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Disulfide cross-linking in crude embryonic lysates reveals three complexes of the *Drosophila* morphogen dorsal and its inhibitor cactus

(embryonic polarity/nuclear localization/Rel/Nf-kB)

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ABSTRACT **In *Drosophila* embryos dorsoventral polarity is determined by a concentration gradient of dorsal (dl) protein in the nuclei formed by the differential regulation of nuclear localization of dl protein. cactus (*cact*) represses the nuclear localization of dl protein. By introducing intermolecular disulfide bonds in homogenates of embryos, we detected three complexes of dl and/or cact proteins. Complex 1 (190 kDa) is a dl protein homodimer (dl_2). Complex 2 (270 kDa) consists of one complex 1 and one cact molecule (dl_2cact). Complex 3 (200 kDa) is a cact protein complex that does not contain dl protein. In wild-type embryos dl_2cact was detected as the major form of dl protein, and dl_2 was minor. With this assay virtually no dl monomer is detected. Analysis of the dl protein complexes in ventralized and dorsalized mutant embryos indicates that dl_2cact is a cytoplasmic form, whereas dl_2 is localized mainly in the nuclei. It seems that a small amount of dl_2 is also present in the cytoplasm.**

In *Drosophila*, the dorsoventral polarity of the embryo is determined by a concentration gradient of dorsal (dl) protein in the nuclei, which is formed by the regulation of its nuclear uptake along the dorsoventral axis (1–3). On the dorsal side, all dl protein remains in the cytoplasm, and no detectable dl protein is found within the nuclei. In contrast, in the ventral-most part of the embryo, most dl protein enters the nuclei, giving a peak level of nuclear dl concentration. dl protein binds to specific DNA sequences, and in the same nuclei activates and represses specific zygotic genes (4–7). This dl gradient is induced by transduction of a signal originating outside the embryo. Inside the oolemma, the products of the maternal genes, tube (*tub*) and pelle (*pll*), and cactus (*cact*) are involved in the formation of the dl gradient (1, 8–11). cact protein is necessary for the retention of dl protein in the cytoplasm, and association of dl and cact proteins was shown (12, 13). In contrast to cact protein, the products of *tub* and *pll* activate the uptake of dl protein into the nuclei.

The dl protein is a member of the Rel family of transcription factors that share a homologous domain of ≈300 aa, named the Rel homology domain (14–16). It was shown by immunoprecipitation that dl protein self-associates *in vivo* (17). Among the Rel family of proteins, transcription factor NF-κB is best understood with respect to its activation. In most tissues, NF-κB is stored in the cytoplasm as an inactive complex with an inhibitor protein, IκB (18). Extracellular signals result in the release of NF-κB dimer from IκB and its translocation into nuclei, where it activates target genes (15, 19). The inhibitors, the IκB family, share a common structural motif, the ankyrin repeat (14, 15). cact protein, which binds dl protein and inhibits its nuclear targeting, also contains six ankyrin repeats (12, 13).

To further analyze the molecular mechanism of dl gradient formation, in particular the role of cact protein, we identified and characterized complexes of dl protein and complexes of cact protein by a cross-linking method.

Cross-linking, the introduction of covalent bridges between neighboring subunits in complexes, is a simple and rapid technique to analyze quaternary structure (20). To identify complexes of dl and cact present *in vivo*, intermolecular disulfide bonds were introduced between endogenous cysteine residues of the proteins in a crude lysate immediately after disruption of the embryos. We optimized oxidation conditions and detected two complexes of dl protein and two of cact protein. We detected a dl homodimer as well as a complex of dl_2 with cact. In dorsalized mutant embryos the dl_2cact form prevails, whereas ventralized embryos have more of the dl_2 complex. This result suggests that dl_2cact is the cytoplasmic and dl_2 is the nuclear form of the dl protein.

MATERIALS AND METHODS

Buffers and Fly Strains. One molar glycine buffer, pH 10.0 (21), was stored at −20°C and before use diluted 10-fold with double-distilled water saturated with air. The 2× reducing sample buffer [0.125 M Tris·HCl, pH 6.8/4.6% SDS/20% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol] was stored at 4°C. The 2× oxidation sample buffer was 2× reducing sample buffer without 2-mercaptoethanol, saturated with air by extensive shaking and stored at room temperature.

The wild-type strain used is Oregon R, and the mutant strains are described in ref. 22. The genotypes of females used were *$dl^1/Df(2L)TW119$, dl^{U5}/CyO, $Tl^{10b}/TM3$, $Tl^{10b}/lT(1;3)OR60$, snk^{073}/snk^{229}*, and *$cact^{011}/cact^{011}$*.

Oxidation, Gel Electrophoresis, Immunoblotting, and *N*-Ethylmaleimide (NEM) Treatment. Embryos were collected 0–4 hr after egg deposition, frozen in liquid nitrogen, and stored at −20°C. The embryos were homogenized in 0.1 M glycine buffer at a concentration of 80 mg of embryos per ml in Eppendorf tubes with a Teflon pestle by hand at room temperature, and shaken (mixer 5432; Eppendorf) for 15 min, unless otherwise described. The oxidized samples were mixed with an equal volume of 2× oxidation sample buffer and analyzed by standard Laemmli SDS/PAGE. An extract of 0.6 mg of embryos was loaded in each lane unless otherwise described. Detection of immunoanalysis was performed with the ECL system (Amersham). Anti-dl antiserum (1) was preabsorbed with fixed *dl^-* embryos. Anti-cact antiserum was from S. Hagenmaier (Max-Planck-Institut, Tübingen). The signal intensity of dl or cact monomer cannot be com-

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Abbreviations: NEM, *N*-ethylmaleimide; dl, dorsal; cact, cactus.

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pared directly with that of their complexes, due to different efficiencies of transfer from the gel to the membrane.

For NEM treatment, wild-type embryos were homogenized in 0.1 mM, 1 mM, and 10 mM NEM solution in 0.1 M Tris·HCl, pH 6.8, and shaken for 15 min.

Partial Dissociation and Two-Dimensional Oxidizing/Reducing Gel Electrophoresis. We made 2^n-fold (2^{10}–2^2) dilutions of 2-mercaptoethanol present in reducing sample buffer (5%) with oxidation sample buffer. The oxidized embryonic lysate was dispensed into aliquots, and each aliquot was mixed with an equal volume of these buffers or with oxidation sample buffer. Two blots were prepared. One blot was probed with anti-dl antibody, and the other was probed with anti-cact antibody.

For two-dimensional oxidizing/reducing gel electrophoresis, the oxidized embryonic lysate was electrophoresed, and a lane of the gel was cut out, soaked in 2× reducing sample buffer for 20 min, and then placed on a second SDS/polyacrylamide gel. The second electrophoresis was performed, followed by blotting and detection with the antibodies.

RESULTS

Detection of Two dl Protein Complexes. We investigated whether protein association can be detected by the introduction of artificial disulfide bonds between endogenous cysteines of dl and any associated proteins in a crude embryonic extract in the absence of purification. We homogenized *Drosophila* embryos in 0.1 M glycine buffer, pH 10, shook the homogenate for 15 min to air-oxidize it, and divided the homogenate in half. One aliquot was mixed with SDS/PAGE sample buffer without any reducing agents (oxidation sample buffer), and the other was mixed with SDS/PAGE sample buffer containing the reducing agent 2-mercaptoethanol (reducing sample buffer). Then the protein extracts were examined by SDS/PAGE, and dl protein was detected by immunoanalysis (Fig. 1). Two bands absent in the reduced extract were observed in the oxidized extract (compare lanes 1 and 3), indicating that these two bands depend on the presence of disulfide bonds. If these bands result from intramolecular disulfide bonds of dl protein, their apparent molecular masses are expected to be smaller than that of the reduced form. The molecular masses of the two bands are 190 kDa and 270 kDa, far higher than that of the dl monomer (90 kDa). We therefore conclude that these are the complexes of dl protein and call them complex 1 (190 kDa) and complex 2 (270 kDa). Although a smear-like signal would be expected were dl protein forming disulfide bonds at random with other proteins, we observe two discrete sharp bands (lane 1). We believe, therefore, that complexes 1 and 2 reflect two complexes present *in vivo*.

Interestingly, the monomer form of dl protein is hardly detectable in the oxidized extract. Because all the dl protein was found to be solubilized, essentially all dl protein forms complexes *in vivo*.

We tested whether the disulfide bonds that stabilize these complexes in the SDS solution are made *in vivo* or artificially *in vitro*. A thiol alkylating agent, NEM, blocks free SH groups and "freezes" the *in vivo* redox state of proteins if it is added during cell lysis (23). If the disulfide were produced by oxidation after disruption of cells, disulfide formation should be inhibited by NEM. We homogenized wild-type embryos in different concentrations of NEM (Fig. 2). Because NEM is unstable in basic solution (23), we used a buffer of pH 6.8 for this experiment, instead of pH 10. At pH 6.8, the major complex is complex 1 and not complex 2 (Fig. 2, lane 1), a pattern distinct from that at pH 10 (analysis of the pH dependence is shown below). At a concentration of 1 mM, a decrease in the amount of the complexes and the appearance of the monomer were observed (lane 3). This change was more pronounced in 10 mM NEM (lane 4). This clear inhibition indicates that the disulfides in complexes 1 and 2 are formed *in vitro*, not *in vivo*.

Relationship Between complex 1 and complex 2. Relatively weak reduction of the oxidized extract should cause partial dissociation of the complexes, and the number and molecular mass of the intermediates could give important information about the composition of the complexes. The oxidized embryonic extract was mixed with SDS/PAGE sample buffer containing increased amounts of 2-mercaptoethanol and examined by SDS/PAGE and immunoblot analysis (see Fig. 4*C*). In the range of 2^{10}- to 2^5-fold dilution the amount of complex 2 decreased, and finally it disappeared at 2^5-fold dilution, while complex 1 increased in amount concomitantly (see Fig. 4*C*, lanes 1–7), and no other bands were detected. This result indicates that complex 2 dissociates into complex 1—i.e., complex 2 contains complex 1.

Although a 2^5-fold dilution of reducing sample buffer is sufficient for the full reduction of complex 2 (see Fig. 4*C*, lane 7), stronger reduction at 2^2-fold dilution is required for complete dissociation of complex 1 (see Fig. 4*C*, lane 10). This clear difference in susceptibility to reduction gives us a good criterion to optimize oxidation conditions for this method.

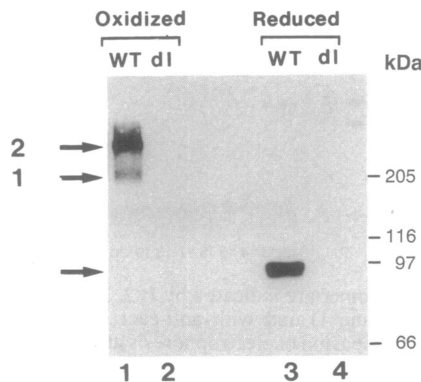

FIG. 1. dl protein complexes. The oxidized lysate of the embryos was mixed with oxidation sample buffer (lanes 1 and 2) or with reducing sample buffer (lanes 3 and 4), examined by SDS/PAGE, and the dl protein was detected on an immunoblot with anti-dl antibody. Lanes: 1 and 3, wild-type (wt) embryos; 2 and 4, *dl*⁻ embryos.

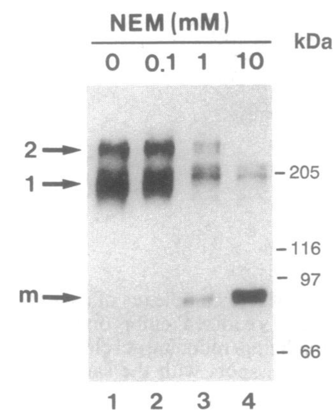

FIG. 2. Inhibition of disulfide bond formation by NEM. Wild-type embryos were homogenized and oxidized in the absence (lane 1) and in the presence (lanes 2–4) of different concentrations of NEM. Complexes 1 and 2 and dl monomer are indicated by 1, 2, and m, respectively.

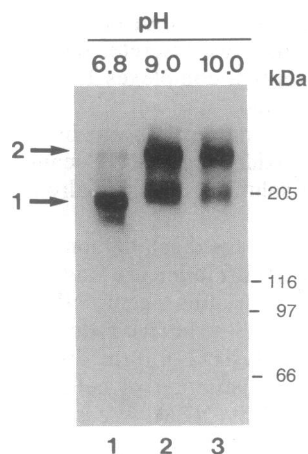

FIG. 3. pH dependence of the abundance of complexes 1 and 2. Wild-type embryos were homogenized and air-oxidized with shaking for 15 min at different pH values. Lanes: 1, 0.1 M Tris·HCl, pH 6.8; 2, 0.1 M Tris·HCl, pH 9; 3, 0.1 M glycine buffer, pH 10. The apparent decrease in abundance of the dl complexes in lane 1 in comparison with lane 2 or 3 was caused by low reproducibility at neutral pH.

Optimization of Oxidation Conditions. In this approach, the oxidation procedure is crucial. The degree of oxidation can be monitored by the relative abundance of complex 2 to complex 1. Because complex 2 contains complex 1 and is less easily oxidized than complex 1, insufficient oxidation would increase complex 1 and decrease complex 2. On the other hand, if the oxidation conditions are too strong, disulfide bonds will be formed by random collisions of proteins in the extract, resulting in a smear-like signal on immunoblots or in a reduction in signal intensity due to aggregation of most proteins into huge complexes. We tested different oxidation methods such as glutathione disulfide and H_2O_2 and found that simple air oxidation by shaking was the best (data not shown).

For high reproducibility, we found that the pH of the lysis buffer and oxidation time were crucial. The amount of complex 2 increased and that of complex 1 decreased when the lysis buffer was more basic (Fig. 3). This result is readily explained by the facilitation of disulfide bond formation at high pH because thiols are active only in the ionized thiolate form and pK values of most thiols are between 8 and 9 (24). At pH 10, the amounts of complexes 1 and 2 showed little change when shaken for 5, 15, and 45 min, indicating that this oxidized state is very stable. The high stability of the abundance of complexes 1 and 2 at pH 10 over time makes this a suitable condition for reproducible experiments. Therefore, we used as standard oxidation conditions a buffer at pH 10 and shaking for 15 min. The fact that longer oxidation does not significantly change the relative abundance of complexes 1 and 2 suggests that disulfide bond formation between the components of complexes 1 and 2 is nearly complete under these conditions.

Composition of complexes 1 and 2. The association of dl and cact proteins *in vitro* and *in vivo* was shown by an electrophoretic mobility-shift assay and immunoprecipitation (12, 13). We tested whether complex 1 or 2 contains cact protein. Two bands of molecular masses of 270 kDa and 200 kDa were detected by anti-cact antibody, in addition to cact monomer band (Fig. 4*A*, lane 2). We used two-dimensional oxidizing/reducing gel electrophoresis to confirm that these bands contain cact protein. Three spots with the same migration rate were detected by immunoanalysis (Fig. 4*B*). Two of them were dissociation products of the two high-molecular mass complexes, and one came from cact monomer, demonstrating that the two complexes contain cact protein.

The molecular mass of complex 2 is the same as that of the larger cact-containing complex. Complex 1 and the lower-molecular mass cact-containing complex show a very similar

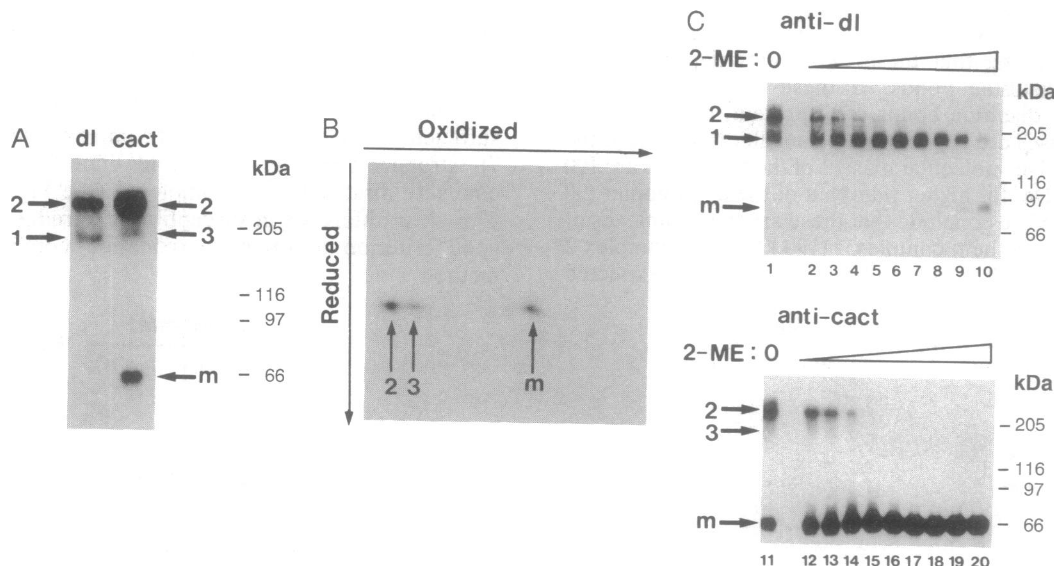

FIG. 4. Detection of two complexes of cact protein. Complexes 1, 2, and 3 and cact monomer are indicated by 1, 2, 3, and m, respectively. (*A*) Blots of the same oxidized embryonic lysate were probed with anti-dl antibody (lane 1) and with anti-cact antibody (lane 2). (*B*) Two-dimensional oxidizing/reducing gel electrophoresis of cact complexes. In the second dimension of electrophoresis after complete reduction of the complexes, three spots with the same molecular mass are observed; two are derived from the two complexes, and one is from cact monomer. This result confirms that the two complexes shown in *A* really do contain cact protein. (*C*) Comparison of the susceptibility of dl protein complexes and cact protein complexes to reduction using progressive reduction of the same lysate. The oxidized lysate of the wild-type embryos was mixed with SDS/PAGE sample buffer containing increased concentrations of 2-mercaptoethanol (2-ME), and two immunoblots were made from the same sample. Detection with anti-dl antibody (*Upper*) and with anti-cact antibody (*Lower*) revealed that complex 2 and the bigger complex of cact protein shows the indistinguishable sensitivity to reduction, whereas complex 1 and the smaller cact complex dissociate in a completely different pattern. Lanes 1 and 11 are without 2-mercaptoethanol; lanes n and $10 + n$ are 2^{12-n}-fold dilution of 2-mercaptoethanol ($n = 2, 3, \ldots, 10$; see *Materials and Methods* for details).

migration rate on SDS/PAGE. To test whether these cact-containing complexes are identical to complexes 1 and 2, respectively, we performed a partial reduction experiment and compared the dissociation pattern of complexes 1 and 2 and cact-containing complexes (Fig. 4C). Complex 2 and the corresponding cact-containing complex showed indistinguishable sensitivity to reduction. In contrast, the dissociation pattern of complex 1 was completely different from that of its cact-containing counterpart, indicating clearly that these complexes are distinct from each other. We name this cact-containing complex (200 kDa) complex 3. We conclude that complex 2 contains cact protein, but complex 1 does not, and that complex 3 does not have dl protein as a component.

The apparent difference in molecular mass between complex 2 and complex 1 is 80 kDa and close to the molecular mass of cact protein [76 kDa (25)], suggesting that complex 2 is composed of complex 1 plus one cact molecule. In this case, there would be no intermediate dissociation product between complexes 2 and 1 in the partial dissociation experiment, as observed (Fig. 4C).

Complex 1 may be a dl homodimer as it does not contain cact protein, its molecular mass (190 kDa) is approximately twice that of dl monomer (90 kDa), and its reduction does not yield intermediates. To test this possibility we examined dl protein complexes in $dl^{U5}/+$ embryos. dl^{U5} has a nonsense mutation (Gln-488 to a stop codon) that results in a truncated dl protein [60 kDa (11)]. If complex 1 is a dl homodimer, it is expected that in addition to a 190-kDa wild-type complex 1 (dl-dl), two bands corresponding to dl-dl^{U5} and dl^{U5}-dl^{U5} are observed. We detected three bands at ≈190, 155, and 125 kDa (data not shown). This result confirms that complex 1 is a homodimer of dl protein.

Complexes 1 and 2 in Mutant Embryos. In the completely dorsalized embryos caused by loss-of-function alleles of genes acting upstream of *dl*, all dl protein is located in the cytoplasm (1–3). In contrast, nuclear targeting of dl protein is enhanced in ventralized mutant embryos (1, 10). Using ventralizing and dorsalizing mutants, we tested whether there is a correlation between the localization of dl protein and the pattern of dl_2 and dl_2cact. A gain-of-function allele of the Toll (*Tl*) gene, Tl^{10b}, shows a strong ventralization phenotype where most dl protein is localized in the nuclei on both the ventral and dorsal sides of the embryo (1, 3, 26). In Tl^{10b} embryos most dl protein is in the dl_2 form, whereas dl_2cact is the major form in wild-type embryos (Fig. 5A). Weaker ventralization is caused by an allele of *cact* ($cact^{011}$), and nuclear targeting of dl protein is facilitated in the mutant embryos (10). dl_2cact is decreased, and dl_2 is increased in amount in the $cact^{011}$ embryos (Fig. 5B). Interestingly, although the cause of facilitation of nuclear transfer of dl protein in these two ventralizing mutants is genetically distinct, an increase in the amount of nuclear dl protein correlates with an increase in that of dl_2. In contrast, the amount of dl_2 is reduced in completely dorsalized snake mutant embryos, where dl protein is absent from the nuclei and remains in the cytoplasm (Fig. 5C). These results suggest that dl_2cact is a cytoplasmic form, and dl_2 is a nuclear form.

DISCUSSION

To detect association of dl protein and/or cact protein with itself or with other factors *in vivo*, we introduced another method, disulfide cross-linking in crude lysates. This approach uses artificial introduction of disulfide bonds between the endogenous cysteines in the complex components in a crude embryonic lysate without any purification. With this method we detected two complexes of dl protein and two of cact protein that were clearly different from the respective monomers. Complex 1 of dl protein (190 kDa) is a dl homodimer (dl_2). The simplest interpretation of the dissociation pattern and size of complex 2 is that complex 2 consists of one complex 1 and one cact molecule—i.e., complex 2 is dl_2cact. Although the lower-molecular mass cact-containing complex showed a similar migration rate on SDS/PAGE to that of dl_2, they are distinct from each other because their susceptibility to reduction was completely different. We refer to the lower-molecular mass cact-containing complex as complex 3 (200 kDa). Complex 3 may be involved in a zygotic function of cact protein.

Disulfide Cross-Linking in Crude Lysates. The validity of this approach is indicated principally by the sharpness and small number of the complex bands itself. These bands are unlikely to be produced by random collision of proteins but probably result from disulfide formation between proteins that are closely localized to each other *in vivo*. Because the C^a atoms of two cysteine residues must be 0.4–0.9 nm apart for the formation of a disulfide bond (27), disulfide bond formation between two proteins demonstrates that they contact each other very tightly. In addition, a small number of the bands also indicates that specific combinations of cysteine residues are preferred to the many other possible combinations. In particular, in the case of dl and cact proteins, each complex of dl protein and/or cact protein is represented by only one band, indicating clearly that these bands reflect specific complexes present *in vivo*.

For this disulfide-crosslinking approach, the strength of the oxidation is most critical. If oxidation is too weak, a large proportion of the complexes do not form disulfide bonds. If the oxidation conditions are too strong, random collision of proteins in the homogenate causes disulfide formation between proteins that do not naturally associate. We found that air oxidation with shaking was strong enough and mild enough at the same time. Two factors, pH and time, were found to be crucial. At pH 10, disulfide-bond formation increased with time; then a plateau appeared where little change was observed over a long span of time. We used conditions producing this oxidation plateau because it was stable and thus suitable for a reproducible oxidation procedure.

Our approach may be of general use, as its particular advantage is that we can test protein association *in vivo*

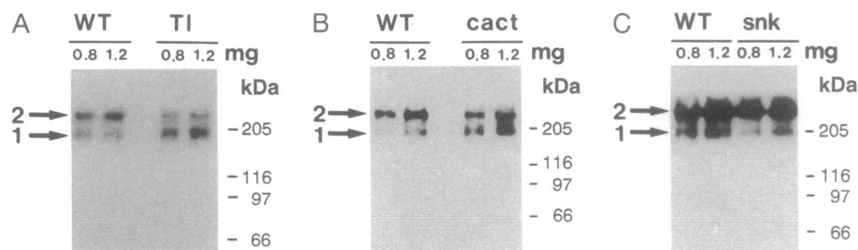

FIG. 5. Complexes 1 and 2 in ventralized and dorsalized mutant embryos. In each figure, the homogenate of two different amounts (0.8 and 1.2 mg) of embryos was tested to compare the intensity of the bands. (*A*) Strongly ventralized embryos from $Tl^{10b}/+$ females (Tl). (*B*) Moderately ventralized embryos derived from the females homozygous for $cact^{011}$. (*C*) Completely dorsalized embryos produced by *snk* females.

because the proteins in question are not purified. It is also fast and simple, and because very small amounts of protein extracts are required, it is suitable for the analysis of mutants. High stability of the complexes mediated by disulfide bonds may also offer an easier purification procedure for protein complexes.

The Role of dl Protein Complexes in Nuclear Localization. There is a strong correlation between the localization of dl protein and the pattern of the dl_2 and dl_2cact complexes. Most dl protein is in the dl_2cact form in the wild-type embryo, where dl protein is cytoplasmic in most of the embryonic regions except the ventral-most part (1–3). In the embryos where most of the dl protein enters the nuclei, dl_2 is the major form. In contrast, dl_2 decreases in amount in the dorsalized embryos where all the dl protein is retained in the cytoplasm. These data indicate that dl_2cact is the cytoplasmic form, and dl_2 is the nuclear form. However, a question arises about the presence of dl_2 in the dorsalized mutants because no dl protein is detected in the nuclei of the dorsalized embryos (1–3). Although the amount of dl_2 in the dorsalized embryos is smaller than that in the wild-type embryos, dl_2 is still clearly present in those mutant embryos. This might be an artifact caused by dissociation of dl_2cact, presumably due to inadequate oxidation. Although we tried longer oxidation, it was not possible to remove all the dl_2 signal in the dorsalized embryos. Accordingly, we suggest another possibility that a small amount of dl_2 is always present in the cytoplasm as a candidate form for entry into the nuclei, and the cytoplasmic dl_2 is translocated into the nuclei in response to a signal from the outside of the embryo. In this hypothesis, cact protein reduces the cytoplasmic concentration of candidate nuclear dl protein (dl_2), and the target for activation is dl_2. It is different from the activation mechanism proposed for the NF-κB system, where an extracellular signal through phosphorylation or proteolysis of IκB releases NF-κB from IκB, and this free NF-κB enters the nuclei (14, 15, 28, 29). However, further experiments are required to distinguish between these two models.

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