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# CKA, a Novel Multidomain Protein, Regulates the JUN N-Terminal Kinase Signal Transduction Pathway in *Drosophila*

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**The *Drosophila melanogaster* JUN N-terminal kinase (DJNK) and DPP (*decapentaplegic*) signal transduction pathways coordinately regulate epithelial cell sheet movement during the process of dorsal closure in the embryo. By a genetic screen of mutations affecting dorsal closure in *Drosophila*, we have now identified a multidomain protein, *connector of kinase to AP-1* (*cka*), that functions in the DJNK pathway and controls the localized expression of *dpp* in the leading-edge cells. We have also investigated how CKA acts. This unique molecule forms a complex with HEP (DJNKK), BSK (DJNK), DJUN, and DFOS. Complex formation activates BSK kinase, which in turn phosphorylates and activates DJUN and DFOS. These data suggest that CKA represents a novel molecule regulating AP-1 activity by organizing a molecular complex of kinases and transcription factors, thus coordinating the spatial-temporal expression of AP-1-regulated genes.**

Morphogenetic movements play a central role in the establishment of the overall body organization and shape during metazoan development. Most types of morphogenetic movements involve epithelial cell invagination and spreading (epiboly). In *Drosophila melanogaster*, the morphogenetic movement that occurs during dorsal closure (DC) of the embryonic epidermis has provided an excellent system for characterizing the molecular mechanisms underlying the coordinated movements of epithelial cell layers (8, 13, 25, 26, 38).

DC is a major morphogenetic event that occurs during midembryogenesis. DC relies predominately on cell shape changes within the postmitotic epidermis. These changes are initiated in two symmetric dorsalmost rows of epidermal cells, known as the leading-edge (LE) cells, followed by the stretching of the more lateral epidermal cells, and are finished with the fusion of the two rows of LE cells at the dorsal midline (4, 23, 43).

Embryos that fail to complete DC due to mutations in the genes required for this process die with a characteristic dorsal-open phenotype (14, 27, 29, 41). Based on molecular data, two types of genes for DC have been identified. The first class identifies cytoskeletal and extracellular matrix components. These molecules may form the mechanical machinery that responds to signaling and drives cell shape changes. The second class identifies signaling molecules, including components in the *D. melanogaster* JUN N-terminal kinase (DJNK) signal transduction pathway and the *dpp* signal transduction pathway (8, 13, 25, 26, 38).

Mutations in DJNK cascade genes block cell shape changes and disrupt organization of the cytoskeleton components at the LE. In addition, *dpp* gene expression is lost from the LE at the onset of DC. The *Drosophila* homologue of c *-jun* (*Djun*) is a central player in this signal transduction pathway (12, 16, 32). *Djun* mutations completely block epithelial cell sheet movement in DC. In doing so, DJUN is a target of the DJNK signal transduction pathway. DJNK may function by phosphorylating and activating DJUN, which in turn induces transcription of *dpp* in the LE cells during DC (12, 32). Expression of a dominant form of *Drosophila* Rac (*Drac*) (9) and mutations in genes that inactivate this pathway, such as the Ste20-related kinase *misshapen* (*msn*) (39), DJNK kinase *hemipterous* (*hep*) (7), DJNK *basket* (*bsk*) (31, 35), and *Djun* (12, 16, 32), cause a reduction of *dpp* expression in the LE cells. Mutations in the gene that down-regulates DJNK activity (such as the DJNK phosphatase *puckered* [*puc*]) (22) or in the gene that inhibits DJUN activity (such as the ETS repressor AOP/YAN) (32) cause expansion of *dpp* expression. The *Drosophila* homologue of c-*fos* (*Dfos*) is also required for DC (33, 44). Mutations in the *Dfos* (*kayak* or *kay*) gene generate a strong dorsal-open phenotype similar to mutations in the *Djun* gene. In *Dfos* null alleles, *dpp* expression in the LE cells is abolished; the cell shape changes that normally occur in the LE cells and in more ventrally located epithelial cells are blocked during DC.

However, the detailed mechanism is still unclear regarding the way in which the DJNK signal transduction pathway is activated and then turned on DFOS and DJUN for activating *dpp* transcription. In this study, we report the characterization of a novel gene associated with a dorsal-open mutant phenotype. Based on its presumed function in the DJNK signal transduction pathway, we named it *connector of kinase to AP-1* (*cka*). CKA functions in the DJNK signal transduction path-

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way and regulates *dpp* gene expression in the LE cells. Our data suggest that CKA represents a novel molecule regulating AP-1 activity by organizing a molecular complex of kinases and transcription factors.

## MATERIALS AND METHODS

***Drosophila* stocks.** *cka*$^{1}$ was identified in a large screen for autosomal zygotic lethal mutations associated with specific maternal effect phenotypes (30). *cka*$^{1}$, l(2)05836, originated from the collection of second-chromosome lethal mutations generated by Karpen and Spradling (15).

To determine whether the insertion of the enhancer trap P element was responsible for the mutant phenotype, we mobilized the P element associated with the *cka*$^{1}$ mutation using the *y w*; Δ*2-3*, *Sb*/*TM6* strain (34). The excision lines fell into two classes: 97% (97 of 100) were wild type and 3% (3 of 100) had a lethal mutation and were named *cka*$^{2}$, *cka*$^{3}$, and *cka*$^{4}$ (data not shown). *cka*$^{2}$ and *cka*$^{3}$ are protein null and have dorsal-open phenotypes similar to *cka*$^{1}$ (data not shown).

*bsk*$^{1}$, *kay*$^{2}$, *HS-Djun*$^{\Delta SP}$, *UAS-tkv*$^{QD}$, and mutant lines have been described elsewhere (FlyBase 1999) (13). *UAS-hep*$^{CA}$ was from T. Adachi-Yamada.

Flies were raised on standard *Drosophila* medium at 25°C, unless otherwise indicated. Chromosomes and mutations that are not described in the text can be found in the work of Lindsley and Zimm (18) and in FlyBase (1999).

**Germ line clones.** Females carrying germ line clones of *cka* were generated using the FLP-DFS technique (6, 11).

**Molecular cloning.** Genomic DNA flanking the *l(2)05836* P element was recovered by plasmid rescue and used to screen a 0- to 4-h embryonic cDNA library (3) and to search the *Drosophila* expressed sequence tag database. One full-length 3.3-kb clone and several shorter cDNA clones corresponding to one open reading frame were identified. The longest 3.3-kb clone was selected for further analysis. DNA sequencing was carried out on both strands on an ABI model 310 DNA sequencer with consecutive oligonucleotide primers synthesized to extend the sequences. The *cka* genomic organization and the P-element insertion site were deduced from the BDGP database (http://www.fruitfly.org). Resequencing confirmed the P-element insertion point and the exon-intron joints. The *cka* gene is encoded by four exons, and the P element, inserted into a 5′ untranslated sequence in the first exon, is 469 bp away from the ATG translation-starting site (data not shown).

**Rescue of *cka* embryonic mutant phenotype and lethality.** A P-element rescue plasmid was constructed by inserting the 3.3-kb cDNA into the vector pCasperhs (40). Germ line transformants were produced by standard methods (37), following injection of the rescue into *y w*; Δ*2-3*, *Sb*/*TM6* embryos. A second-chromosome line was recombined onto the *FRT*$^{40A}$ *cka*$^{1}$ chromosome and was used for rescue of *cka* embryonic mutant phenotype and lethality. To rescue the *cka* embryonic mutant phenotype, *y w FLP*$^{12}$/+; *FRT*$^{40A}$ *cka*, *HS-cka*/*FRT*$^{40A}$ [*ovo*$^{D1}$]$^{13X13}$ females carrying *cka* homozygous germ line clones were crossed to males of the genotype *cka*, *HS-cka*/*Cyo*. Embryos were collected for 2 h and aged to 5 to 7 h after egg laying prior to a 60-min heat shock. Following the heat shock, the embryos were aged for another 48 h at 25°C before cuticle preparation. A total of 1,273 embryos were examined; more than 80% of *cka* embryos were rescued to wild-type cuticle.

To rescue *cka* lethality, heat shock at 37°C was administered for 30 min to *cka*, *HS-cka*/*Cyo* flies every 12 h from the second instar larval stage; more than 20 straight-winged flies were obtained from each vial.

**Antibody production and immunohistochemistry in embryos.** Peptides corresponding to the N-terminal sequence of CKA and to the C-terminal sequences of DJUN and DFOS were used to produce antibodies in rabbits. Antiserum was purified by using the peptides as affinity reagents. Mouse antihemagglutinin (anti-HA) (12CA5) and anti-AU5 monoclonal antibodies were purchased from Babco (Richmond, Calif.). CKA staining was performed using the purified antisera at 1:2,000 dilutions. Anti-HA and anti-AU5 were used at 1:1,000 dilutions. Embryos aged 10 to 14 h at 25°C were used to study the process of DC.

In situ hybridization to whole-mount embryos with a digoxigenin-labeled antisense of the *dpp*-encoding DNA probe was performed as described elsewhere (12).

**DNA constructs and cell transfection.** pAP1-Luc and pcDNA3–β-galactosidase have been described previously (20). DJUN, DFOS, HEP, and BSK cDNAs were amplified by the PCR technique using corresponding cDNAs as the template. The sequences of the oligonucleotides utilized are available upon request. The amplified DNAs were subcloned as a *Bgl*II/*Eco*RI fragment in pCEFL, a modified pcDNA3 expression vector containing the elongation factor 1 promoter driving the expression of an in-frame N-terminal tag of either 9 amino acids (aa) derived from the influenza virus HA1 protein (HA) (42) or the AU5 epitope (21). To obtain the green fluorescent protein (GFP)-CKA construct, the PCR-amplified CKA cDNA was cloned into a pCEFL-GFP vector.

Transient transfections of NIH 3T3 and 293T cells were performed with the Lipofectamine Plus reagent (GIBCO BRL) according to the manufacturer's instructions. After 24 h, cells were collected and the lysates were used for assaying luciferase and β-galactosidase activities or used for coimmunoprecipitation experiments.

**Direct and indirect immunofluorescence in cultured cells.** 293T cells were seeded on glass coverslips and transfected with Lipofectamine Plus reagents (Life Technologies, Inc.) as described previously (21). Twenty-four hours later, serum-starved cells were washed twice with 1× phosphate-buffered saline (PBS) and fixed and permeabilized with 4% formaldehyde and 0.5% Triton X-100 in 1× PBS for 10 min. After being washed with PBS, cells were blocked with 1% bovine serum albumin and incubated with the indicated primary antibodies for 1 h. Mouse anti-AU5 and anti-HA antibodies (1:200 dilution) were used to detect HA- or AU5-tagged molecules. Following incubation, cells were washed three times with 1× PBS and then incubated with the corresponding secondary antibodies (1:200 dilution) conjugated with tetramethylrhodamine B isothiocyanate (Jackson ImmunoResearch Laboratories, Inc.). GFP-tagged CKA was directly visualized. Coverslips were washed three times, mounted in Vectashield mounting medium with 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.), and viewed using a Zeiss Axiophot photomicroscope equipped with epifluorescence. Immunofluorescence was photographed using Kodak TMAX 3200 film.

**Kinase assay.** Cells were seeded at 70 to 80% confluence and transfected with expression vectors for HA-tagged BSK alone or in combination with HEP and CKA. After transfection, cells were cultured for 24 h, incubated in serum-free medium for 2 h, washed with cold PBS, and lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 1 mM vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin/ml, and 20 μg of leupeptin/ml. Cleared lysates containing HA-tagged BSK were immunoprecipitated at 4°C for 2 h with anti-HA monoclonal antibody (Babco), and the kinase reaction was carried out essentially as previously described (20) using as substrate 1 μg of purified, bacterially expressed glutathione *S*-transferase (GST)–ATF2 or GST-DJUN (data not shown). Samples were analyzed by sodium dodecyl sulfate gel electrophoresis on 12% acrylamide gels, and autoradiography was performed with the aid of an intensifying screen.

**PP2A dephosphorylation assay.** Total lysates were incubated with 0.25 U of protein phosphatase 2A (PP2A; Upstate Biotechnology Inc.) at 30°C for 1 h according to the manufacturer's instructions.

**Preparation of embryonic cell lysates, immunoblotting, and immunoprecipitation.** Embryonic protein extracts were prepared in extraction buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin [0.15 U/ml], 20 μM leupeptin, 5 mM sodium vanadate) and centrifuged at 4°C for 10 min at 14,000 × *g* to remove insoluble material. Equal amounts of protein were used in immunoblottings and immunoprecipitations. For immunoprecipitations, equal amounts of primary antibodies were added to the embryo lysates. Bound proteins were analyzed by immunoblotting with anti-CKA antibodies.

**Western blotting and immunoprecipitation in cultured cells.** Extracts from cells transfected with epitope-tagged HEP, BSK, DJUN, or DFOS were immunoprecipitated with an anti-HA specific antibody, and the immunocomplexes were recovered with protein G-Sepharose (Pharmacia). Beads were washed three times with PBS containing 1% NP-40 and 2 mM vanadate and loaded in 10% polyacrylamide gels. The presence of CKA in the complexes was analyzed by Western blotting using an anti-CKA specific antibody (1:1,000 dilution), and the other molecules were detected with anti-HA and anti-AU5 monoclonal antibodies (Babco) (1:500 dilution). Proteins were visualized by enhanced chemiluminescence detection (Amersham Corp.) using goat anti-mouse and anti-rabbit immunoglobulin G coupled to horseradish peroxidase as the secondary antibody (Cappel).

**Reporter gene assays.** Cells were transfected with different expression plasmids together with 0.1 μg of an AP-1-driven reporter plasmid (Stratagene) and 0.01 μg of pRL-null (a plasmid expressing the enzyme *Renilla* luciferase from *Renilla reniformis)* as an internal control. In all cases, the total amount of plasmid DNA was adjusted with pcDNA3–β-galactosidase. Firefly and *Renilla* luciferase activities were measured as previously described (21).

**Nucleotide sequence accession number.** The GenBank accession number for the sequence reported in this paper is AF364305.

## RESULTS

**Identification of a new locus required for DC.** In a genetic screen for autosomal zygotic lethal mutations associated with specific maternal effect lethal phenotypes, we identified a P-element-induced mutation, l(2)05836, located at 28E1-2 (30), which showed a maternal effect dorsal-open phenotype. Based on its presumed role in the DJNK signal transduction pathway described below, we have named this novel gene *connector of kinase to AP-1* (*cka*). Animals zygotically homozygous for *cka* survive until pupal stages. However, embryos lacking both maternal and zygotic activity of *cka* (referred to as *cka* embryos throughout the text) die during embryogenesis with a dorsal-open phenotype. They display head defects and lack the dorsal epidermis, as indicated by a large dorsal hole in the cuticle preparations (Fig. 1; compare panels B and C with panel A). The mutant phenotype of *cka* embryos is fully rescuable by a wild-type paternal chromosome, indicating that the gene product is required only after the onset of zygotic gene expression. The P-element-induced mutation, which we refer to as $cka^1$, may represent a null mutation. In a Western blotting experiment using anti-CKA antibodies, no protein was detected in the *cka* embryonic lysates compared to wild-type embryonic lysates (see Fig. 4A; compare lane 3 with lanes 2 and 1); precise excision of the P element leads to homozygous viable flies, indicating that the original chromosome did not contain additional zygotic lethal mutations. In addition, we isolated three independent deletion alleles due to imprecise excisions in generating the precise P-element excisions. Two of these alleles, which we refer to as $cka^2$ and $cka^3$, are protein null and exhibit the same DC defect as $cka^1$ in germ line clone assays (data not shown).

***cka* is a novel component of the DJNK signal transduction pathway.** To more directly test the function of *cka* in the DJNK signal transduction pathway, we examined embryonic epithelial cell shape changes and *dpp* gene expression in *cka* mutant embryos. Like *Djun* and *Dfos* null mutations, *cka* null alleles completely blocked cell shape changes (Fig. 1I) that normally occur in the LE and lateral epithelial cells (Fig. 1H). However, *dpp* expression in the $cka^1$ embryo (Fig. 1; compare panel F with panel D and panel G with panel E) is strongly reduced but not abolished, as in *Djun* and *Dfos* null alleles. Like mutations of other components of the DJNK signal transduction pathway, the *cka* mutation affects *dpp* expression only in the LE cells. *dpp* expression in other tissues (including the visceral mesoderm and the lateral ectoderm) is normal.

To establish the *cka* function in the DJNK pathway, we examined genetic interactions between *cka* and components of the DJNK pathway. $kay^2$ and $bsk^1$ are weak hypomorphic alleles of *Dfos* and *DJNK*. Embryos zygotically homozygous for $kay^2$ and $bsk^1$ exhibit weak dorsal-open phenotypes (Fig. 2A and C); these phenotypes are strongly enhanced by removal of zygotic *cka* activity (Fig. 2B and D). We further introduced constitutively activated *Djun* or *hep* under the control of the heat shock promoter and enhancer ($HS\text{-}Djun^{ASP}$ or $UAS\text{-}hep^{CA}/HS\text{-}Gal4$) into *cka* embryos. The heat shock treatment significantly rescued the dorsal-open phenotype of *cka* embryos in both cases (compare Fig. 2F and G with 2E). All of these results suggest that *cka* positively regulates the DJNK signaling and functions upstream of or parallel to *hep*.

The DPP signal transduction pathway acts downstream of DJUN in regulating the DC mechanism. Taking advantage of the availability of transgenic flies expressing activated type I DPP receptor, we then asked whether expression of this dominant-acting molecule could bypass the requirement for CKA and rescue a *cka* mutant phenotype. We expressed a constitutively activated form of type I DPP receptor ($tkv^{QD}$) (10) in $cka^1$ mutant embryos, by using a $UAS\text{-}tkv^{QD}$ transgene driven by an *HS-Gal4* line (2) that is ubiquitously expressed. Indeed, when supplied in this manner, $tkv^{QD}$ dramatically rescued the *cka* mutant phenotype (compare Fig. 2H with 2E). These data provided further evidence that CKA functions upstream of the DPP signal transduction pathway.

In the late development stages the DJNK signal transduction pathway plays a crucial role in eliminating deleterious cells caused by distortion of proximodistal information in imaginal disks (1). A fraction of flies expressing a constitutively activated form of HEP ($hep^{CA}$) are missing wing tissues due to elimination of cells by DJNK-dependent apoptosis (1) (Fig. 2J). Reduction of *cka* activity by half restores the $hep^{CA}$ fly wing to normal (Fig. 2K), suggesting that *cka* activity is required for HEP-regulated apoptosis.

In summary, the above genetic data indicate that *cka* functions in two DJNK pathway-regulated developmental processes: embryonic DC and apoptosis in wing imaginal disk.

***cka* encodes a novel multidomain protein.** Genomic DNA flanking the P-element insertion site associated with $cka^1$ was recovered following plasmid rescue and used to screen a *Drosophila* embryonic cDNA library and search the *Drosophila* expressed sequence tag database (for details see Materials and Methods). The longest cDNA recovered was a full-length 3,274-bp clone which encodes a putative protein of 730 aa (Fig. 3).

Several lines of evidence argue that the cDNA that we isolated corresponds to *cka* function. The P element is inserted in the 5′ untranslated region, and this insertion leads to the absence of detectable levels of CKA protein in *cka* embryos (Fig. 4A, lane 3). A transgene containing the full-length cDNA driven by the heat shock promoter was introduced into the *Drosophila* germ line. Upon heat shock induction (for details see Materials and Methods), the transgene rescued the $cka^1$ embryonic mutant phenotype. Further, the heat shock transgene fully rescued the lethality of homozygous $cka^1$ animals.

Searches of databases revealed that CKA shares a distinct homology with the products of a recently identified gene family (5), including the human SG2NA, zinedin, and striatin and an as yet uncharacterized *Caenorhabditis elegans* gene (GenBank accession no. CEK07C5). Protein sequence analysis revealed that CKA and its homologues share several known protein-protein interaction motifs (Fig. 3). From the N to the C terminus of CKA, these motifs include a putative caveolin-binding domain (aa 105 to 113), a putative coiled-coil motif (aa 120 to 166), a putative calmodulin (CaM)-binding domain (aa 204 to 221), and seven WD40 repeats (aa 413 to 730). In addition, several potential sites of phosphorylation by casein kinase II and protein kinase C were identified.

**CKA forms a complex with HEP, BSK, DJUN, and DFOS.** Information from the above-mentioned experiments and the multidomain structure of CKA prompted us to test whether there is physical interaction of CKA with known components

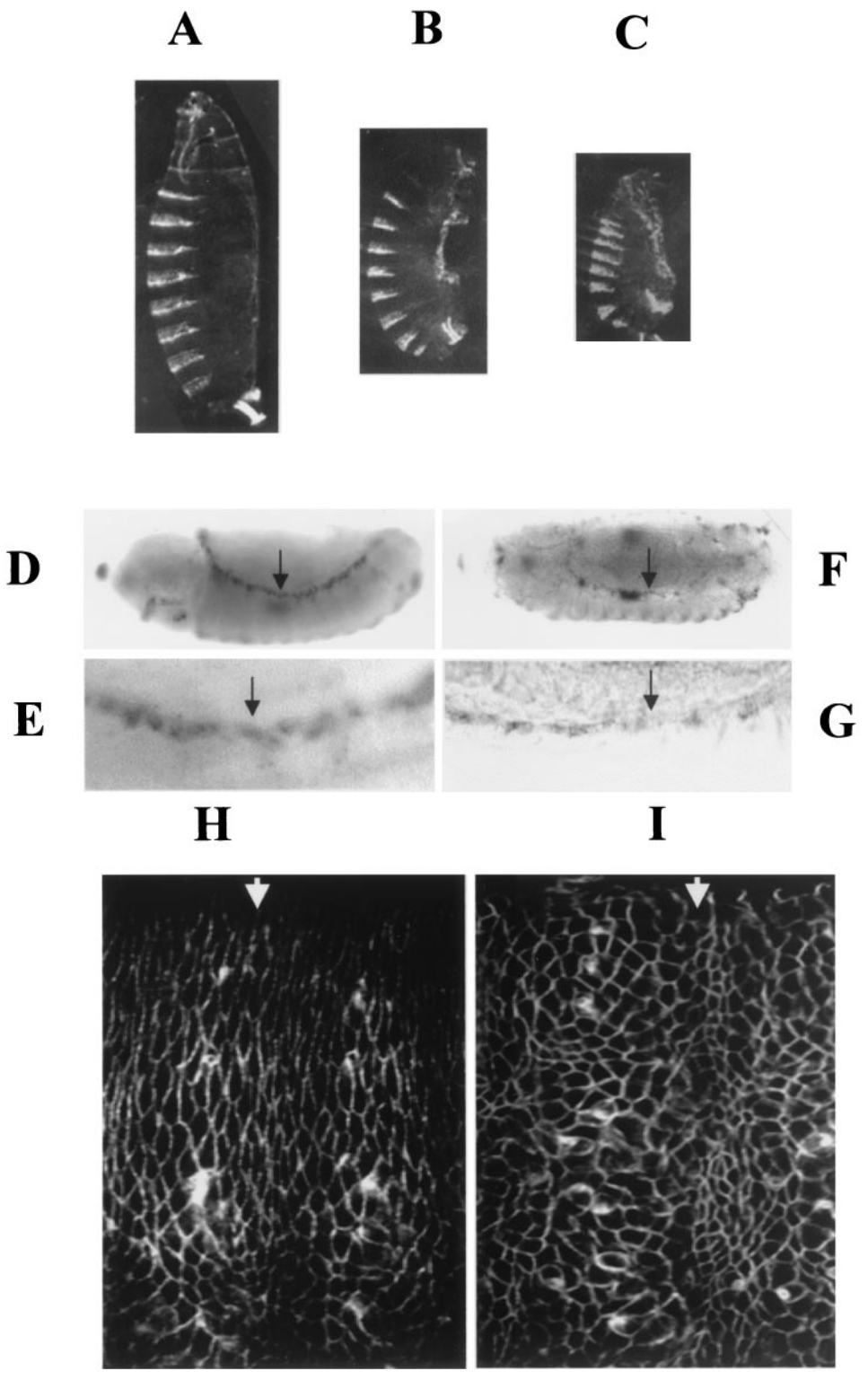



FIG. 1. The *cka* mutation blocks the DC process in the embryo. (A) Dark-field cuticle patterns of wild-type embryo. (B and C) $cka^1$ embryos from females with $cka^1$ germ line clones exhibit the dorsal-open cuticle phenotype; 534 out of 1,064 (~50%) embryonic cuticles show the DC phenotype (lateral views). (D to G) *dpp* in situ hybridizations of wild-type (D and E) or $cka^1$ mutant (F and G) embryos. After germ band retraction, the *dpp* stripe of expression in the LE cells (arrows in panels F and G) is significantly disrupted. Panels E and G are enlarged views of panels D and F, respectively. (H and I) Epithelial cell shape changes during DC are revealed in wild-type and $cka^1$ mutant embryos, respectively. Cell profiles are illustrated with anti-α-spectrin antibodies. In the wild-type stage 14 embryo, epidermal cells in both the dorsalmost (arrow) and lateral positions are elongated (H), but the dorsalmost cells (arrow) in the $cka^1$ mutant embryo remain polygonal (I) (dorsal views).

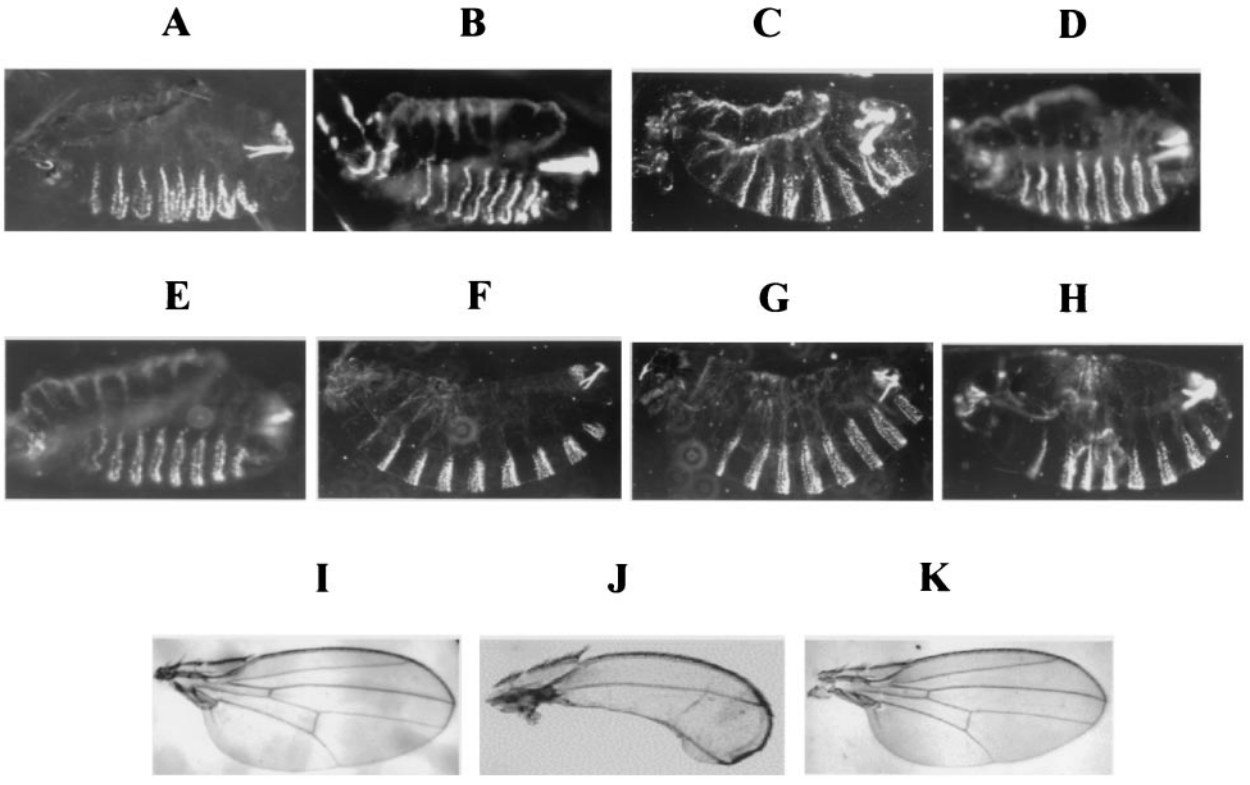
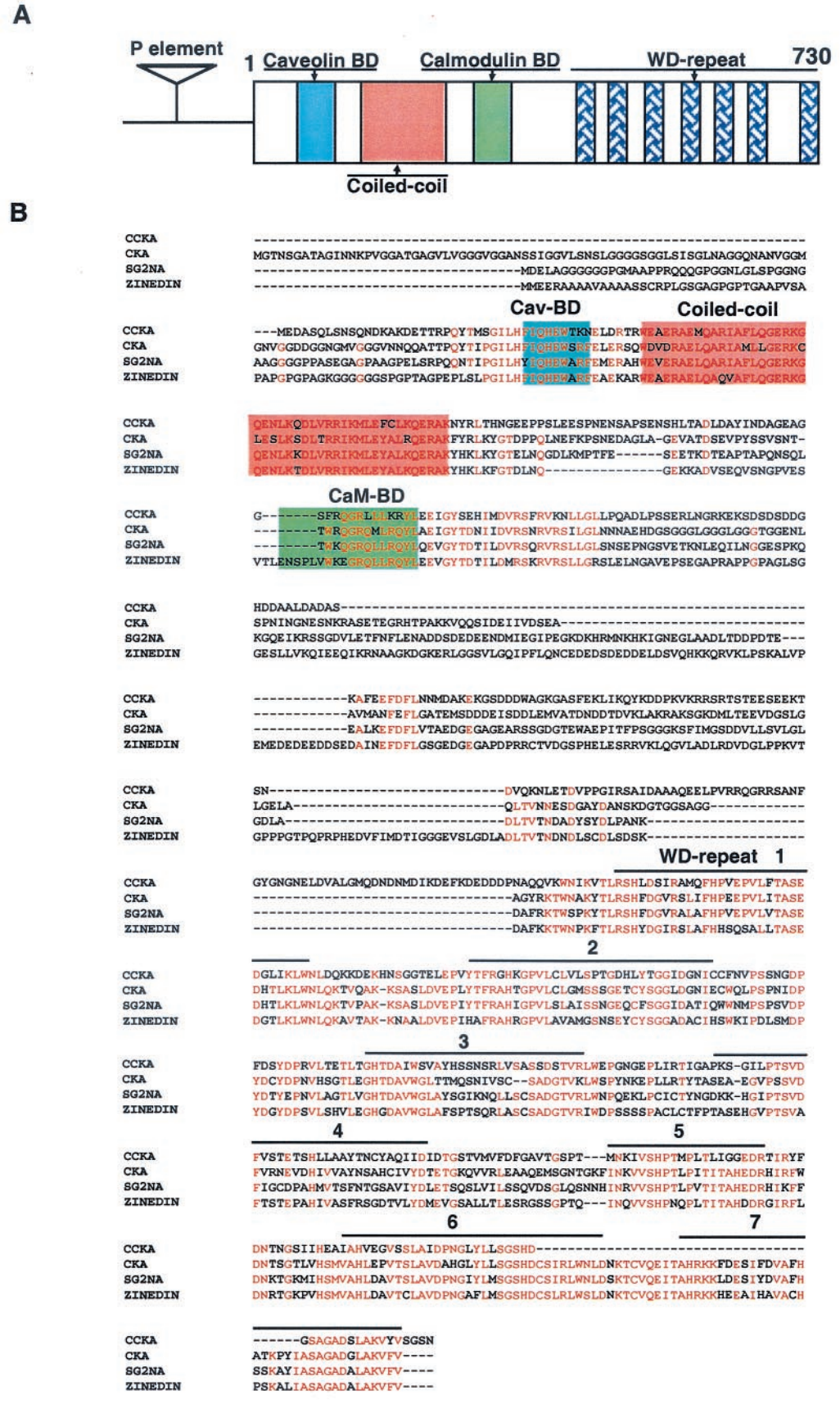



FIG. 2. *cka* genetically interacts with components of the DJNK signal transduction pathway. The figure shows the dark-field cuticle pattern of embryos of the following genotypes. (A) $kay^2/kay^2$ (zygotic). $kay^2$ cuticle phenotypes are variable. (B) $cka^1/+$ (zygotic); $kay^2/kay^2$ (zygotic). Of the 156 cuticles examined, 80 had a bigger dorsal-open phenotype (B), whereas 76 had a $kay^2$-like phenotype (A). This distribution corresponds statistically to a 1:1 ratio, consistent with half of the mutant $kay^2$ embryos having received a mutant *cka* allele and the other half having received a balancer chromosome carrying wild-type *cka*. (C) $bsk^1/bsk^1$ (zygotic) cuticles with dorsal-open phenotype. (D) $bsk^1cka^1/bsk^1cka^1$ (zygotic) cuticles had a bigger dorsal-open phenotype than did $bsk^1/bsk^1$ cuticles. (E) $cka^1/cka^1$ (germ line clone). (F) $cka^1$ $+/cka^1$ $HS\text{-}DJUN^{ASP}$ (germ line clone). (G) $UAS\text{-}hep^{CA}$ $cka^1/HS\text{-}Gal4$ $cka^1$ (germ line clone). (H) $cka^1$ $UAS\text{-}tkv^{QD}/cka^1$ *HS-Gal4* (germ line clone). Embryos with $HS\text{-}Djun^{ASP}$ and *HS-Gal4* were heat shocked for 30 min at 5 to 7 h after egg laying. In all panels, dorsal is up and anterior is right. $HS\text{-}Djun^{ASP}$ is the constitutively activated form of *Djun* under the heat shock promoter. More than 100 mutant embryonic cuticles were examined for each of the panels F to H. All embryos were rescued but to different degrees. Representative phenotypes for each of panels F to H are shown. (I) Normal fly wing. (J) $hep^{CA}/+$ fly wing (10% of flies show this phenotype). (K) $hep^{CA}/+$; $cka^2/+$ fly wing. *cka* suppresses wing tissue loss caused by constitutively activated *hep* ($hep^{CA}$).

of the DJNK signal transduction pathway. Cell lysates were prepared from wild-type *Drosophila* embryos, immunoprecipitated with anti-DJUN and anti-DFOS antibodies, and then probed using an anti-CKA antibody. We found that CKA coimmunoprecipitated with endogenous DJUN (Fig. 4A, lane 7) and DFOS (Fig. 4A, lane 9) in the wild-type embryonic lysates but not in the *cka* embryonic lysates (data not shown). To demonstrate that the identified band corresponds to CKA, we amplified the CKA cDNA and subcloned it in an AU5-tagged mammalian expression vector. Then we cotransfected 293T cells with the AU5-tagged form of the protein and blotted total lysates with anti-AU5 and anti-CKA antibodies (1:1,000 dilution). As shown in Fig. 4A (lower panel), CKA antibody specifically recognizes AU5-CKA. In parallel, we tested the specificity of DJUN and DFOS antibodies by cotransfecting 293T cells with AU5-tagged forms of the factors and blotted the total lysates with anti-AU5, anti-DJUN, and anti-DFOS antibodies (1:1,000 dilutions). Indeed, the antibodies were shown to be specific for the *Drosophila* transcription factors, since they recognized only the bands corresponding to the AU5-tagged forms with the same intensity as the AU5 antibody, when used at the same dilution.

Due to the lack of good-quality antibodies to detect interactions among CKA, HEP, and BSK in embryonic lysates, we tested interactions among CKA, HEP, and BSK in tissue culture cells.

FIG. 3. *cka* encodes a multidomain protein. (A) Diagram of positions of the caveolin-binding domain, the coiled-coil domain, the CaM-binding domain, and the C-terminal WD40 repeats. The P element is inserted into the 5′ untranslated sequence of the *cka* gene in the $cka^1$ mutation. (B) *cka* encodes a multidomain protein related to SG2NA, zinedin, and a *C. elegans* open reading frame that we renamed *ccka*. Alignment of the amino acid sequences of CCKA, CKA, SG2NA, and zinedin. Identical amino acids are shown as red letters on a white background. The conserved domains are indicated above the sequences. The caveolin-binding domain (Cav-BD) is shaded blue; the coiled-coil domain is shaded pink; the CaM-binding domain (CaM-BD) is shaded light green; solid lines above the sequences indicate the seven copies of the WD40 repeats in all four proteins.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WD-repeat
730
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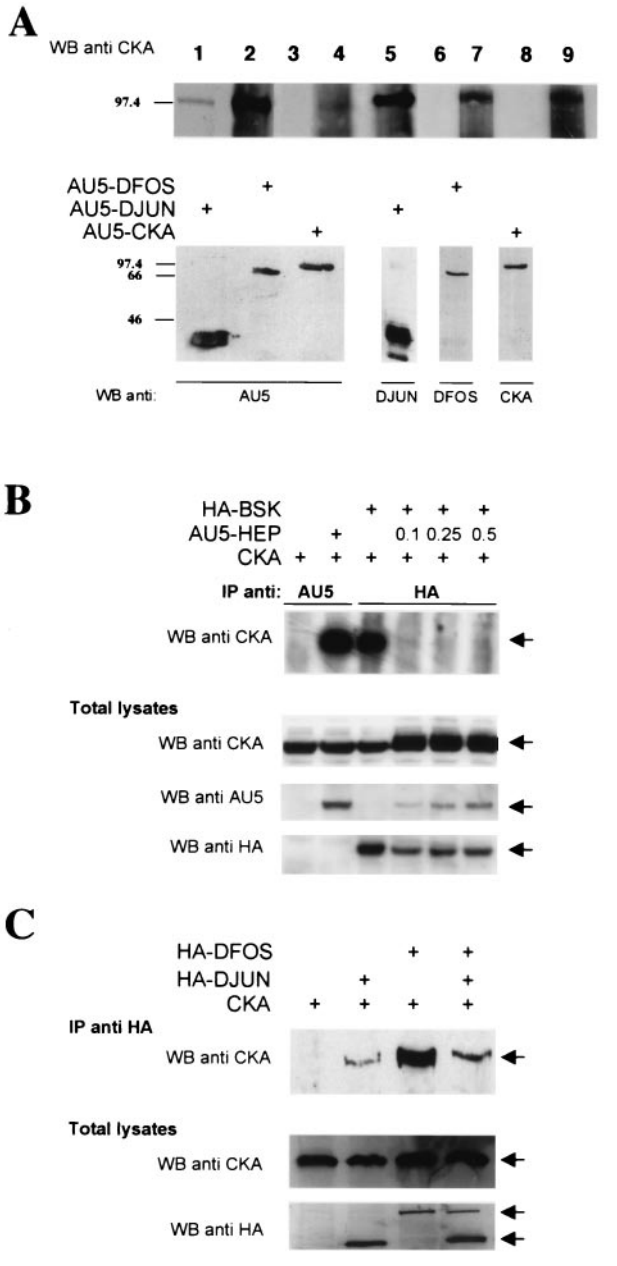
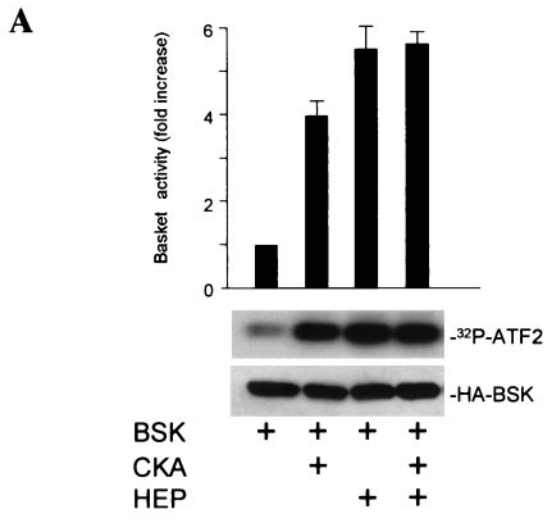



FIG. 4. CKA interacts with BSK, HEP, and DJUN-DFOS. (A) (Upper panel) Wild-type *Drosophila* embryos (0 to 12 h) (lanes 4 to 9) were lysed and immunoprecipitated using antibodies (anti-CKA [lane 5], anti-DJUN [lane 7], and anti-DFOS [lane 9]) or their preimmune sera (anti-CKA preimmune serum [lane 4], anti-DJUN preimmune serum [lane 6], and anti-DFOS preimmune serum [lane 8]). Lane 1 is 0- to 1.5-h wild-type embryo lysate, lane 2 is 0- to 12-h wild-type embryo lysate, and lane 3 is 0- to 1.5-h embryo lysate from female flies with $cka^1$ germ line clones. Immunoprecipitates (lanes 4 to 9) and lysates (lanes 1 to 3) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting using anti-CKA antibodies. (Lower panel) 293T cells were transfected with expression vectors for AU5-tagged DJUN, DFOS, or CKA. Total lysates were analyzed by Western blotting experiments with different antibodies. Molecular mass (kilodaltons) is indicated at the left of both blots (Rainbow markers; Amersham Corp.). (B) 293T cells were transfected with expression vectors for CKA alone or in combination with pCEFL-HA-tagged BSK and different concentrations of AU5-HEP as indicated. After 24 h, cellular lysates were immunoprecipitated with anti-HA or anti-AU5 antibodies and blotted with a specific anti-CKA antibody (top panel). Blotting of parallel samples with anti-HA, anti-AU5, and anti-CKA antibodies confirmed the expression of all transfected proteins (middle and bottom panels). (C) Cells were transfected as described above with CKA and HA-tagged DJUN and DFOS. Lysates were immunoprecipitated with anti-HA antibody and blotted with a specific anti-CKA antibody (top panel). Blotting of parallel samples with anti-HA and anti-CKA antibodies confirmed the expression of all transfected proteins (middle and bottom panels). Similar results were obtained in three different experiments. WB, Western blotting; IP, immunoprecipitation.

We prepared cell lysates from 293T cell lines expressing CKA with AU5-tagged HEP or HA-tagged BSK, DJUN, or DFOS (42). The lysates were immunoprecipitated using an anti-HA or anti-AU5 antibody and then probed using an anti-CKA antibody. We found that CKA coimmunoprecipitated with HEP and BSK (Fig. 4B, lanes 2 and 3). Interestingly, this association seems to be dynamic, since when HEP and BSK are added together, the interaction between CKA and BSK is almost abolished (Fig. 4B, lanes 4 to 6), whereas the interaction with HEP remains the same (data not shown). One putative explanation is that, when BSK is activated by HEP, BSK is released from the complex to phosphorylate specific substrates. Of note, in the presence of activated BSK, CKA seems to be phosphorylated as suggested by a shift in the molecular mass of the protein (Fig. 4B, middle panel, lanes 4 to 6). This apparent phosphorylation may be indicative of a change in the charge and/or shape of the protein that could lead to the release of BSK. CKA also coimmunoprecipitates with DJUN and DFOS when expressed together or individually, although the interaction between CKA and DFOS is stronger than that between CKA and DJUN (Fig. 4C). Together, these data indicate a physical connection between CKA and the DJNK pathway members although, surprisingly, CKA does not bind DTAK1, a proposed upstream activator of the DJNK signal transduction route (data not shown).

**CKA and HEP activate BSK, which in turn binds and phosphorylates both DJUN and DFOS.** To explore whether the physical interaction between CKA and these molecules has functional consequences, we studied whether the kinase activity of BSK was stimulated in the presence of CKA. We cotransfected 293T cells with an HA epitope-tagged BSK along with CKA, with its direct upstream activator, HEP, being used as a positive control. As expected, this kinase was clearly activated by the transfected HEP compared to the β-galactosidase-transfected control. Surprisingly, CKA itself activated BSK, probably by bringing it closer to endogenous JNKKs, as CKA itself does not phosphorylate BSK in vitro (data not shown). The combination of HEP plus CKA did not cause more activation since, in the presence of high levels of transfected HEP, BSK is probably activated to its maximum capacity (Fig. 5A). Even lower amounts of transfected HEP (10 ng/well) or CKA (10 ng/well) fully activated BSK in 293T cells. We then decided to do similar experiments using NIH 3T3 cells, since they are transfected with a much lower efficiency. In kinase assay experiments using different concentrations of HEP (5 to 500 ng/well) we found that, at 50 ng/well, the system was already saturated (data not shown). Thus, we decided to coexpress different amounts of CKA along with 5 ng of HEP per well. As we expected, at these low concentrations, the



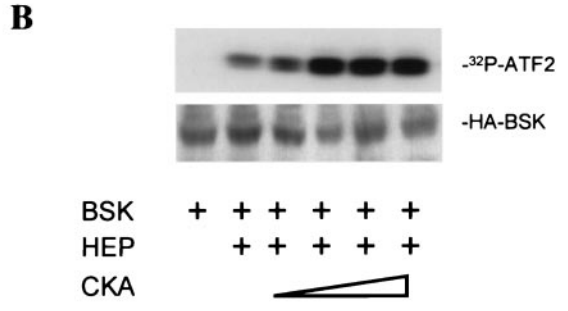




FIG. 5. Activation of BSK kinase activity by CKA. (A) 293T cells were transfected with HA-tagged BSK along with CKA and HEP, as indicated. After 2 h of serum starvation the samples were lysed, immunoprecipitated with anti-HA antibody, and used for kinase reactions. The histogram represents the kinase activity relative to that of cells transfected with a plasmid expressing β-galactosidase, which was used as a control and whose value was taken as 1. Data represent the means ± standard errors of triplicate samples from a typical experiment. Similar results were obtained in three additional experiments. (B) The experiment was carried out as described above except that NIH 3T3 cells were transfected with HA-tagged BSK along with HEP (5 ng/well) and CKA (10, 25, 75, and 375 ng/well). Similar results were obtained in three different experiments.

combination of CKA with HEP resulted in a higher BSK activity than that with HEP alone (Fig. 5B). To further explore the effects of BSK activation on its downstream target, DJUN, we investigated whether CKA could induce in vivo phosphorylation of this transcription factor. As DFOS is an integral component of the JNK pathway in DC (33, 44), we also included it in this analysis. Thus, 293T cells were transfected with AU5-tagged DJUN and DFOS together with CKA, alone or in combination with BSK or HEP. As shown in Fig. 6A (top panel), activated BSK induced phosphorylation of both DJUN and DFOS, as judged by a strong shift in their apparent molecular mass, which likely represents phosphorylated states of these proteins. Interestingly, when BSK was transfected along with CKA, an upper band of DFOS appeared in lane 7 whereas the lowest band of DJUN (lane 3) almost completely disappeared in lane 7. Next, we investigated if the observed shift in the molecular weight of the factors was due to changes in the phosphorylation state. We incubated lysates from cells cotransfected with HEP and BSK, which showed the most dramatic change, with the phosphatase PP2A, which specifically removes phosphate groups from phosphorylated serine/threonine residues. The shift induced by HEP and BSK was reversed by treatment of the same cell lysates with the phosphatase for 1 h at 30°C (Fig. 6B). These observations parallel the results obtained in the kinase assays and suggest that CKA may be increasing BSK activity, resulting in DJUN and DFOS phosphorylation.

**CKA regulates AP-1 transcription activity in tissue culture cells.** Based on our previous results, we next investigated whether the effect of CKA on BSK activity and phosphorylation of DJUN and DFOS resulted in an increase in the AP-1-dependent transcription. For these experiments we transfected

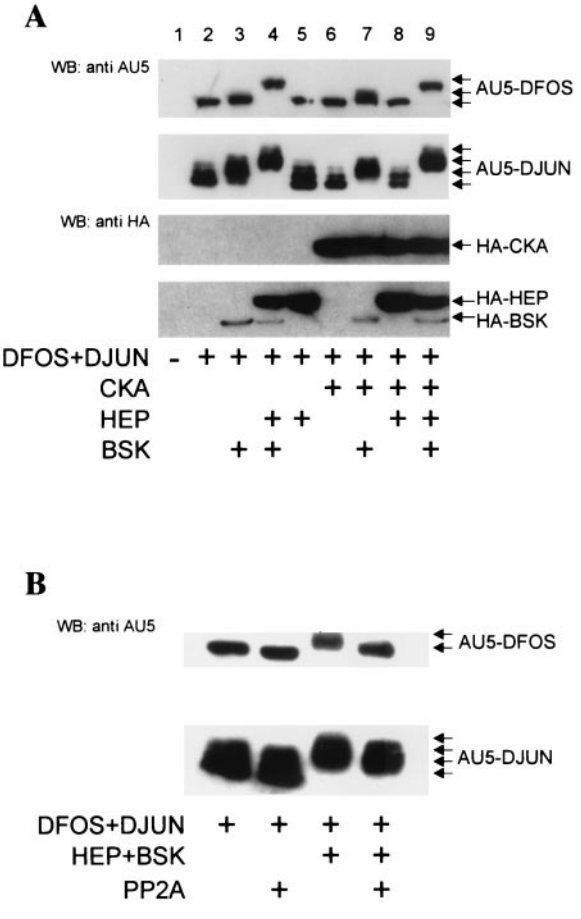



FIG. 6. Phosphorylation of both DJUN and DFOS by BSK is regulated by CKA. (A) Cells were transfected with pCEFL-AU5 DJUN and DFOS alone or in combination with HA-tagged BSK, HEP, and/or CKA as indicated. Total lysates were analyzed by Western blotting experiments. DJUN and DFOS were detected by a mouse monoclonal anti-AU5 antibody. HA-tagged BSK, HEP, and CKA were detected by an anti-HA specific antibody. (B) Cells were transfected as indicated. Total lysates were treated with PP2A as described in Materials and Methods before the Western blot analysis. The blots shown are representative of three different experiments. WB, Western blotting.

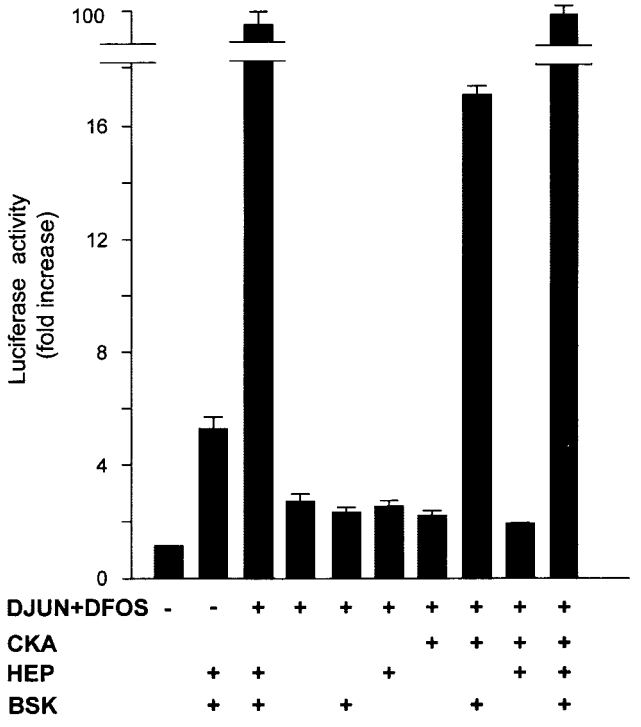



FIG. 7. CKA stimulates the transcriptional activity of DJUN and DFOS. NIH 3T3 cells were cotransfected with pAP1-Luc and pRL-SV40 reporter plasmid DNAs (0.1 and 0.01 μg each per plate), along with expression vectors for CKA, BSK, HEP, DJUN, and DFOS (0.5 μg each). In all cases, the total transfected DNAs were adjusted to the same amount with pcDNA3–β-galactosidase. The data represent firefly luciferase activity normalized to *Renilla* luciferase activity present in each sample, expressed as fold induction relative to the control, whose value was taken as 1. Values are the averages ± standard errors of triplicate samples from a typical experiment. Nearly identical results were obtained in three additional experiments.

NIH 3T3 cells with an AP-1-driven luciferase reporter gene (pAP1-Luc) in the presence or absence of DJUN and DFOS with CKA alone or along with HEP, BSK, or both. Lysates obtained from transfected cells were assayed for luciferase activity. As shown in Fig. 7, in the absence of DJUN and DFOS, HEP and BSK stimulated AP-1 activity by nearly six-fold, probably due to the effect on some endogenous AP-1 factors. However, in the presence of DJUN and DFOS, which by themselves cause a very limited activation of pAP1-Luc, HEP and BSK strongly activated the activity by nearly 90-fold. These data indicate the requirement for specific *Drosophila* transcription factors downstream of BSK to achieve a full AP-1 reporter activity. This activation was not increased further by CKA, which is consistent with the fact that, in the presence of overexpression of HEP, BSK is activated to its maximal extent. However, when BSK was transfected along with CKA the AP-1 activity was increased by nearly 20 times. In contrast, the co-expression of CKA with HEP did not significantly affect the reporter activity. This difference is probably due to the fact that DJUN and DFOS are specific targets for BSK, since in our experiments activated human JNK was not able to phosphorylate significantly the *Drosophila* transcription factors (data not shown). These observations are also consistent with the pattern of phosphorylation of DJUN and DFOS shown in Fig. 6A, suggesting a direct correlation between DJUN-DFOS phosphorylation and AP-1 activity, as expected. Altogether, these data are consistent with our previous findings and indicate that CKA may affect the functional activity of BSK, which in turn phosphorylates DJUN (and likely also DFOS), thus leading to an enhanced AP-1 activity.

**The components of the DJNK pathway regulate CKA subcellular localization.** To study the subcellular localization of CKA in relation to other components of the DJNK pathway, we fused the coding sequence of GFP to that of CKA, as well as engineered tagged forms of HEP, BSK, DJUN, and DFOS. For these experiments, we used a readily transfectable cellular system, which facilitated the detection of GFP-CKA and the coexpressed molecules by immunofluorescence using specific antitag antibodies. As shown in Fig. 8, GFP-tagged CKA was detectably expressed when transfected alone or in combination with HA-tagged forms of HEP, BSK, DJUN, and DFOS in mammalian 293T cells. Although there was a small fraction localized to the nucleus, the main fraction of CKA was found in the cytoplasm when transfected alone or in combination with BSK. However, upon cotransfection with HEP, which localizes mainly to the cytosol, an important fraction of CKA translocated to the nucleus (Fig. 8, middle panels). Of note, in the presence of DJUN and DFOS (Fig. 8, rightmost panels, and data not shown), a fraction of CKA also translocated to the nucleus. This finding implies that increasing expression of DJUN and DFOS and their affinity for CKA may shuttle some protein to the nucleus. It is likely that this translocation of CKA to the nucleus in cultured cells represents a tightly regulated mechanism, which may ensure the proper control of its biological activity.

## DISCUSSION

In this study, we have characterized *cka*, a novel gene for a multidomain protein. Our genetic data indicate that *cka* functions in the DJNK signal transduction pathway. In the embryo, *cka* controls the localized expression of *dpp* in the LE cells for epithelial cell sheet movement. *cka* also functions in the DJNK signal transduction pathway in regulating apoptosis in the wing imaginal disk. CKA forms a complex with HEP, BSK, DJUN, and DFOS. This interaction seems to be dynamic and leads to the activation of BSK kinase and subsequent phosphorylation and activation of DJUN and DFOS transcriptional activity. Our data suggest that CKA represents a unique molecule that regulates AP-1 activity by organizing a complex of kinases and transcription factors.

**CKA is a component of the DJNK signal transduction pathway.** Several lines of evidence support the view that CKA is a component of the DJNK signal transduction pathway. As with other mutants in the DJNK signal transduction pathway, *cka* embryos have a dorsal-open cuticle phenotype, *dpp* expression in the LE cells is significantly reduced in *cka* embryos, and *cka* null alleles completely block cell shape changes of the LE and lateral epidermal cells during DC. Furthermore, a constitutively active form of *hep* and *Djun* rescues the dorsal-open cuticle phenotype of *cka* embryos. In addition, zygotic *cka* mutants enhance the mutant phenotypes of weak alleles of *Dfos* (*kay*) and *DJNK* (*bsk*).

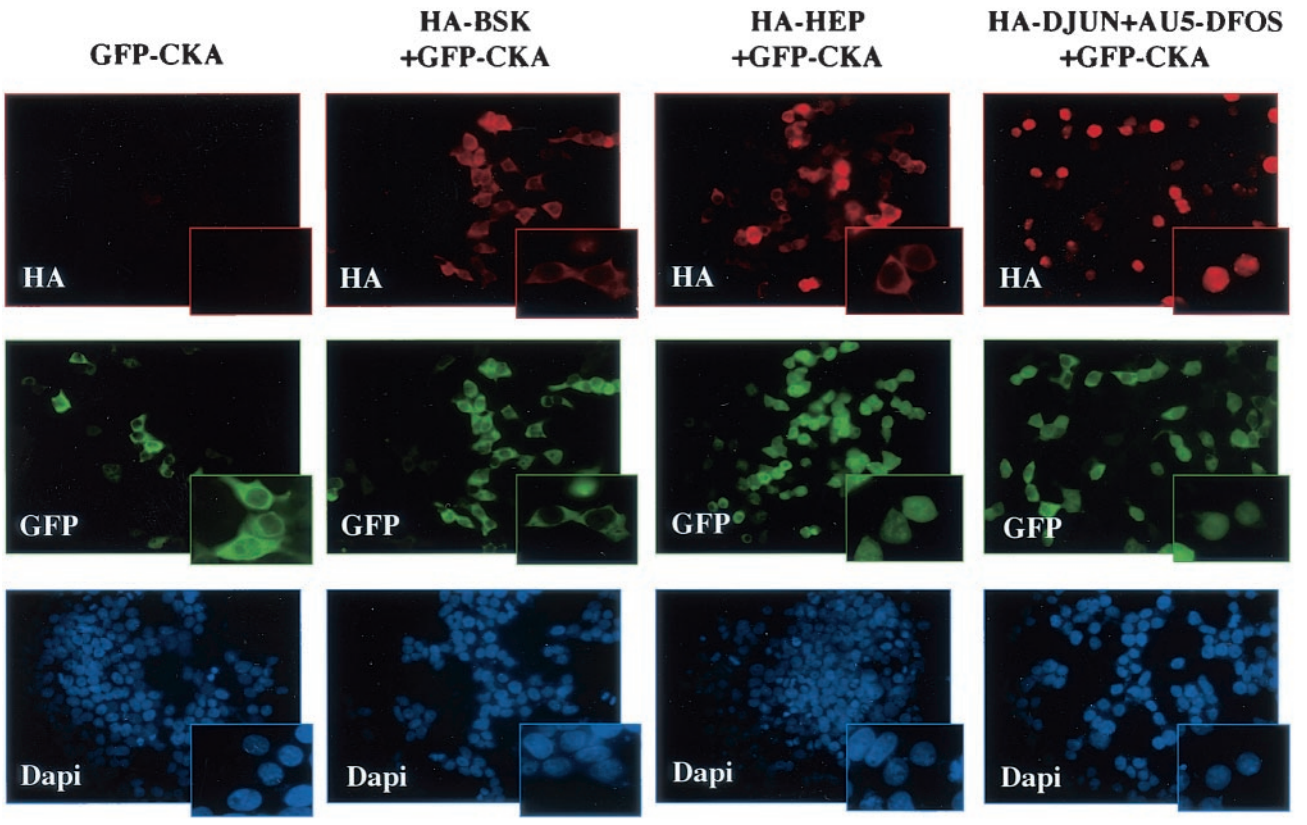



FIG. 8. The components of the DJNK pathway regulate CKA subcellular localization. 293T cells were seeded on coverslips and transfected with GFP-tagged CKA alone or together with HA-tagged-BSK, HEP, DJUN, and DFOS as indicated. Twenty-four hours after transfection, cells were fixed and analyzed by immunofluorescence for BSK, HEP, and DJUN with anti-HA specific antibodies (top panels), GFP-CKA (middle panels), and nuclear staining (DAPI; bottom panels).

CKA also functions in the DJNK signal transduction pathway at other developmental stages. In wing imaginal disks, loss of *cka* activity blocks DJNK-dependent apoptosis.

**Regulation of DJNK signal transduction pathway by CKA.** The CKA protein sequence contains four known protein-protein interaction motifs. From the N terminus to the C terminus of CKA, they are a putative caveolin-binding domain, a putative coiled-coil motif, a putative CaM-binding domain, and seven WD40 repeats. The caveolin-binding domain and the CaM-binding domain are found in a variety of proteins involved in signal transduction. Caveolin binding may be responsible for some CKA membrane localization. Caveolin binding generally parks the associated signaling molecules in an inactivated state (28). It remains to be tested whether CKA is also regulated by this mechanism. The WD40 repeats are found in a variety of proteins involved in signal transduction, RNA processing, gene regulation, vesicle fusion, cytoskeletal assembly, and protein degradation (36). Crystal structure analysis predicts that this domain forms a circular β-propeller structure. The WD-repeat propeller structures create a stable platform that can coordinate sequential and/or simultaneous protein-protein interactions. Several WD-repeat proteins have been shown elsewhere to form complexes reversibly with other proteins (36).

We found that CKA interacts with DJUN and DFOS in embryonic lysates. In tissue culture cotransfection assays we demonstrated that HEP and BSK strongly bind CKA independently of one another. Although HEP can directly phosphorylate BSK when overexpressed, it is possible that, under physiological conditions and upon certain stimuli, CKA regulates the DJNK module by acting as a scaffolding protein that brings BSK into close proximity with its direct activator, HEP. Available data suggest that, upon BSK activation, CKA is phosphorylated and releases the DJNK from the complex, which in turn enters into the nucleus, where it binds and phosphorylates both DJUN and DFOS. The phosphorylation of these nuclear factors stimulates their transcriptional activity, and consequently they turn on the AP-1-regulated gene transcription. A similar mechanism is found in yeasts, where the scaffolding protein Ste5 interacts with the kinases Ste7 (a mitogen-activated protein kinase [MAPK] kinase) and Fus3 (a MAPK) in an independent fashion, and this binding is crucial for the function of the MAPK cascade in vivo. Although STE7 can directly activate Fus3, the presence of Ste5 is required for the correct organization of the pathway. This physical interaction facilitates both activation and attenuation of cross-interactions between these kinases and those of other MAPK modules (17). Notably, CKA also interacts physically with the nuclear proteins DJUN and DFOS, although the functional consequences of this interaction are still not fully elucidated. Since in the

presence of HEP a fraction of CKA goes to the nucleus, it is possible then that the binding of CKA to DJUN and DFOS facilitates the phosphorylation of these transcription factors by activated BSK. Thus, CKA would act as a two-step scaffolding molecule that temporally and spatially organizes the cytosolic and nuclear components of the DJNK pathway, thereby promoting an enhanced activation of these molecules. Similarly, it has been demonstrated previously that the yeast scaffolding protein Ste5 shuttles to the nucleus and that its translocation is necessary to prevent kinase activation in the cytosol under certain physiological conditions (19).

Interestingly, all CKA homologues from *C. elegans* to mammals can replace the *Drosophila* CKA in activating the DJNK signal transduction pathway, suggesting that they are not just structural homologues and that their functions are also conserved (data not shown). CKA human homologues striatin and SG2NA have recently been shown to form a complex with the PP2A A/C heterodimer, CaM, and several other cellular proteins (24); this indicates that CKA binding partners are not limited to JNKK and JNK. They may form a multiple protein functional module, and this module may respond to extracellular signals and regulate AP-1 transcriptional activity. Identification of additional components in the CKA complex will shed more light on the functional module.

**Concluding remarks.** The multidomain protein CKA provides a new level of regulation in the DJNK signal transduction pathway. The emerging picture is that CKA regulates DJNK signaling by organizing a protein complex or functional module of kinase, phosphatase, and transcription factors. These findings raise the possibility that similar modules may regulate other signal transduction pathways, a possibility that warrants further investigation. Current studies are aimed at elucidating in detail the molecular mechanisms whereby CKA exerts its profound biological effects.

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