric amounts of label and thus, if stable cactus protein is only present as complexes the observed ratio should correspond closely with the ratio of methionine residues in the sequences of cactus and dorsal. It is known that the complexes are heterotrimers with two molecules of dorsal and one molecule of cactus (13). The primary sequences of dorsal and cactus have ten and nine methionine residues respectively and therefore the ratio expected is 0.45, a figure that agrees closely with the experimental value. Interestingly, in Figure 5 above where SLDL cells were labelled for just 10 min the cactus:dorsal ratio at 1.35 is even higher than the 0 time in this experiment. The simplest interpretation of these results is that newly synthesised cactus is either rapidly degraded or becomes stabilised by incorporation into complexes with dorsal. The data suggests that unassociated and bound cactus have half lives of ~40 min and in excess of 24 h respectively.

Figure 5. Rate of synthesis of cactus in SL2 and SLDL cells. SL2 (lane 2) or SLDL cells (lane 3) were metabolically labelled with [^{35} S] methionine for 10 min and cell extracts were immunoprecipitated using anti-cactus antibody. Samples were fractionated by 7% Low-SDS-PAGE and analysed by fluorography (4 days exposure). Apparent molecular weights of dorsal and cactus isoforms are indicated as in Figure 3. Lane 1, as lane 2 but with pre-immune serum.

Figure 6. Northern blot analysis of RNA prepared from SL2 and SLDL cells. Approximately 15 μ g of whole RNA was separated on 1.1% agarose formaldehyde gel. Duplicate samples (B,D = SL2; A,C = SLDL) were run on the same gel and blotted at the same time. Blots were hybridised with probes to either ribosomal protein L18a (C,D) or cactus (A,B). The blots were autoradiographed for either 16 h (L18a) or 3 days (cactus). For quantitation the blots were exposed to a phosphor-imager screen for 18 h.

A second interesting feature of the pulse-chase experiment is that the newly synthesised cactus protein in both SL2 and SLDL cells is present predominantly as the zygotic and maternal-zygotic forms with little of the phosphorylated maternal-zygotic isoform being detected (Fig. 7, cf Fig. 4). However during the period of the chase the phosphorylated maternal/zygotic protein accumulates so that by 8 h the distribution of isoforms closely resembles that seen in Western blots of dorsal immunoprecipitates (Fig. 4A). Thus the phosphorylation events that generate the slowest migrating form of cactus protein appear to accumulate slowly subsequent to association with dorsal.

DISCUSSION

In this paper we have shown that the presence of dorsal protein enhances biosynthesis of cactus. This finding could be explained if dorsal stimulates the expression of the cactus gene and our finding that cactus mRNA levels are elevated in SLDL cells

supports such an explanation. For example, excess dorsal protein could migrate into the nucleus, bind to dorsal sites in the cactus promoter and activate expression. In the embryo, dorsal target genes are acted upon over a wide range of nuclear dorsal concentrations depending upon the affinity of their dorsal binding sites (23). Thus cactus expression could be induced by low levels of nuclear dorsal protein that do not effect other dorsal target genes. A similar conclusion has been drawn in the case of mammalian NF κ B p65 subunit. Scott *et al.* (24) showed that transfection of p65 into 293 cells induced synthesis of mammalian I κ B α and that this correlated with increased levels of transcription of the the corresponding gene. Such a model predicts that the promoter of the cactus gene contains binding sites for dorsal and would be consistent with the finding in the I κ B α (MAD3) promoter of a functional κ B site (25). However this site is required for restoring I κ B α levels subsequent to stimulation of the cell by factors that activate NF κ B and the evidence suggests that it is not needed to maintain resting levels of I κ B. Furthermore, if the cactus gene is activated by dorsal, cactus protein should accumulate ventrally during pre-cellular embryogenesis and this is not seen (20). We also find that pAcd1561 (18), which encodes a truncated form of dorsal is effective at enhancing the level of cactus in transient transfection assays (data not shown). This mutant dorsal protein is known to be defective in transcriptional activation (18,26) and should not be able to positively regulate the cactus promoter.

A second explanation for the enhancement of cactus biosynthesis is that unassociated dorsal stimulates translation of the cactus mRNA. Indeed the observed 5-fold increase in cactus production cannot be accounted for solely by a 2-fold increase in gene expression. Furthermore, the higher level of cactus transcript could be a consequence of RNA stabilisation rather than enhanced gene expression. It is possible that part of the difference is caused by increased lability of the unassociated cactus in SL2 cells. However the half life of unassociated cactus in SL2 cells is about the same as in SLDL cells and because only about one-third of the labelled cactus has bound dorsal in SLDL cells by the end of the 10 min labelling period, the contribution of this factor to the enhancement is probably negligible. Translational regulation could be caused directly or indirectly. In a direct model, dorsal dimer would bind to the cactus mRNA and cause both stabilisation and translational enhancement. It is not unprecedented for transcription factors to bind specifically to both DNA and RNA molecules, for example TF111A binds to a regulatory site in the 5S RNA gene and the 5S RNA itself (27). Such a model can explain the puzzling finding of Isoda and Nüsslein-Volhard (13) that a small fraction of dorsal is present in the cytoplasm as a dimer rather than a heterotrimer with cactus even in dorsalized mutant embryos, the dorsal dimers could be sequestered in the cytoplasm by binding with the cactus mRNA. It can also explain why dorsal is retained in the cytoplasm when multiple copies are expressed from an actin promoter in transient transfection experiments (18) as such a control point would allow dorsal to be buffered in the cytoplasm over a wide range of concentrations. In the case of the NF κ B system, Scott *et al.* (24) conclude that activation of I κ B biosynthesis by NF κ B p65 occurs wholly or mainly at the level of gene expression. However, their results do not exclude a role for mRNA stabilisation and translational enhancement. Indeed, they were not able to make a direct comparison of I κ B biosynthesis and mRNA levels of the kind reported here in their experiments. Le Bail *et al.* (25) have

Figure 7. Pulse-chase of cactus in SL2 and SLDL cells. SL2 (A) and SLDL cells (B) were pulse labelled with [^{35} S]-methionine for 60 min and chased in the presence of excessive non-labelled methionine. Cell extracts were prepared at the indicated times (h) of chase and immunoprecipitated using anti-cactus antibody. As a control, cell extracts pulse labelled for 60 min were immunoprecipitated using preimmune serum (PI). Immunoprecipitated samples were fractionated by 7% Low-SDS-PAGE and visualised by fluorography (5 days exposure). Dorsal and cactus isoforms are indicated on the right. Apparent molecular weights are indicated as in Figure 3. (C) Time course of the cactus:dorsal ratio. The amount of cactus and dorsal protein purified by immunoprecipitation at each time point was quantified using a phosphor-imager and the cactus:dorsal ratio was plotted as a function of time.

investigated whether κ B-like sequences in the promoter region of I κ B α (MAD3) are required for the restoration of I κ B protein after stimulation of cells with agents that act on NF κ B. They concluded that the only κ B-like sequence required was one which lies in the 5' untranslated region of the transcriptional unit, a highly unusual position for such regulatory sites to lie. It could be argued *a priori* that these results, which relied on luciferase reporter constructs,

were in consequence of translational enhancement rather than transcriptional regulation as the κ B site resides within the mRNAs of the reporter gene. The hypothesis of translational regulation could be tested directly by *in vitro* translation of cactus mRNA in the presence and absence of purified dorsal protein.

We have also shown in this paper that binding with dorsal stabilises the cactus protein. The half life of the free form is measured to be 40 min, similar to values reported for I κ B α (24,28) but that of bound cactus is at least 30-fold longer. This result is consistent with those of Whalen and Steward (20) who showed that in embryos lacking dorsal the level of cactus protein is very much diminished. However, our results suggest that this effect may be due not solely to stabilisation but also to reduced biosynthesis. We also find that bound cactus is modified by protein kinases either during or subsequent to association with dorsal and forms into slower mobility phosphorylated isoforms. This finding is consistent with the situation found during the 7 days of oogenesis and early embryogenesis when both cactus and dorsal are synthesised continuously. Only the maternal/zygotic form of cactus protein is detected and this accumulates as a phosphorylated isoform of slower electrophoretic mobility towards the end of oogenesis. Our experiments do not determine whether phosphorylation is required for association or stabilisation as some phosphorylation events do not cause alterations in electrophoretic mobility (29,30). Indeed, I κ B is phosphorylated constitutively and this modification does not cause a shift in mobility. It should now be possible to address this question directly by labelling SLDL cells with ^{32} P phosphate and then purifying the bound and free forms of cactus by immunoprecipitation of lysate with firstly anti-dorsal and subsequently anti-cactus antibody.

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REFERENCES

- Kidd, S. (1992) *Cell*, **71**, 623–635.
- Geisler, R., Bergmann, A., Hiromi, Y. and Nüsslein-Volhard, C. (1992) *Cell*, **71**, 613–621.
- Steward, R. and Govind, S. (1993) *Curr. Opin. Genet. Dev. Biol.*, **3**, 556–561.
- Ip, Y.T., Reach, M., Engstrom, Y., Kadalayil, L., Cai, H., Gonzalez Crespo, S., Tatei, K. and Levine, M. (1993) *Cell*, **75**, 753–763.
- Stylianou, E., O'Neill, L.A.J., Bird, T., Rawlinson, L., Edrooke, M.R., Woo, O. and Saklatvala, J. (1992) *J. Biol. Chem.*, **267**, 15 836–15 841.
- Gay, N.J. and Ntwasa, M. (1993) *FEBS Lett.*, **335**, 155–160.
- Rogers, S., Wells, R. and Rechsteiner, M. (1986) *Science*, **234**, 361–368.
- Henkel, T., Zabel, U., Zee, K., Müller, J.M., Fanning, E. and Baeuerle, P.A. (1992) *Cell*, **68**, 1121–1133.
- Mellits, K.H., Hay, R.T. and Goodbourn, S. (1993). *Nucleic Acids Res.*, **21**, 5059–5066.
- Frantz, B., Nordby, E.C., Bren, G., Steffan, N., Paya, C.V., Kincaid, R.L., Tocci, M.J., O'Keefe, S.J. and O'Neill, E.A. (1994) *EMBO J.*, **13**, 861–870.
- Brown, K., Park, S., Kanno, T., Franzoso, G. and Siebenlist, U. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 2532–2536.
- Beg, A.A., Finco, T.S., Nantermet, P.V. and Baldwin, A.S. (1993) *Mol. Cell. Biol.*, **13**, 3301–3310.

- 13 Isoda, K. and Nüsslein-Volhard, C. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 5350–5354.
- 14 Gay, N.J. and Keith, F.J. (1991) *Nature*, **351**, 355–356.
- 15 Roth, S. (1994) *Curr. Biol.*, **4**, 755–757.
- 16 Schneider, D.S., Hudson, K.L., Lin, T. and Anderson, K.V. (1991) *Genes Dev.*, **5**, 797–807.
- 17 Kubota, K., Keith, F.J. and Gay, N.J. (1993) *Biochem. J.*, **296**, 497–503.
- 18 Rushlow, C.A., Han, K., Manley, J.L. and Levine, M. (1989.) *Cell*, **59**, 1165–1177.
- 19 Keith, F.J. and Gay, N.J. (1990) *EMBO J.*, **9**, 4299–4306.
- 20 Whalen, A.M. and Steward, R. (1993) *J. Cell Biol.*, **123**, 523–534.
- 21 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn, Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY.
- 22 Ntwasa, M., Buchanan, S.G.S.C. and Gay, N.J. (1994) *Biochem. Biophys. Acta*, **1218**, 210–212.
- 23 Jiang, J. and Levine, M. (1993) *Cell*, **72**, 741–752.
- 24 Scott, M.L., Fujita, T., Liou, H., Nolan, G.P. and Baltimore, D. (1993) *Genes Dev.*, **7**, 1266–1276.
- 25 LeBail, O., Schmidt-Ullrich, R. and Israël, A. (1993) *EMBO J.*, **12**, 5043–5049.
- 26 Isoda, K., Roth, S. and Nüsslein-Volhard, C. (1992) *Genes Dev.*, **6**, 619–630.
- 27 Geiduschek, E.P. and Tocchini-Valentini, G.P. (1988) *Annu. Rev. Biochem.*, **57**, 873–915.
- 28 Sun, S.C., Ganchi, P.A., Ballard, D.W. and Greene, W.C. (1993) *Science*, **259**, 1912–1915.
- 29 Gay, N.J., Poole, S.J. and Kornberg, T.B. (1988) *Nucleic Acids Res.*, **16**, 6637–6647.
- 30 Tanaka, M. and Herr, W. (1990) *Cell*, **60**, 375–386.