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A *Drosophila* Homolog of the Rac- and Cdc42-Activated Serine/Threonine Kinase PAK Is a Potential Focal Adhesion and Focal Complex Protein That Colocalizes with Dynamic Actin Structures

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Changes in cell morphology are essential in the development of a multicellular organism. The regulation of the cytoskeleton by the Rho subfamily of small GTP-binding proteins is an important determinant of cell shape. The Rho subfamily has been shown to participate in a variety of morphogenetic processes during *Drosophila melanogaster* development. We describe here a *Drosophila* homolog, DPAK, of the serine/threonine kinase PAK, a protein which is a target of the Rho subfamily proteins Rac and Cdc42. Rac, Cdc42, and PAK have previously been implicated in signaling by c-Jun amino-terminal kinases. DPAK bound to activated (GTP-bound) *Drosophila* Rac (DRacA) and *Drosophila* Cdc42. Similarities in the distributions of DPAK, integrin, and phosphotyrosine suggested an association of DPAK with focal adhesions and Cdc42- and Rac-induced focal adhesion-like focal complexes. DPAK was elevated in the leading edge of epidermal cells, whose morphological changes drive dorsal closure of the embryo. We have previously shown that the accumulation of cytoskeletal elements initiating cell shape changes in these cells could be inhibited by expression of a dominant-negative DRacA transgene. We show that leading-edge epidermal cells flanking segment borders, which express particularly large amounts of DPAK, undergo transient losses of cytoskeletal structures during dorsal closure. We propose that DPAK may be regulating the cytoskeleton through its association with focal adhesions and focal complexes and may be participating with DRacA in a c-Jun amino-terminal kinase signaling pathway recently demonstrated to be required for dorsal closure.

The Rho subfamily of Ras-related small GTPases comprises a group of homologous proteins that are crucial components in the regulation of cell morphology. Members of the subfamily have been identified in a wide range of species and are classified as Rho, Rac, or Cdc42 on the basis of sequence. In *Saccharomyces cerevisiae*, Cdc42 interacts with the actin cytoskeleton to regulate bud formation (1, 27). Studies of mammalian fibroblasts have demonstrated that the Rho subfamily proteins may act in a hierarchical manner to modify the actin cytoskeleton. Activation of Cdc42 promotes the formation of filopodia and also the activation of Rac (29, 43), which can induce lamellipodia (53). Subsequently Rac activates Rho (43), leading to the assembly of focal adhesions and actin stress fibers (51). Multimolecular focal complexes associated with the plasma membrane are found in the filopodia and lamellipodia induced by Cdc42 and Rac. These focal complexes contain components found in focal adhesions but are distinct from the focal adhesions induced by Rho (43).

The changes in the cytoskeleton coordinated by the Rho subfamily produce alterations in morphology that are likely to be required for cell movement (43). Cell shape change and cell movement are essential in the development of any multicellular organism, and it is likely that the Rho subfamily has numerous developmental roles. We and others have recently begun to characterize the participation of this group of proteins in the development of *Drosophila melanogaster*, an organism for which the developmental requirements for Ras have already been extensively studied. *Drosophila* homologs of Rac are implicated in axon outgrowth and myoblast fusion (33), germ band retraction and dorsal closure (20), and the assembly of actin at adherens junctions in epithelial cells of the wing imaginal disc (12). A *Drosophila* Cdc42 homolog is involved in the outgrowth of dendrites and axons (33), the determination of muscle fiber morphology (33), and polarized cell shape changes in the wing imaginal disc (12). Overexpression of the *Drosophila* Rho1 product disrupts eye development (21). These studies indicate that the Rho subfamily has wide-ranging roles in development.

The signal transduction pathways by which members of the Rho subfamily exert their effects are being characterized, with recent work indicating that protein kinases are involved. The tyrosine kinase inhibitor genistein prevents the formation of focal adhesions and stress fibers by activated Rho (52), and the broad-spectrum kinase inhibitor staurosporine blocks the Rho-mediated assembly of focal adhesions (43). A tyrosine kinase, $p120^{ACK}$, which binds to the activated GTP-bound form of Cdc42 has been isolated (36). The serine/threonine protein kinase $p65^{PAK}$ (PAK) binds activated Rac and Cdc42 and is itself activated by this binding, making this protein a likely downstream target of the p21s (37). PAK is strikingly similar to the *S. cerevisiae* protein Ste20 (30, 49), which is involved in the transmission of the pheromone signal from G protein βγ subunits to a mitogen-activated protein kinase cascade (reviewed in reference 23), a process that requires Cdc42 (58, 66). For mammalian cells, it has recently been shown that Rac and

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Cdc42 activate signaling by the mitogen-activated protein kinase family members c-Jun amino-terminal kinase (JNK) (10, 42, 45) and p38 (65). PAK proteins appear to be involved in the activation of the JNK (47) and p38 (65) cascades, although their precise roles in these pathways are unclear. Ste20 (47) and the Ste20 homologs *S. cerevisiae* Shk1 (47) and human germinal center kinase (48) have also been implicated in JNK activation.

We have isolated a gene encoding a *Drosophila* homolog of PAK. This homolog, DPAK, which can bind activated *Drosophila* Rac (DRacA) and Cdc42 (Dcdc42) proteins, has an embryonic distribution with similarities to the distributions of integrins, extracellular matrix (ECM) molecules, and phosphotyrosine. Integrins and phosphotyrosine are components of focal adhesions, while ECM molecules can function as ligands for integrins. High levels of phosphotyrosine are also found in Cdc42- or Rac-induced focal complexes (43). DPAK may therefore be a focal adhesion and focal complex protein. DPAK is elevated in the epidermal cells of the leading edge during dorsal closure of the epidermis over the amnioserosa. We have previously shown that expression of a dominant-negative DRacA transgene disrupts the formation of an actomyosin structure at the dorsal end of these cells (20). This actomyosin structure is believed to drive the cell shape changes of dorsal closure (63), and expression of dominant-negative DRacA inhibits these cell shape changes. The leading-edge cells during dorsal closure present a promising system for the study of the Rho subfamily of p21s in development. We have analyzed in detail the distributions of DPAK and other leading-edge components in these cells and present evidence that DPAK is involved in cytoskeletal regulation by association with focal adhesions and focal complexes. The elevated levels of DPAK at the leading edge during dorsal closure suggest that DPAK may be participating with DRacA in a JNK signaling pathway required for dorsal closure (15).

MATERIALS AND METHODS

Standard molecular biology procedures were done as described previously (55). Low-stringency library screening was performed as described previously (20).

DNA sequencing. cDNA restriction fragments were subcloned into Bluescript vectors (Stratagene) and sequenced on both strands by the dideoxy chain termination method with Sequenase (United States Biochemical). Sequences were completed by using specific 17-mer oligonucleotide primers.

In situ hybridization to polytene chromosomes. DNA for in situ hybridization was labeled with digoxigenin by using the Boehringer Mannheim nonradioactive DNA labeling kit. The probe was hybridized to wild-type (Canton S) polytene chromosomes as described previously (2) except that the acetylation step was omitted. Detection of signals was done by the glucose oxidase-diaminobenzidine-nickel method (24) with a peroxidase-conjugated antidigoxigenin antibody (Boehringer Mannheim).

Whole-mount in situ hybridization to embryos. Hybridization with digoxigenin-labeled DNA probes was performed as described previously (2). Stages of embryos were determined as described previously (7).

p21-binding analysis. A 0.5-kb fragment encoding N-terminal sequences of DPAK was generated by PCR with the oligonucleotides indicated in Fig. 1A and cloned into the pGEX-2T vector (Pharmacia) for expression as a glutathione *S*-transferase (GST) fusion protein. The overlay protocol used to assay the p21-binding properties of this fusion protein has been described previously (35). Briefly, GST fusion proteins of N-terminal sequences of DPAK and controls were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and blotted onto nitrocellulose. Filters were incubated with [γ-^{32}P]GTP-p21, and binding was detected by autoradiography. pGEX constructs of human Rac, Cdc42, RhoA, and Rab3A were described previously (8, 35).

Preparation of Dcdc42 and DRacA fusion proteins. *Dcdc42* was isolated in a PCR screen for Rho subfamily members in *D. melanogaster*. First-strand cDNA synthesis was performed with total RNA prepared from adult flies by using a Pharmacia kit. PCR was performed with the synthesis by using the degenerate oligonucleotides GA(T/C)GG(T/C/A)GC(N)GT(N)GG(T/C/A)AA(A/G)AC and (A/G)TC(T/C)TC(T/C)TG(A/G/T)CC(N)GC(N)GT(A/G)TC, corresponding to the conserved amino acid sequences DGAVGKT and DEQGATD, respectively. Thirty cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min were carried out. Appropriately sized PCR products were cloned into Bluescript vectors (Stratagene) and sequenced. A clone with strong homology to existing Cdc42 sequences was identified and was used to screen a 2- to 14-h embryonic cDNA library (Stratagene). The cDNA sequence obtained from the isolated clones was identical to the *Dcdc42* sequence previously reported (33). One of the clones isolated in the screen, which had a complete *Dcdc42* open reading frame, was subcloned into the pGEX-2T vector (Pharmacia) for expression as a GST fusion protein. A *DRacA* cDNA (20) was similarly expressed in pGEX-2T.

Antibodies. The N-terminal DPAK-GST fusion protein described above was used to immunize rabbits and mice. Antibodies were purified by the low-pH method (55). The anti-nonmuscle myosin antibody (28) was a gift from Dan Kiehart. A monoclonal antiphosphotyrosine antibody was obtained from Upstate Biotechnology Inc.

Western blot (immunoblot) analysis. Embryonic protein extracts were prepared by homogenization in SDS sample buffer (3% SDS, 100 mM Tris [pH 8.0], 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 1% Triton X-100, 10 mM $MgCl_2$, 100 mM NaCl, 50 μg of aprotonin per ml). Samples were boiled for 5 min, centrifuged to remove cell debris, and electrophoresed on an SDS–8% polyacrylamide gel. The gel was electroblotted onto a nitrocellulose membrane and incubated with the primary antibody and then with peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Signal detection was by chemiluminescence with the Amersham ECL Western reagents.

Immunocytochemistry. Antibody and phalloidin staining and confocal microscopy of embryos were as described previously (20). Embryos aged 10 to 14 h at 25°C were used to study dorsal closure, with the exception of the heat shock studies (see below). At least 50 embryos were examined for any circumstance described in this paper.

Heat shock induction of Rac transgenes. Transgenic Rac lines and the method of heat shock have been described previously (20). In the present study the dominant-negative Rac N17-104 and wild-type Rac WT-10 transgenic lines were used. Four-hour collections of embryos were aged at 25°C until they were 8 to 12 h old and heat shocked for 30 min at 37°C. Following the heat shock, the embryos were aged for 6 to 8 h at 21°C and then fixed for antibody staining. Since the embryos were already undergoing dorsal closure at the time of heat shock, all embryos showed a degree of epidermal cell elongation, regardless of the severity of effects on the leading edge. We found that this regimen produced high yields of embryos "captured" during dorsal closure and that the N17-104 embryos had frequent leading-edge defects.

Nucleotide sequence accession number. The nucleotide sequence presented in this report has been submitted to GenBank under accession number U49446.

RESULTS

Cloning of a *Drosophila* homolog of PAK. A *Drosophila* 2- to 14-h embryonic cDNA library (Stratagene) was screened under low-stringency hybridization conditions, and a single strongly hybridizing clone of 2 kb was isolated. DNA sequence analysis of this clone indicated that it encoded a putative protein with homology to PAK but was clearly truncated. The clone was used to probe a plasmid embryonic 4- to 8-h cDNA library (6), and further clones were isolated. The longest of these clones, containing an insert of 3.2 kb, was selected for DNA sequence analysis. The clone contained an open reading frame encoding a putative protein, DPAK, with high degree of homology to PAK. The open reading frame appeared to be complete on the basis of an alignment with PAK and the presence of stop codons in all three reading frames on either side of the open reading frame. The nucleotide and predicted amino acid sequences of the *DPAK* cDNA clone are given in Fig. 1A. Sequencing of the ends of other cDNAs identified a clone in which an alternate polyadenylation signal had been used. A polytene chromosome in situ hybridization performed with a *DPAK* cDNA localized the *DPAK* gene to cytological position 83E on chromosome 3R (data not shown).

The DPAK amino acid sequence was aligned against the sequences of homologous PAK and Ste20 proteins. In previous alignments performed with this family of proteins, two blocks of highly conserved sequences were identified (37, 40): the serine/threonine kinase domain and a stretch of amino acids identified as the p21-binding domain (37). The DPAK p21-binding domain was most homologous to that of the original rat PAK sequence (37) and to domains from *Caenorhabditis elegans* (GenBank accession number CELC098B) and human

A

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  AGTGGCAGTGACACTCGAAGGTGGGAGACACGCTGACGGAACAACTGAGCAGCGGGCCCACAGTCCGAATTAGTTCGCGGACGGCCTA 88
TATTGATAAATTTATAACTGCGGCTATATGTCTCCTCTCCTGCTGCAGCCAACCAGTGCGTGTGTGTGTGTCTGTTTTCTGGGCGTAG 178
AACAGCTGTGCATAACGGGCCGCTAGACGAAGCGGAGGAGGAGGAAGCAGGAGCTAACCAGAACAGCCAGAAGCACACAAAGACGAATCC 268
M  S  S  E  E  D  K  P  P  A  P  P  V  R  L  T  S  N  R  G  G  N  E  R  S  G  G  G  V  G   30
ATGTCCAGCGAGGAAGACAAACCGCCGGCGCCGCCTGTGCGTCTCACCAGCAACCGGGGCGGCAATGAACGATCGGGAGGAGGAGTGGGC 35
V  G  G  G  G  L  G  G  G  G  M  G  D  V  P  P  D  M  R  P  L  P  K  E  P  D  D  S  D  R  ·  60
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K  K  K  T  L  K  S  K  I  K  G  S  K  P  S  H  T  D  S  K  P  N  I  S  Y  P  T  N  F  E   90
AAGAAGAAAACGCTGAAGAGCAAGATCAAAGGATCGAAGCCCTCGCACACGGACTCCAAGCCGAACATCTCCTATCCCACGAACTTTGAG 538
H  T  V  H  V  G  F  D  A  V  T  G  E  F  T  G  M  P  E  A  W  A  R  L  L  M  N  S  N  I   120
CACACGGTCCATGTGGGATTCGATGCTGTCACCGGCGAGTTTACCGGCATGCCGGAGGCATGGGCGCGACTCCTGATGAACTCCAACATC 628
S  K  Q  E  Q  K  K  N  P  Q  A  V  L  D  V  L  K  W  F  D  N  T  T  K  Q  R  P  S  S  K   150
AGCAAGCAGGAGCAGAAGAAGAATCCCCAGGCCGTGCTGGATGTGCTCAAGTGGTTCGACAACACTACCAAACAGAGGCCAAGTTCCAAG 718
Y  M  T  N  A  I  T  T  H  S  G  S  S  L  S  R  V  S  S  S  S  P  S  S  T  P  T  D  S  E   180
TACATGACAAATGCCATTACGACGCATTCAGGTTCATCGCTCAGCCGCGTCAGCAGCAGCAGTCCGAGCAGCACGCCAACGGACTCGGAG 808
L  H  G  S  N  S  G  G  N  L  I  G  V  Q  L  G  S  M  T  L  G  P  N  A  N  N  V  A  V  A   210
CTGCACGGCTCCAACAGCGGAGGTAACCTGATTGGCGTCCAGTTGGGCAGTATGACTCTGGGTCCCAACGCCAACAACGTGGCCGTTGCT 898
G  Q  I  L  G  N  H  Y  Q  Q  Q  Q  Q  H  L  L  Q  Q  Q  Q  P  L  L  H  Q  N  H  N  Q  H   24
GGGCAAATTCTGGGCAACCACTACCAACAGCAGCAGCAGCACCTCCTTCAGCAGCAGCAGCCACTTTTGCATCAGAACCACAACCAGCAT 988
H  M  G  I  S  Q  S  H  S  Y  N  F  V  G  H  T  V  S  S  S  T  S  Q  H  S  S  A  N  E  D   270
CACATGGGCATAAGTCAATCGCACTCGTACAACTTTGTCGGACACACGGTCTCCTCATCCACATCGCAACATTCATCCGCTAACGAGGAC 1078
D  M  L  G  P  Q  H  P  Q  Q  Q  P  P  P  P  P  V  A  S  R  P  E  R  T  K  S  I  Y  T  R   300
GATATGCTGGGACCACAGCACCCGCAGCAACAGCCGCCACCTCCACCGGTGGCCTCTAGGCCTGAGCGCACGAAATCCATCTACACGCGG 1168
P  I  E  D  L  Q  P  A  I  I  P  M  P  V  A  P  A  T  T  P  A  T  P  L  Q  N  H  R  T  P   330
CCAATCGAGGATCTACAGCCAGCCATCATCCCAATGCCAGTGGCGCCAGCAACCACGCCTGCAACGCCGCTGCAAAATCATAGGACGCCA 1258
G  G  I  S  A  P  A  T  S  P  M  M  H  N  N  A  T  T  T  L  D  K  N  K  N  N  A  N  L  Y   360
GGCGGAATATCAGCACCAGCGACATCGCCAATGATGCACAACAATGCCACGACGACGCTGGACAAGAATAAAAACAATGCAAATCTGTAC 1348
T  P  E  P  T  V  A  Q  V  S  A  G  G  P  S  S  Q  V  A  G  N  Q  I  A  V  P  Q  A  A  V   390
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A  P  A  A  T  P  N  T  R  A  A  N  A  K  K  K  K  M  S  D  E  E  I  L  E  K  L  R  T  I   420
GCTCCGGCTGCAACGCCCAACACGAGGGCAGCCAACGCCAAGAAGAAAAAGATGTCCGACGAGGAGATCTTGGAGAAACTGCGCACCATT 1528
V  S  V  G  D  P  N  R  K  Y  T  K  M  E  K  I  G  Q  G  A  S  G  T  V  Y  T  A  I  E  S   450
GTCTCTGTGGGCGATCCAAATCGAAAGTACACCAAGATGGAGAAGATTGGCCAGGGTGCCTCTGGCACAGTGTATACTGCAATTGAATCC 1618
S  T  G  M  E  V  A  I  K  Q  M  N  L  S  Q  Q  P  K  K  E  L  I  I  N  E  I  L  V  M  R   480
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E  N  K  H  P  N  V  V  N  Y  L  D  S  Y  L  V  S  E  E  L  W  V  V  M  E  Y  L  P  G  G   510
GAGAACAAACACCCAAATGTAGTCAACTATCTTGACAGCTACCTCGTGTCTGAGGAACTGTGGGTGGTCATGGAGTACCTGCCCGGCGGC 1798
S  L  T  D  V  V  T  E  T  C  M  D  E  G  Q  I  A  A  V  C  R  E  V  L  Q  A  L  E  F  L   540
TCTCTTACCGACGTCGTCACCGAGACCTGCATGGACGAGGGCCAGATCGCAGCTGTTTGCCGAGAAGTGCTGCAAGCTCTGGAGTTCCTT 1888
H  A  N  Q  V  I  H  R  D  I  K  S  D  N  I  L  L  G  L  D  G  S  V  K  L  T  D  F  G  F   570
CACGCCAACCAGGTTATCCATCGCGACATCAAGAGTGATAACATTCTGCTGGGCCTTGACGGCTCTGTCAAGCTGACTGACTTTGGTTTC 1978
C  A  Q  I  S  P  E  Q  S  K  R  T  T  M  V  G  T  P  Y  W  M  A  P  E  V  V  T  R  K  Q   600
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Y  G  P  K  V  D  L  W  S  L  G  I  M  A  I  E  M  V  E  G  E  P  P  Y  L  N  E  N  P  L   630
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K  A  L  Y  L  I  A  T  N  G  K  P  E  I  K  E  K  D  K  L  S  S  A  F  Q  D  F  L  D  Q   660
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C  L  E  V  E  V  D  R  R  A  S  A  L  D  L  L  K  H  P  F  L  K  L  A  R  P  L  A  S  L   690
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T  P  L  I  M  A  A  K  E  A  T  K  G  N  *                                              704
ACTCCCTTGATCATGGCCGCAAAGGAGGCTACCAAGGGCAACTGATTGACAACCAAGAAGAAACCGCTTATTTAACCACTACTGATGAAT 2428
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TTTTTATATGATTTTAAAAAAAAAAA                                                              3175
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FIG. 1. Nucleotide and predicted amino acid sequences of *DPAK*. (A) Nucleotide sequence and conceptual translation of *DPAK* cDNA. The oligonucleotide sequences used for PCR cloning of the N-terminal region of the *DPAK* coding sequence into the pGEX-2T vector are underlined. Predicted polyadenylation signals are in boldface, as is the alternate position of the poly(A) tail. (B) Alignment of the predicted amino acid sequences of DPAK, PAK (37), and a *C. elegans* clone (CePAK) (GenBank accession number CELC098B). Matches to the consensus sequence have a black background. Dashes in the sequences indicate gaps introduced to maximize alignment. The predicted p21-binding domains are boxed with a dashed line; the kinase domains are boxed by a solid line.

B

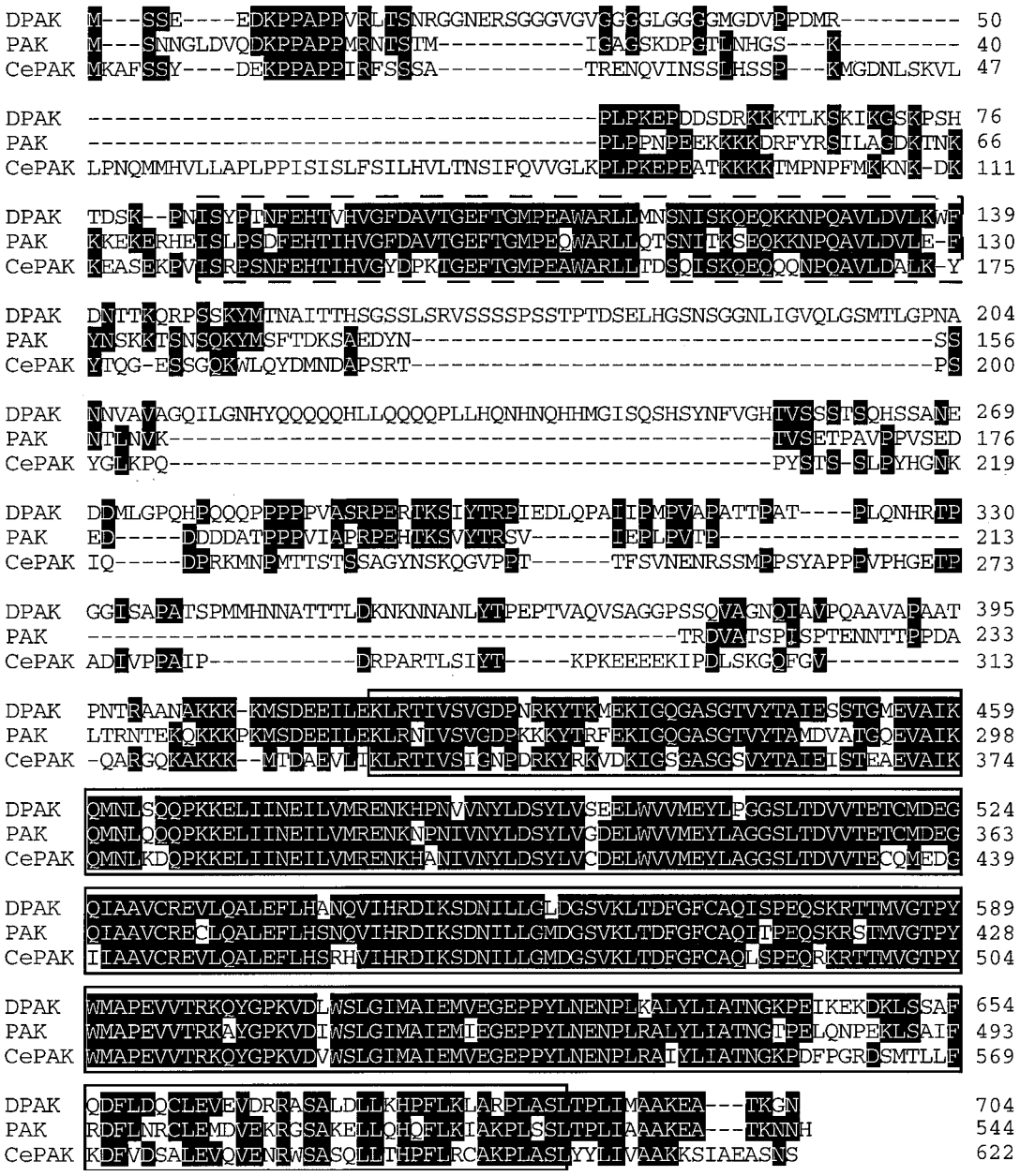

FIG. 1—*Continued.*

(40) PAK-Ste20 family members, with identities of 79, 74, and 79%, respectively. There was considerably less homology to the corresponding domains of Ste20 (30, 49) and two other *S. cerevisiae* proteins, Cla4 (11) and a putative protein we call ScPAK (Genbank accession no. Z48149), as well as to that of the Shk1 protein from *Schizosaccharomyces pombe* (39), with identities of 55, 50, 46, and 53%, respectively. Similarly, in a comparison of the kinase domains, DPAK was most like the rat, *C. elegans*, and human proteins, with identities of 82, 79, and 79%, respectively. Over the entire length of the protein, DPAK was most homologous to the rat and *C. elegans* proteins (Fig. 1B), with blocks of proline-rich sequences being conserved in addition to the p21-binding and kinase domains. The predicted DPAK protein (704 amino acids) is larger than PAK (544 amino acids). This is due to the presence of additional amino acids N terminal to the p21-binding domain and between the p21-binding and kinase domains. The lengths of these regions vary considerably among the PAK-Ste20 family members.

DPAK binds Rac and Cdc42 proteins. To investigate the p21-binding properties of DPAK, an N-terminal 0.5-kb fragment of the cDNA containing the entire coding sequence for the predicted p21-binding domain (Fig. 1A) was PCR amplified into the pGEX-2T vector and expressed as a GST fusion

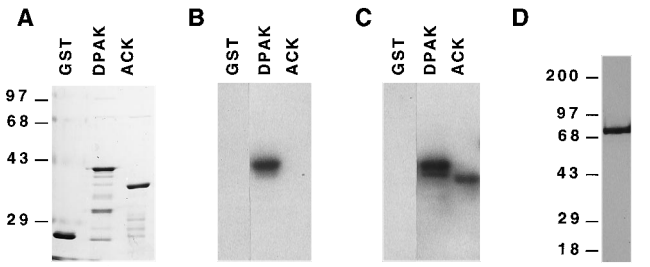

FIG. 2. p21-binding studies and detection of DPAK protein. Molecular masses of protein markers (in kilodaltons) are indicated. (A to C) Binding of the N-terminal region of DPAK to DRacA and Dcdc42. GST fusion proteins of the N-terminal region of DPAK (see text) and the p21-binding domain of $p120^{ACK}$ (36) were run along with GST on SDS-polyacrylamide gels and Western blotted. (A) Coomassie-blue stained gel of the GST fusion proteins and GST. (B) Western blot overlaid with [γ-^{32}P]GTP-labeled DRacA, showing binding by DPAK. (C) Western blot overlaid with [γ-^{32}P]GTP-labeled Dcdc42, showing binding by DPAK and $p120^{ACK}$. $p120^{ACK}$ had previously been shown to bind only Cdc42 of the Rho subfamily members (36). (D) An affinity purified anti-DPAK antibody detects a single band of about 75 kDa in extracts prepared from embryos.

protein. By using an overlay assay (35), the GST-DPAK fusion protein was assessed for its ability to bind the following [γ-^{32}P]GTP-bound p21s: *Drosophila* Rac (DRacA) and *Drosophila* Cdc42 (Dcdc42) (Fig. 2A to C) and human Rac1, Cdc42, RhoA, Ras, and Rab3A (data not shown). The GST-DPAK fusion protein showed binding only to the *Drosophila* and human Rac and Cdc42 proteins, suggesting that its p21-binding specificity is similar to that of the rat PAK protein.

***DPAK* RNA expression pattern.** Hybridization of a *DPAK* cDNA probe to a Northern (RNA) blot containing total RNA from various developmental stages revealed a diffuse band of about 4.3 kb, which was expressed throughout development (data not shown). This diffuse band may represent two transcripts of similar sizes, as suggested by the cDNA sequence data.

The distribution of *DPAK* RNA during embryonic development was assessed by whole-mount in situ hybridization with a *DPAK* cDNA probe. *DPAK* transcripts were detected throughout embryonic development, apparently ubiquitously. However, *DPAK* RNA levels were elevated in particular cells. The first obvious elevation was seen in stage 11 in the epidermal cells immediately flanking the amnioserosa. This elevation was particularly pronounced in stage 14 embryos around the time of dorsal closure initiation (Fig. 3A). Dorsal epidermal cells in the first row of cells adjacent to the amnioserosa showed an increased *DPAK* RNA level relative to the rest of the epidermis, with at least two cells flanking each segment border having particularly high levels. Expression was greater in the abdominal segments than in the thorax, but in later embryos increased RNA levels were seen in the thorax and in more ventrally located epidermal cells flanking the segment borders (data not shown). Following the completion of dorsal closure, an elevation in *DPAK* transcript levels was found in the dorsal vessel (Fig. 3B), in the central nervous system (CNS), and in the epidermal cells at three thoracic muscle attachment sites in the epidermis (Fig. 3C and D). At the end of embryogenesis, elevated *DPAK* transcript levels were seen in muscle attachment sites throughout the epidermis (Fig. 3E).

The DPAK protein expression pattern indicates that the kinase is a potential focal adhesion and focal complex protein. The GST-DPAK fusion protein that had been tested for p21 binding was used to inoculate rabbits and mice to produce polyclonal antisera. Affinity-purified rabbit antibodies against the GST-DPAK fusion protein detected a protein estimated to

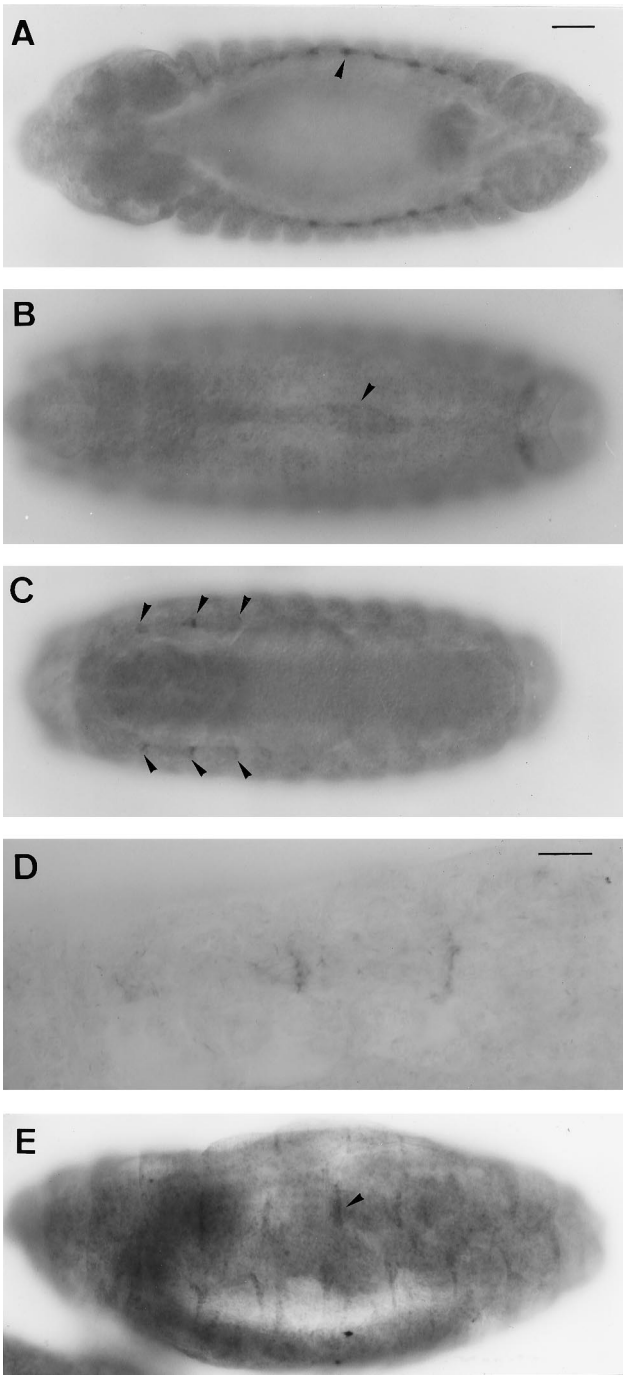

FIG. 3. Whole-mount RNA in situ hybridizations to wild-type (Canton S) embryos with a digoxigenin-labeled *DPAK* cDNA probe. Anterior is to the left. (A) Dorsal view of a stage 14 embryo, showing elevated *DPAK* RNA in epidermal cells flanking the amnioserosa. A particularly high signal is seen in a few cells flanking the segment borders in each abdominal segment (arrowhead). (B) Dorsal view of a stage 15 embryo following the completion of dorsal closure. There is elevated *DPAK* RNA in the dorsal vessel (arrowhead). (C) Ventral view of a stage 15 embryo, showing elevated *DPAK* RNA in the epidermal cells at three muscle attachment sites located ventrally in the thorax (arrowheads). (D) Higher magnification of thoracic muscle attachment sites. (E) Lateral view of a stage 17 embryo, showing elevated *DPAK* RNA in muscle attachment sites throughout the epidermis (arrowhead). Bars, 25 μm (A) and 10 μm (D).

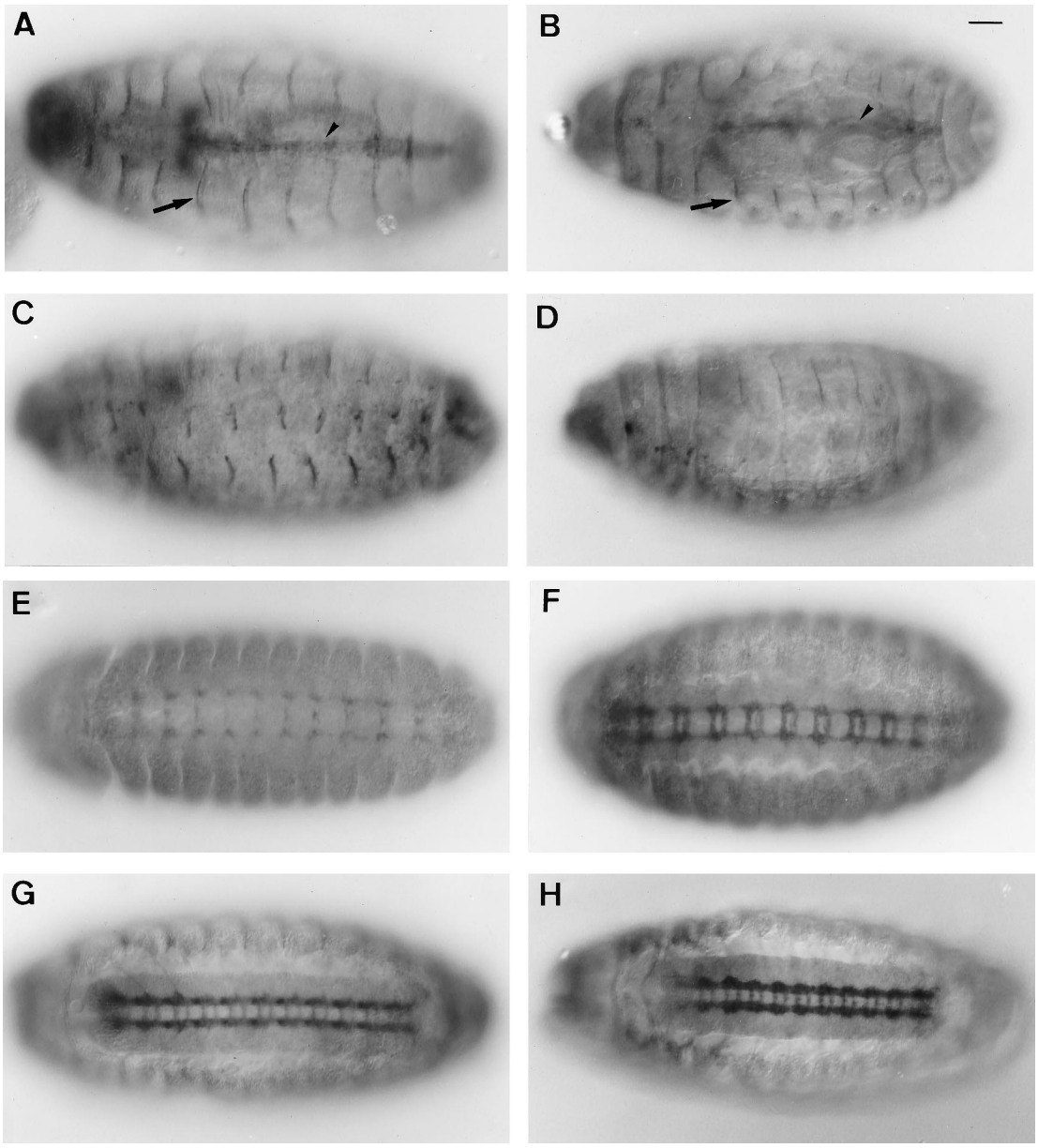

FIG. 4. Distributions of DPAK protein and phosphotyrosine during late embryogenesis in wild-type embryos. Anterior is to the left. Staining was with an anti-DPAK antibody (A, C, and E to G) or an antiphosphotyrosine antibody (B, D, and H). (A) Dorsal view of a stage 16 embryo, showing concentrations of DPAK in the dorsal vessel (arrowhead) and muscle attachment sites (arrow). (B) Dorsal view of a stage 17 embryo, showing that, as with DPAK, phosphotyrosine is heaviest in the dorsal vessel (arrowhead) and muscle attachment sites (arrow). (C) Lateral view of a stage 16 embryo, showing accumulation of DPAK in muscle attachment sites. Although only the major areas of somatic muscle attachment can be readily distinguished in this embryo, all somatic muscle attachment sites appear to stain with anti-DPAK antibodies. (D) Lateral view of a stage 17 embryo, showing phosphotyrosine in muscle attachment sites. (E to G) Progressively later embryos, showing emergence of DPAK staining in the CNS coincident with the development of the commissures and longitudinal connectives. (H) Phosphotyrosine staining in commissures and longitudinal connectives of a stage 16 embryo. Bar, 25 μm.

be 75 kDa by Western blot analysis, which is in good agreement with the predicted molecular mass of 76 kDa for DPAK (Fig. 2D). Rabbit and mouse anti-DPAK antibodies were used in whole-mount staining of embryos, with the two antisera giving identical results. As with *DPAK* RNA, the protein was found widely distributed throughout the embryo during embryogenesis, perhaps in every cell. There was heavy DPAK staining in the dorsal vessel (Fig. 4A) and muscle attachment sites (Fig. 4A and C), consistent with the RNA in situ expression pattern. Beginning at stage 12, there was staining of the commissures and connectives of the CNS, which was heaviest in the CNSs of late embryos (Fig. 4E to G).

The presence of DPAK in the muscle attachment sites is intriguing. These sites are points of adhesion between muscles and gut or epidermal cells, and they contain high levels of integrins (3, 31), which are heterodimeric transmembrane receptors for extracellular ligands. The extracellular ligands of integrins may be in the ECM or on other cell surfaces. A number of *Drosophila* ECM molecules localize to muscle attachment sites and are candidate ligands for integrins (13, 14).

One of these molecules, tiggrin, appears to be a ligand for $\alpha_{PS2}\beta_{PS}$ integrins and shows staining of the CNS similar to that of DPAK (14).

The similarities between the DPAK distribution and those of integrins and ECM molecules suggested that DPAK may associate with integrin molecules as part of a complex. In cultured vertebrate cells integrins are components of focal adhesions (reviewed in reference 32). Integrins in cultured *Drosophila* cells localize to structures reminiscent of focal adhesions (18, 61, 64), which are likely to exist in the intact fly. *Drosophila* homologs of the proteins found specifically in focal adhesions have not been isolated. The existing *Drosophila* integrin antibodies are unlikely to detect all focal adhesions, as different integrin isoforms often show some tissue specificity and new *Drosophila* integrins are still being discovered (16). A notable feature of nontransformed fibroblasts is that their focal adhesions have much higher levels of phosphotyrosine than other areas in the cell (34). An antiphosphotyrosine monoclonal antibody detected focal adhesions as specifically and effectively as antibodies against the focal adhesion proteins focal adhesion kinase (FAK) and talin (19). Focal adhesion-like focal complexes induced by Rac and Cdc42 are also rich in phosphotyrosine (43). We therefore stained *Drosophila* embryos with an antiphosphotyrosine monoclonal antibody in the belief that this would reveal focal adhesions and focal complexes.

As can be seen in Fig. 4 and 5, there were striking similarities in the distributions of phosphotyrosine and DPAK. As with DPAK, phosphotyrosine was elevated in the dorsal vessel and muscle attachment sites (Fig. 4B and D) and in the commissures and connectives of the CNS (Fig. 4H). The distributions of DPAK and phosphotyrosine in precellular embryos were not characterized in detail, but they appeared to colocalize with F-actin. In the syncytial blastoderm, DPAK and phosphotyrosine were associated with the F-actin caps which overlie the nuclei (62) (Fig. 5).

DPAK accumulates in the cells of the leading edge during dorsal closure, a process disrupted by dominant-negative Rac. As with *DPAK* RNA (Fig. 3A), DPAK protein levels were elevated in the first row of epidermal cells flanking the amnioserosa (Fig. 6A to D and G). During dorsal closure of the epidermis over the amnioserosa, large quantities of F-actin and nonmuscle myosin (referred to as myosin below) accumulate at the leading edge, which is composed of the dorsal edges of these cells. This accumulation of F-actin and myosin is thought to constitute a contractile apparatus producing a dorsoventral elongation of epidermal cells that propels the epidermis over the amnioserosa (63). Induction of a dominant-negative DRacA transgene during dorsal closure causes a loss of F-actin and myosin from the leading edge, accompanied by a loss of cell elongation in the epidermis and a failure of dorsal closure (20). The enrichment of DPAK along the leading edge provides further evidence that the Rho subfamily plays a major role in the cytoskeletal changes required for dorsal closure.

Early in dorsal closure, DPAK levels were elevated at the leading edge in one to six cells flanking each segment border (Fig. 6A and B); these cells are hereafter referred to as segment border cells. These DPAK-enriched cells often had a lopsided distribution with regard to the segment border; for example, the group of cells marked with the arrow in Fig. 6A has more members anterior to the segment border than posterior. This group also exhibits another notable feature of DPAK along the leading edge: apparent projections of staining beyond the leading edge out over the amnioserosa. Embryos at a later stage of dorsal closure showed a punctate accumulation of DPAK in a narrow band along the leading edge (Fig. 6C), and the elevation of DPAK in segment border cells was seen to extend to more ventrally located cells (Fig. 6D).

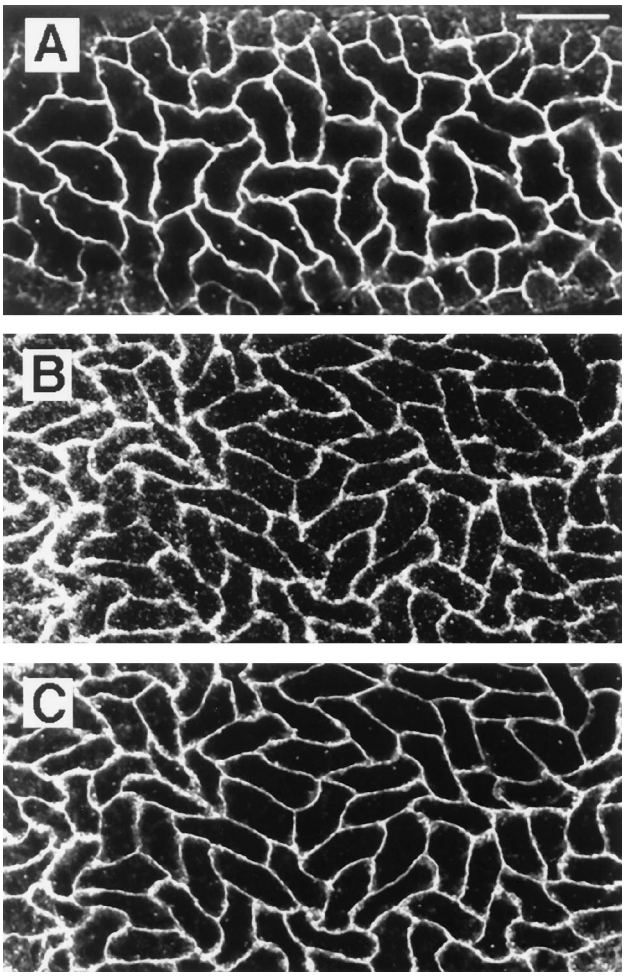

FIG. 5. Confocal fluorescence micrographs of the distributions of F-actin, DPAK, and phosphotyrosine in early wild-type embryos. Staining was with phalloidin (A), an anti-DPAK antibody (B), or an antiphosphotyrosine antibody (C). (A) Surface view of a syncytial blastoderm, showing extended F-actin caps (62). (B and C) Embryo similar to that in panel A, double stained to show association of both DPAK (B) and phosphotyrosine (C) with F-actin caps. Bar, 25 μm.

Phosphotyrosine also accumulated along the leading edge during dorsal closure and was found at the highest levels in triangular nodes located at the cell boundaries (Fig. 6E). The level of phosphotyrosine at the leading edge increased dramatically in embryos nearing the completion of dorsal closure (Fig. 6F). Phosphotyrosine staining was found along plasma membranes, with very little staining visible in the cytoplasm. Throughout embryonic development, phosphotyrosine stained in this manner, as would be expected for the staining of focal adhesions and focal complexes. The clarity with which cell outlines were distinguished by phosphotyrosine staining was comparable to that seen with antibodies against the membrane skeleton protein α-spectrin (46). A comparison of DPAK and phosphotyrosine stainings from double-stained embryos (Fig. 6G and H) indicated that DPAK was probably more widely distributed in the cell. DPAK appeared to be in the cytoplasm but was excluded from the nucleus. Thus, in addition to the DPAK proposed to be associated with focal adhesions and focal complexes at membranes, there may be a cytoplasmic pool of the protein.

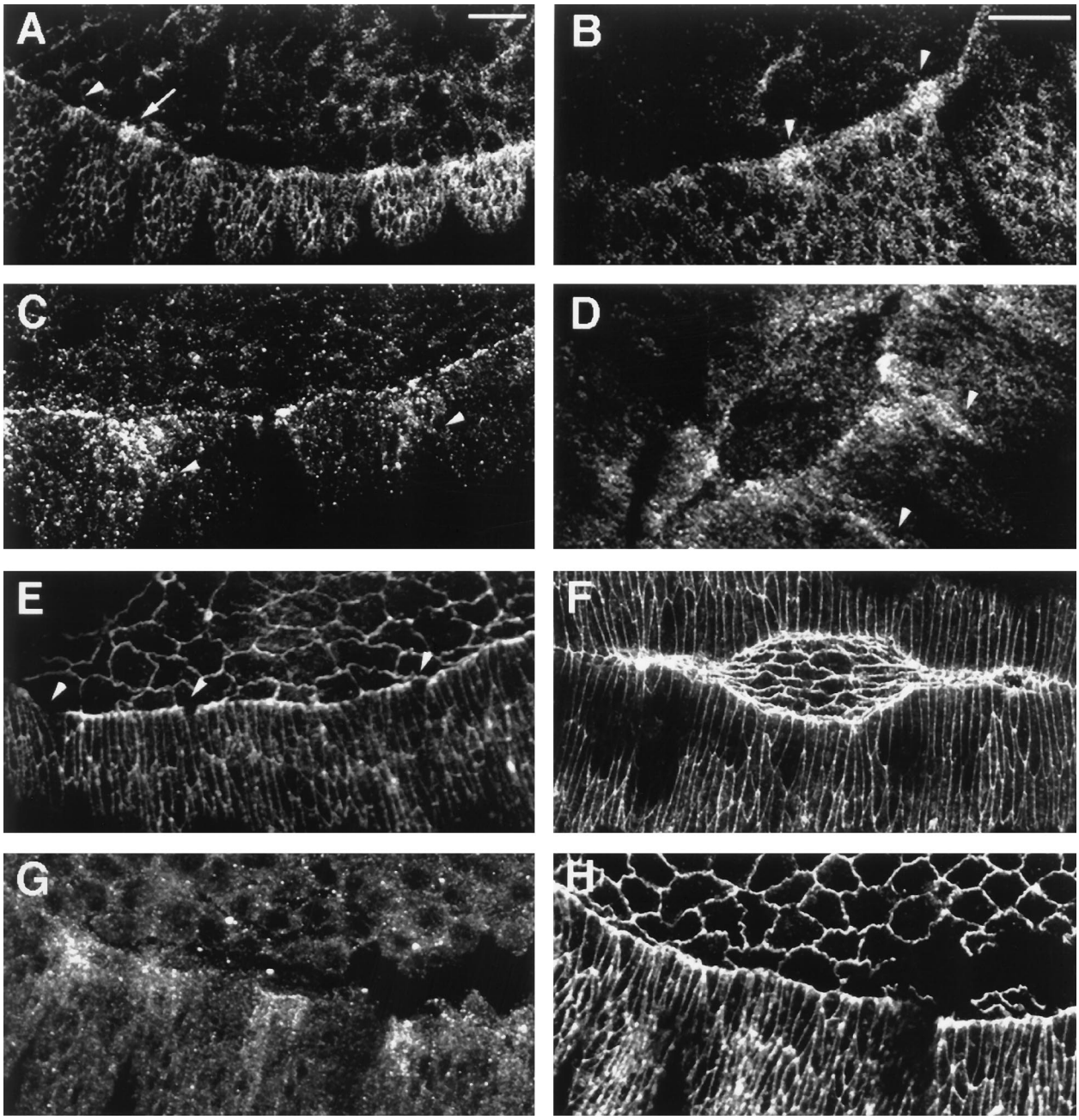

FIG. 6. Confocal fluorescence micrographs of the distributions of DPAK and phosphotyrosine at the leading edge during dorsal closure in wild-type embryos. Staining was with an anti-DPAK antibody (A to D and G) or an antiphosphotyrosine antibody (E, F, and H). The amnioserosa is at the top of each micrograph, with the exceptions of panels D and F, in which it is in the center. All images were taken with a 63× objective except the one in panel A, which was taken with a 45× objective. (A) Distribution of DPAK along the leading edge early in dorsal closure. DPAK accumulates at the dorsal ends of groups of cells flanking the segment borders (arrowhead and arrow). The group of cells marked by the arrow shows triangular projections of DPAK staining beyond the leading edge. (B) Embryo at a stage of closure similar to that in panel A, showing DPAK accumulation in single cells at segment borders (arrowheads). (C) Embryo later in closure than in panel A or B, showing a narrow band of punctate DPAK staining along the leading edge. Elevated DPAK is seen in cells flanking segment borders (arrowheads). (D) DPAK distribution in an embryo late in closure. The dark, elliptical patch in the center of the image is the amnioserosa. DPAK staining persists along the leading edge and extends down the sides of the embryo at the segment borders (arrowheads). (E) Distribution of phosphotyrosine during dorsal closure. Phosphotyrosine is elevated along the leading edge, occurring at the highest levels in triangular nodes found at the cell boundaries. Elsewhere in the epidermis and the amnioserosa, phosphotyrosine is found along cell peripheries. Arrowheads mark breaks in the leading-edge staining of phosphotyrosine at segment borders (see text). (F) Distribution of phosphotyrosine at the end of closure. There is intense phosphotyrosine staining along the leading edge. (G and H) Embryo double stained for DPAK (G) and phosphotyrosine (H) during dorsal closure. DPAK shows much greater cytoplasmic staining than phosphotyrosine. The dark patches in the epidermis and amnioserosa in both micrographs are due to damage to the embryo. Bars, 25 μm.

Cells expressing high levels of DPAK appear to experience transient losses of cytoskeletal elements at the leading edge. To understand better the role of the high levels of DPAK in segment border cells, we examined the leading edge of these cells throughout dorsal closure to see if they had any distinctive features. We noticed that embryos about midway through dorsal closure, in terms of the surface area of the exposed amnioserosa, nearly always had at least one and frequently had sev-

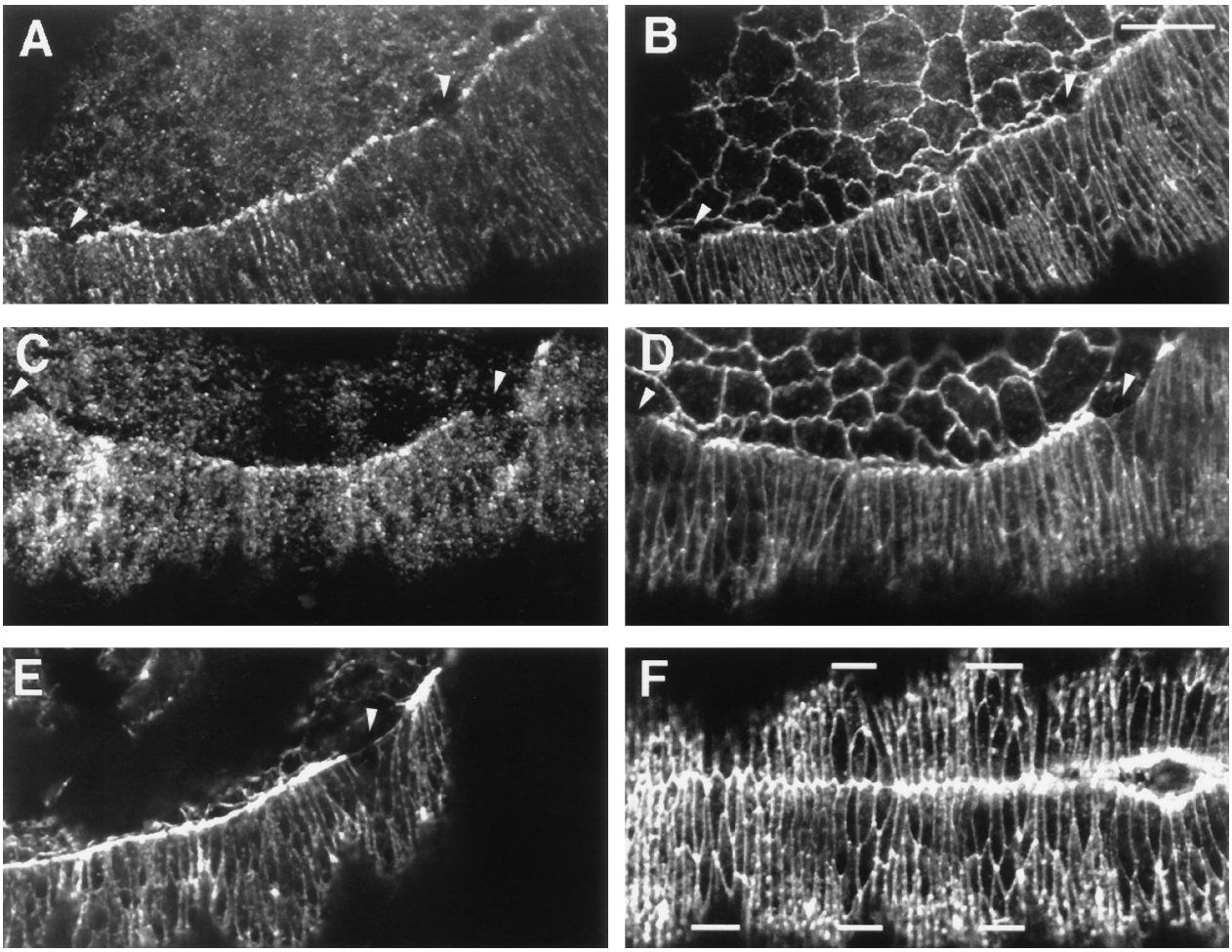

FIG. 7. Confocal fluorescence micrographs of gaps in leading-edge staining at the segment borders during dorsal closure in wild-type embryos. Staining was with an anti-nonmuscle myosin heavy-chain antibody (A), an antiphosphotyrosine antibody (B, D, and F), an anti-DPAK antibody (C), or phalloidin (E). All images were taken with a 63× objective. (A and B) Embryo double stained for myosin (A) and phosphotyrosine (B), showing concomitant loss of these leading-edge components from single cells at two segment borders (arrowheads). (C and D) Embryo double stained for DPAK (C) and phosphotyrosine (D), showing concomitant loss of these leading-edge components from two groups of cells flanking segment borders (arrowheads). (E) Three-cell-wide gap in F-actin accumulation (arrowhead). The leading edge can still be discerned in these cells. (F) At the end of closure, cells flanking the segment borders (bracketed by white bars) are wider along the anterior-posterior axis than their neighbors in the epidermis. Bar in panel B, 25 μm.

eral gaps in the leading-edge staining of actin, myosin, DPAK, and phosphotyrosine at the segment borders (Fig. 6E and 7A to E). These gaps appeared in cells which had had a high level of DPAK at an earlier stage. The gaps ranged from one (Fig. 6E and 7A and B) to several (Fig. 7C to E) cells wide and were not simply due to tears in the embryo at the segment borders, as the intact leading edge could usually be distinguished in the gap (Fig. 7E). Cells in the gap, which from double-staining studies appeared to simultaneously lack actin, myosin, DPAK, and phosphotyrosine at the leading edge, were usually splayed out and wider at their dorsal ends than their neighbors. At the end of dorsal closure, the segment border cells, occurring in a band about four cells across, were wider in the anterior-posterior axis than their neighbors (Fig. 7F).

Heat shock induction of dominant-negative Rac during dorsal closure causes the loss of DPAK and phosphotyrosine from the leading edge. We have previously shown that heat shock induction of dominant-negative DRacA causes the loss of actin and myosin from the leading edge (20). We wondered if the distributions of DPAK and phosphotyrosine at the leading edge would be similarly disrupted. Control heat shocks of wild-type (Canton S) embryos and embryos bearing a wild-type DRacA transgene under control of a heat shock promoter produced leading edges with normal distributions of DPAK, phosphotyrosine, actin, and myosin. Examples of the distributions of DPAK and phosphotyrosine following control heat shocks are given in Fig. 8A and B. Heat shocks performed on embryos bearing the dominant-negative DRacA transgene produced a high frequency (around 50%) of leading edges that exhibited a loss of the various components (Fig. 8C to F). Double staining for the leading-edge components in different combinations indicated that they were concomitantly lost from the leading edge in these embryos. Components remaining in patches at the leading edge occupied shared positions (Fig. 8C and D). When DPAK was lost from the leading edge, the elevated levels of DPAK in the segment border cells persisted (Fig. 8E). When dominant-negative DRacA was induced, there was often a striking loss of phosphotyrosine nodes from the leading edge, with phosphotyrosine staining elsewhere unaffected (Fig. 8F). The appearance of the leading edge following induction of dominant-negative DRacA was identical to

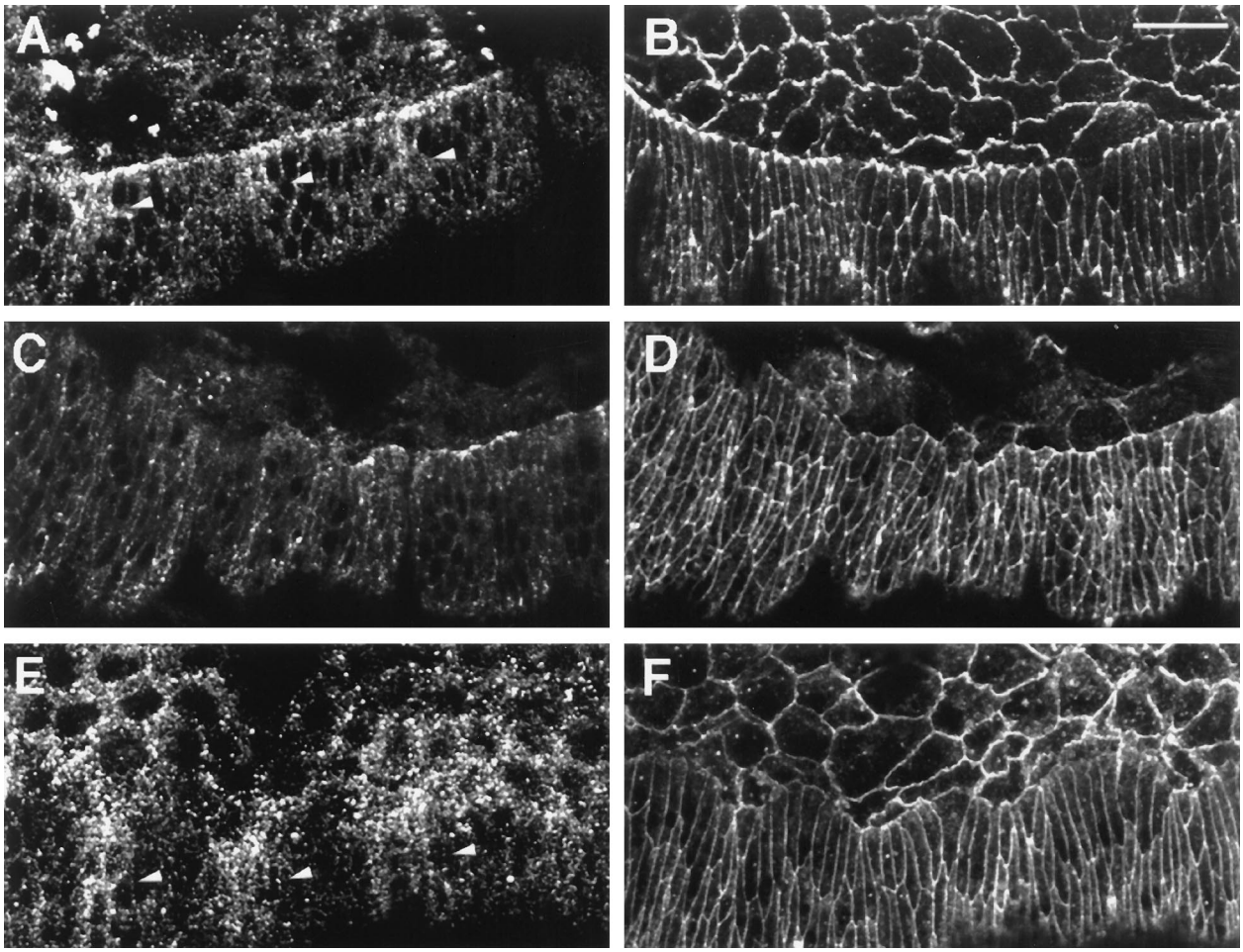

FIG. 8. Confocal fluorescence micrographs of the distributions of leading-edge components following heat shock. Staining was with an anti-DPAK antibody (A and E), an antiphosphotyrosine antibody (B, D, and F), or an anti-nonmuscle myosin heavy-chain antibody (C). All images were taken with a 63× objective. (A) Embryo after heat shock induction of the wild-type Rac transgene, showing normal accumulation of DPAK. Arrowheads show elevated DPAK in segment border cells. (B) Heat-shocked wild-type embryo, showing normal accumulation of phosphotyrosine along the leading edge. (C and D) Embryo in which there has been heat shock induction of a dominant-negative Rac transgene, double stained to show loss of myosin (C) and phosphotyrosine (D) from the leading edge. There are patches of myosin and phosphotyrosine remaining at the leading edge in shared locations. (E and F) Embryo in which there has been heat shock induction of a dominant-negative Rac transgene, double stained to show loss of DPAK (E) (arrowheads show persistence of elevated DPAK in segment border cells) and phosphotyrosine (F) from the leading edge. There is a dramatic absence of the phosphotyrosine nodes at the leading edge in panel F, whereas the phosphotyrosine distribution elsewhere in the view appears normal. Bar, 25 μm.

the gaps in leading-edge staining seen in segment border cells during normal development.

The cells of the leading edge exhibit PAM during dorsal closure, which may contain DPAK at their bases. During our examination of phalloidin-stained leading edges, we noticed that F-actin staining extended beyond the leading edge in peripheral actin microspikes (PAM) (29) (Fig. 9A and B). These PAM were usually narrow and needle-like, but some were wider at the base where they abutted the leading edge. Having seen cases in which DPAK staining also extended beyond the leading edge (Fig. 6A), we wondered if DPAK may be present in these PAM. Double-staining studies revealed that DPAK staining extended into the bases of those PAM which were particularly wide at the base (Fig. 9C and D). Distinguishing DPAK extensions beyond the leading edge was frequently difficult because of heavy DPAK staining in the amnioserosa cells. We could not detect staining in the narrower PAM, nor did we see DPAK staining extending the full length of a PAM. Myosin and phosphotyrosine could not be seen in the PAM. PAM were seen to persist following induction of dominant-negative DRacA (data not shown).

DISCUSSION

We have isolated a *Drosophila* homolog of PAK, a potential downstream effector protein for the Rho subfamily p21s Rac and Cdc42. As with PAK, DPAK binds to GTP-bound Rac and Cdc42. Sequence comparisons between DPAK and other members of the PAK-Ste20 family indicated that DPAK is most similar to the original rat PAK protein but is significantly larger. The regions containing extra amino acids in DPAK are rather repetitive. The members of the PAK-Ste20 family show considerable variation in these areas, which are on either side of the p21-binding domain, and it may be that these regions do not have essential functions and are less constrained evolutionarily than the p21-binding and serine/threonine kinase domains. Alternatively, they may encode species-specific functions. The small blocks of amino acids that are conserved in

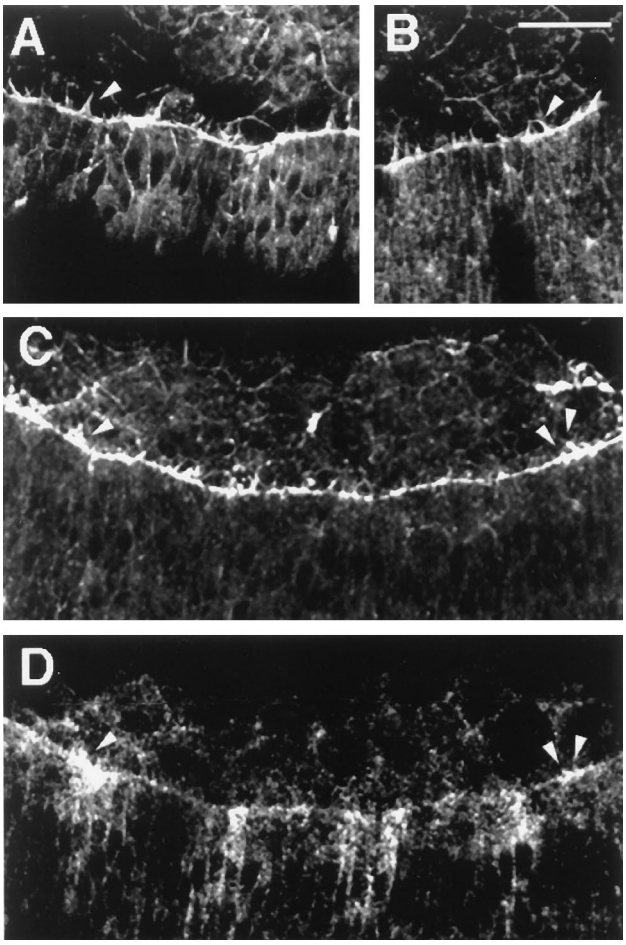

FIG. 9. Confocal fluorescence micrographs of PAM at the leading edge. Staining was with phalloidin (A to C) or an anti-DPAK antibody (D). All images were taken with a 63× objective. (A and B) F-actin at the leading edge extending over the amnioserosa in PAM (arrowheads). (C and D) Embryo double stained for F-actin and DPAK, showing the apparent presence of DPAK in the bases of some PAM (arrowheads). Bar, 25 μm.

these regions between some members of the PAK-Ste20 family, most notably DPAK, PAK, and the *C. elegans* PAK homolog, are proline rich and similar to the consensus sequences derived for motifs binding SH3 domains (17, 50, 59). Interestingly, SH3 domains have been seen in proteins interacting with p21s and in cytoskeletal proteins (reviewed in reference 41).

Studies on the distribution of DPAK during embryonic development suggested that the protein may regulate the organization of focal adhesions and focal complexes, and consequently the organization of the actin cytoskeleton, and may be participating in a JNK cascade required for dorsal closure.

DPAK is a potential focal adhesion and focal complex protein. There were similarities in DPAK distribution during embryogenesis to the staining patterns of integrins and their potential ligands, ECM molecules. Integrins are components of focal adhesions, where they form a link between focal adhesion proteins and extracellular ligands (for reviews, see references 5, 16, and 25). They have important roles in adhesion and in the flow of information into and out of the cell. The protein complexes that constitute focal adhesions are directly linked to the actin cytoskeleton (reviewed in reference 32). There are substantial quantities of tyrosine-phosphorylated proteins in focal adhesions, most notably paxillin, a major phosphotyrosine-containing protein during chicken and rat embryonic development (60). Paxillin phosphorylation is apparently dependent on FAK (57), which is induced by integrins to undergo autophosphorylation on tyrosine (reviewed in reference 56). Antibodies against phosphotyrosine can be used to detect focal adhesions (19, 34) and Cdc42- or Rac-induced focal complexes (43), and we took this approach to visualize focal adhesions and focal complexes in *Drosophila* embryos.

The overall distributions of phosphotyrosine and DPAK in embryos were strikingly similar, suggesting that DPAK associates with focal adhesions and focal complexes. This interpretation is strengthened by the finding that PAK colocalizes with focal adhesions when it is transfected into HeLa cells (38). The potential association of DPAK with focal complexes induced by Cdc42 and Rac is intriguing. These complexes have proteins in common with focal adhesions but are distinct structures (43). When activated Rac protein was injected into serum-starved fibroblasts, lamellipodia with punctate spots of vinculin, paxillin, FAK, and phosphotyrosine along their leading edges were formed. These spots represent focal complexes. Similarly, Cdc42 stimulates the formation of focal complexes along the plasma membrane and in filopodia.

Similar complexes appear to be involved in dorsal closure. There was an accumulation of phosphotyrosine along the leading edge during dorsal closure which was lost following induction of dominant-negative DRacA. Phosphotyrosine staining elsewhere in the embryo appeared to be unaffected. We propose that the phosphotyrosine accumulation along the leading edge represents the presence of Rac-dependent focal complexes. Phosphotyrosine staining elsewhere may mark focal adhesions. DPAK was present along the leading edge during dorsal closure and may be associating with Rac-dependent focal complexes. These complexes at the leading edge are associated with a Rac-dependent accumulation of actin (20), reminiscent of the accumulation of actin along the plasma membrane in lamellipodia. This situation at the leading edge is similar to the association of Rac-induced focal complexes with Rac-induced lamellipodia in cultured cells. Thus, dorsal closure may represent an in vivo, developmental example of the Rac-induced changes seen in cultured cells.

DPAK and dynamic actin structures. Focal adhesions and focal complexes may participate in the organization of F-actin. The broad-spectrum kinase inhibitor stausporine blocks the formation of Rho-induced focal adhesions and prevents the organization of Rho-induced actin filaments into typical stress fibers (43). The organization of the high levels of F-actin at the leading edge during dorsal closure may be dependent on Rac-dependent focal complexes in this region. The accumulation of F-actin at the leading edge is dynamic, persisting only for the duration of dorsal closure. Additionally, we have shown evidence that the leading-edge F-actin may depolymerize transiently in segment border cells during dorsal closure. DPAK may be interacting with the dynamic accumulation of F-actin at the leading edge through its presence in Rac-dependent focal complexes. The first indication during development that DPAK could associate with dynamic actin structures was the colocalization of DPAK with F-actin caps at the syncytial blastoderm stage. Although these embryos were precellular and presumably did not contain focal adhesions or focal complexes, the association of DPAK with F-actin caps may be mediated by focal adhesion and focal complex proteins, given that phosphotyrosine also colocalized with F-actin caps at this stage.

DPAK was elevated in segment border cells prior to the initiation of dorsal closure. These cells later appeared to experience transient, simultaneous losses of actin, myosin, phosphotyrosine, and DPAK from the leading edge. The accumu-

lation of DPAK in the segment border cells did not appear to be very precisely timed: at any given moment one segment could show elevated DPAK in five cells, while another showed elevated DPAK in only one cell. This roughness in timing was also seen for the loss of leading-edge components in segment border cells. No embryos in which losses had occurred simultaneously in every segment were seen, and the size of the leading-edge gap varied from one to several cells. Thus, the timing of the down regulation of the leading-edge cytoskeleton may not be critical; it may suffice that the down regulation occurs at every segment at some point during closure. The result of the transient loss of the leading-edge actomyosin structure appears to be that at the end of closure epidermal cells flanking the segment boundaries, in a band about four cells wide, are wider than their neighbors (Fig. 6F). The purpose for these cells being wider is unknown; this may be necessary for correct closure to occur or may be required for their identity.

DPAK may be involved in down regulating the F-actin accumulation at the leading edge of segment border cells by participating in the disassembly and turnover of focal adhesions and focal complexes. Interestingly, it has recently been shown that FAK is probably involved in focal adhesion turnover in migrating cells (26). The turnover of focal adhesions and focal complexes is likely to be essential in maintaining the actin cytoskeleton in a dynamic condition. The rate of focal adhesion and focal complex turnover could be determined by the relative amounts of DPAK and other proteins involved in assembly and disassembly of focal adhesions and focal complexes. Excessive levels of DPAK would then cause excessive disassembly of focal adhesions and focal complexes, as seen in the segment border cells. The heat shock induction of dominant-negative DRacA caused a phenotype identical to that seen transiently in the segment border cells during normal development: a loss of actin, myosin, phosphotyrosine, and DPAK from the leading edge. This heat shock effect is probably due to an inhibition of Rac signaling (20), which would lead to a loss of Rac-dependent focal complexes. The identical phenotypes may both be a result of the loss of Rac-dependent focal complexes at the leading edge.

We found that F-actin staining extended beyond the leading edge during dorsal closure in PAM. These PAM may represent filopodium-like extensions of leading edge cells and/or retraction fibers (29). In order to distinguish these two possibilities, the PAM would have to be examined through time-lapse microscopy of live embryos. The possibility that at least some of these PAM are filopodia has implications for both the model of dorsal closure and the signaling pathways present in the leading-edge cells. Various roles have been proposed for filopodia in growth cone motility. Filopodia may contribute to the forward movement of growth cones through the exertion of tension (22). They are also used for growth cone navigation in response to environmental signals (9, 44). Thus, the process of dorsal closure may be more complex than was previously believed and may involve a variety of cell movement strategies. The extension of filopodium-like structures from leading-edge cells could involve Dcdc42. DPAK can be detected in the bases of some PAM, where it may be associating with focal complexes, which occur in Cdc42-induced filopodia (43). We have not been able to detect phosphotyrosine in the PAM extending from the leading edge, but this could be due to the small size of these structures. If there are focal complexes in the PAM, DPAK could drive their turnover and maintain the actin cytoskeletons of the PAM in a dynamic state.

DRacA and DPAK may participate in a JNK signaling pathway required for dorsal closure. It has recently been shown that the *hemipterous* (*hep*) locus, loss-of-function mutations of which cause a dorsal closure defect, encodes a protein homologous to JNK (15). This result strongly suggests that a JNK signaling pathway is involved in dorsal closure. The HEP protein is required for the expression of the *puckered* gene product in the cells of the leading edge. *puckered* is also needed for normal dorsal closure to occur (54). HEP may have a specific function in the cells of the leading edge, although it is uniformly expressed in embryos. Given that Rac (10, 42, 45) and PAK (47) may be involved in the activation of JNK, it is conceivable that DRacA and DPAK participate along with HEP in a JNK pathway at the leading edge. This pathway could specifically operate at the leading edge as a result of the enhanced DPAK expression in this region and may be required to confer the identity of the leading-edge cells.

Dorsal closure and DPAK as tools for the further study of the Rho subfamily in development. It will be of interest to determine if Dcdc42 participates in dorsal closure and if the hierarchy of Rho subfamily activity is present in the leading-edge cells. Such findings would make dorsal closure an even more attractive process for the study of the Rho subfamily in development, particularly when coupled with a further examination of the role of DPAK at the leading edge. It should be possible to characterize DPAK function through both mutagenesis screens and transgenic approaches. We have proposed that excessive levels of DPAK in segment border cells lead to the transient loss of leading-edge components. This idea could be tested by overexpressing DPAK in other leading-edge cells, either by heat shock or by using the GAL4 system (4). It will be vital to test for interactions between DPAK, p21s, and HEP. We have concentrated on considering the role of DPAK during dorsal closure, but it will also be of interest to consider its role in the other developmental processes involving the Rho subfamily. The study of the Rho subfamily and its effector proteins can be furthered through a combination of genetic and molecular analyses applied in the context of these processes.

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