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Shark, a Src homology 2, ankyrin repeat, tyrosine kinase, is expressed on the apical surfaces of ectodermal epithelia

(*Drosophila*/signal transduction/invagination)

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ABSTRACT **Tyrosine kinases, ankyrin repeats, and Src homology 2 domains play central roles in developmental processes. The cloning of a cDNA for Shark, a single protein that possesses all three domains, is described. During *Drosophila* embryogenesis, Shark is expressed exclusively by ectodermally derived epithelia and is localized preferentially to the apical surface of these cells. This apical localization persists, even as tissues undergo complex invaginations, moving from the external surface of embryos to form internal structures, but expression is lost when cells lose their polarity. This pattern closely mimics the expression of Crumbs, a protein necessary for proper organization of ectodermal epithelia. Shark's structure and localization pattern suggest that it functions in a signaling pathway for epithelial cell polarity, possibly transducing the Crumbs signal.**

During gastrulation, fruit flies, like nearly all metazoans, generate three germ layers that produce all somatic structures. The outermost layer, the ectoderm, initially exists as a single epithelium of cells. However, as embryogenesis proceeds, this single layer gives rise to all the ectodermally derived tissues of the larva, including the nervous system, the fore- and hindgut, the tracheal system, the outer epidermis, and most head structures (1). The formation of these morphologically complex tissues from a single layer of ectoderm occurs by one of two different processes: delamination or invagination. Most neural tissue forms when ectodermal cells individually delaminate from the epithelium. Upon delamination, these cells continue to differentiate and never again regain their epithelial nature. Conversely, nonneural ectodermal tissues form from invaginating sheets of epithelia, and these tissues retain their epithelial character throughout their development (1, 2). While much work has been done to define the mechanisms by which ectodermal cells "choose" between neural and nonneural fates, little is known about the processes that maintain the epithelial polarity and organization of nonneuronal cells or how this relates to decisions of cell fate.

Work by Knust, Hartenstein, and their coworkers (2–5) suggests that the epithelial organization of nonneural ectoderm is not a default state but requires an active signaling process. They have identified a gene, crumbs, whose product is essential for the proper polarity and organization of ectodermally derived epithelia (3). crumbs encodes a transmembrane protein with a large extracellular domain containing 30 epidermal growth factor-like domains and five G domains of laminin A. Additionally, they have shown that ectodermal tissues that maintain their epithelial organization during development express the Crumbs protein on their apical surface (4). In the absence of Crumbs, nonneuronal ectodermal structures lose their epithelial organization, and in mosaic mutant flies, wild-type Crumbs acts nonautonomously and can induce neighboring Crumbs-deficient cells to maintain their polar nature (3). Given its structure, expression pattern, and the manner in which it acts, Knust and coworkers (4) have proposed that Crumbs serves as an autoregulating signal that maintains the epithelial polarity and organization of ectodermal cells. If Crumbs is such a signal, then there must exist cellular machinery to process this signal. Recent work (5) suggests that several cuticular defective, embryonic lethal mutations isolated in the early 1980s by Wieschaus, Jürgens, and Nüsslein-Volhard might indeed encode some genes for this putative signaling machinery.

In many developmental programs, tyrosine kinases are essential components of the signaling machinery. Among the better studied processes, they are critical for the regulation of eye development in *Drosophila* (6), growth factor-induced proliferation in mammals (7), and vulval development in *Caenorhabditis elegans* (8). Given their central role in so many developmental processes, we began a search for tyrosine kinases in *Drosophila*, hoping to identify one or several kinases that would lend themselves to molecular, genetic, and biochemical analysis and regulate a process common to higher eukaryotes. Among those identified is one that we report here that exhibits an expression pattern consistent with its involvement in a signaling pathway for epithelial cell polarity.

MATERIALS AND METHODS

PCR Amplification and the Cloning of the shark Gene. Genomic DNA extracted from the Oregon R strain of *Drosophila melanogaster* was amplified using several sets of primers. Among the primers used were two corresponding to two well-conserved domains of tyrosine kinases, DVWS(F/Y)G-(GTTGAATTCC(A/G)(A/T)A(A/T/C/G)GACCA(A/T/C/G)AC(G/A)TC) and HRDLAA-(TGTGAAGCTTCA(C/T)CG(A/T/C/G)GA(C/T)TT(A/G)GC(A/T/C/G)GC). Reaction parameters were 10 min at 95°C followed by 3 cycles of 94°C for 1 min, 50°C for 2 min with a 2-min ramp to 76°C, and 76°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 76°C for 2.5 min plus 2 sec per cycle. One hundred-microliter reactions were carried out in a standard buffer (50 mM KCl/10 mM Tris Cl, pH 8.4/0.01% gelatin/1.5 mM $MgCl_2$/0.25 mM of each dNTP) in the presence of 0.1 μg of genomic DNA, 0.25 μM of each primer, and 2.5 units of *Taq* DNA polymerase. The *Eco*RI- and *Hin*dIII-digested reaction products were subcloned into pBluescript II SK vector. Six genomes worth of an EMBL3 Canton S genomic library (kindly provided by R. Karess, New York University School of Medicine) were screened with a randomly primed ^{32}P-labeled probe corresponding to TK17. Several positive clones were purified, and one was sequenced to confirm that it contained the TK17 sequence (9).

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Abbreviations: BSA, bovine serum albumin; ANK repeat, ankyrin repeat; SH2, Src homology 2.

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Developmental Expression of shark mRNA. Five micrograms of poly(A)$^+$ RNA was isolated from embryo, larvae, pupae, imaginal disks, and adults using guanidinium and oligo(dT)-cellulose columns, and a Northern blot was performed using a genomic clone containing the kinase domain of Shark (9). This probe also detected a second unrelated transcript which is expressed uniformly in all fly tissues and stages examined (unpublished results) and provided an excellent control for RNA loading.

Shark Immunostaining. Three peptides corresponding to regions of the kinase domain of Shark were synthesized (QAKISDFGMSRSLRPGSTEYC, SNKQEFLREASVMMRLEC, and RKSGAGEESRLEVAIKMLSC), purified, and individually coupled to keyhole limpet hemocyanin. The coupled peptides were combined and injected into rabbits (Pocono Rabbit Farm, Canadensis, PA). Antibodies were then immunoaffinity purified from the antisera using columns of all three peptides coupled to Sepharose beads (10). Embryos were collected at staged intervals and dechorionated in 50% bleach for 2 min. The dechorionated embryos were washed (0.8% NaCl/0.02% Triton X-100) and incubated for 20 min in a fixative solution (4% paraformaldehyde/50 mM Pipes, pH 6.9/1 mM $MgSO_4$/1 mM EGTA/20% heptane). The fixative was extracted with methanol, and the embryos were rehydrated in 1 ml of phosphate-buffered saline (PBS)/methanol (1:1, vol/vol). The rehydrated embryos were blocked by washing three times with gentle rocking for 0.5 hr in PBS/1% bovine serum albumin (BSA)/0.1% Triton X-100. Blocked embryos were incubated overnight with the primary antibody, diluted 1:50 in PBS/BSA, at 4°C with gentle rocking. The embryos were sequentially washed four times with rocking for 30 min in PBS/BSA at room temperature, incubated at room temperature for 4 hr with rocking in the presence of a biotinylated secondary anti-rabbit IgG antibody (Vector Laboratories), and washed three times with rocking in PBS/BSA for 30 min at room temperature. Staining was carried out using Vectastain kit (Vector Laboratories) according to the manufacturer's instructions. Stained embryos were either temporarily mounted with 80% glycerol or dehydrated and permanently mounted with D.P.X. (Aldrich).

RESULTS AND DISCUSSION

Cloning and Developmental Expression of the shark Gene. Using a simple PCR strategy and primers to well-conserved stretches of amino acids in tyrosine kinases, we amplified *Drosophila* genomic DNA. After subcloning many of these amplification products, several were shown to encode tyrosine kinases. Among them was a clone that we named TK17, which corresponds to a gene that we subsequently designated as shark (see below). We used TK17 to isolate a genomic clone that contained the coding region of the entire kinase domain. Sequencing of this region revealed that TK17 possesses all the hallmarks of a tyrosine kinase. Using a portion of the genomic clone corresponding to the kinase domain as a probe, Northern blots revealed a 3.3-kb mRNA, most strongly expressed by 0- to 12-hr embryos and imaginal disks (Fig. 1).

Isolation of shark cDNA. By screening two embryonic libraries, we purified 13 clones whose inserts were all 3–3.2 kb in size, differing only in the completeness of the 5′ ends (9). The sequence of the longest clone predicts that TK17 is a non-receptor tyrosine kinase with several identifiable subdomains and a molecular mass of 106 kDa (Fig. 2*A*). At its amino-terminal end and similar to ZAP-70 (11), SYK (12), and the recently described HTK16 (13), TK17 possesses two phosphotyrosyl binding Src homology 2 (SH2) domains. The ability of SH2 domains to distinguish among different phosphotyrosyl residues and bind specifically only to some is largely determined by a small number of key amino acids in the SH2 domains. In this way, SH2 domains have been classified based

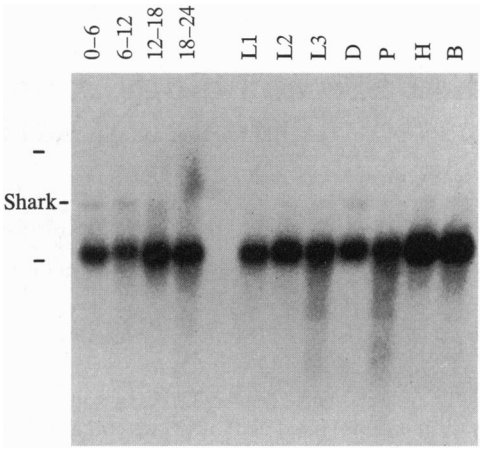

FIG. 1. Expression of the shark gene. Poly(A)$^+$ RNA was extracted from flies at various developmental stages. A Northern blot was prepared and probed with a genomic probe corresponding to the kinase domain. Lanes 1–4, embryos collected in 6-hr intervals; lanes L1–L3, larval stages; lane D, imaginal disks; lane P, pupae; lane H, adult head; lane B, adult body. shark is expressed as a single 3.2-kb message detectable in early embryos (0–12 hr), in larval imaginal disks, adult head, and adult body. The more rapidly migrating band is the 2.2-kb message used as a control for RNA loading. The two bars in the left margin indicate the positions of mouse 18S and 28S rRNA markers.

on the sequence of residues central to their binding specificity (14). By such a classification, both of TK17's SH2 domains belong in the Abl or 1B family, a family that also includes the SH2 domains of GAP, Nck, SYK, and ZAP-70 (Fig. 2*B*).

However, the most striking structural feature of this protein is a series of five repeats sandwiched between the two SH2 domains (Fig. 2*C*). These 33-amino acid repeats exist in about 20 other known proteins, including two *Drosophila* proteins Notch (15) and Cactus (16, 17). They have been given different names but are here called ankyrin repeats (ANK repeats). Unfortunately, by comparison to SH2 domains, much less is known about the structure and function of ANK repeats. However, in the few well-studied proteins—namely, Cactus (16), GABPβ (18), NF-κB/I-κB (19), and ankyrin (20)—ANK repeats mediate regulated protein–protein interactions. Mutant forms of some ANK repeat proteins have been implicated in oncogenesis in mammals (21, 22) and gross cell fate defects in *Xenopus* (23). Most recently, it has been shown that the ANK repeats of the Notch/LIN-12 family proteins have important signaling functions in fly (24) and nematode differentiation (25). Additionally, the TK17 kinase contains a stretch of 54 amino acids rich in proline and basic amino acids (lysine, arginine, and histidine) (Fig. 2*A*). While this region shows no significant homology to any known protein, its proline-rich, charged character is reminiscent of regions that bind SH3 domains (26).

Interestingly, the tyrosine kinase domain of TK17 is most similar to those of other tyrosine kinases that possess two SH2 domains, HTK16, ZAP-70, and SYK (Fig. 2*D*). The tyrosine kinase domains of TK17 and HTK16 share more than 52% of their amino acids and define a new family of tyrosine kinases. Because the most prominent structural features of the predicted TK17 protein are its SH2 domains, Ankyrin Repeats, and its Kinase domain, it was named Shark.

Expression of the Shark Protein. As a first step in the characterization of Shark, we generated antisera to three Shark peptides. Using affinity-purified antibodies, we delineated the expression pattern of Shark in whole-mount embryos. In all embryonic stages, there is no detectable staining with either preimmune serum or anti-Shark antibodies that have been preincubated with peptide antigens (Fig. 3*A*). From fertiliza-

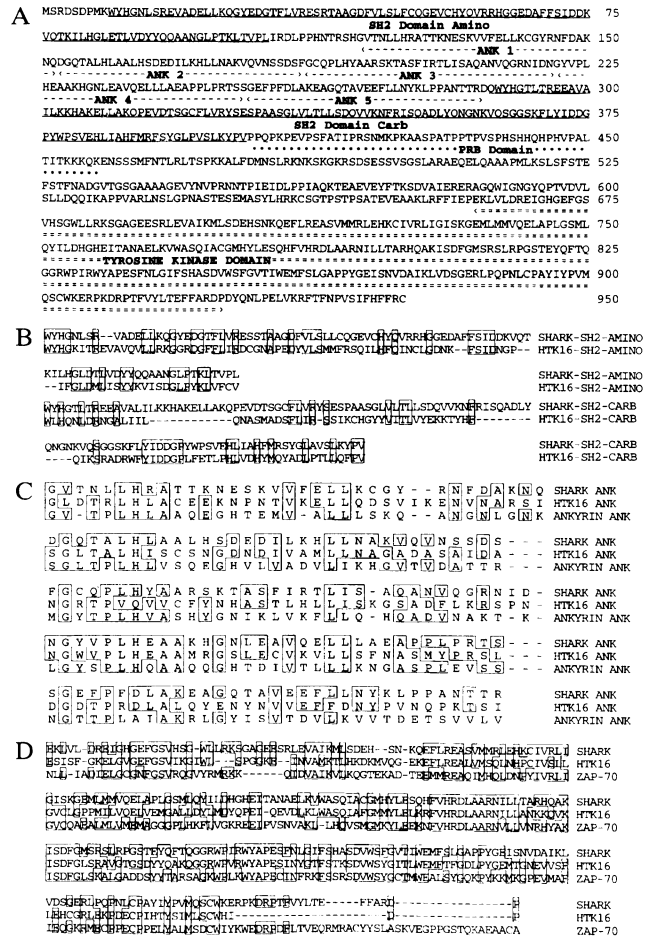

FIG. 2. Structure of the Shark protein. (*A*) The 3207-bp sequence of the longest cDNA clone predicts a protein of 910 amino acids with several identifiable structural motifs including two SH2 domains (SH2 domain amino and SH2 domain carb), five ANK repeats (ANK 1–5), a proline rich-basic (PRB) domain, and a tyrosine kinase domain. (*B*) The SH2 domains of Shark are most similar to each other and the SH2 domains of the hydra protein HTK16. The amino and carboxyl SH2 domains of Shark are aligned with their counterparts from HTK16. (*C*) The ANK repeats of Shark are shown aligned with each other and those of HTK16 and the five repeats from the mouse ankyrin protein. (*D*) Alignment of the Shark tyrosine kinase domain sequence with the sequences of similar tyrosine kinases. The tyrosine kinase domains of Shark and HTK16 share more than 52% of their amino acids in common, whereas the next most closely related kinases are the human ZAP-70 and SYK proteins, which are, respectively, 42% and 38% identical to Shark. Amino acid residues are indicated by the single-letter code.

tion to cellularization and before gastrulation, there is no detectable staining of embryos with anti-Shark antibodies (Fig. 3*B*). However, by the end of gastrulation the outer layer of cells, those destined to form the ectoderm, begin to express Shark at a low but detectable level (Fig. 3*C*). At this time, the most prominently staining structure is the cephalic furrow, which ultimately gives rise to many ectodermal head structures. As is clearly seen among the cephalic furrow cells, Shark expression is limited to the apical surfaces of the epithelia (Fig. 3*E*).

At the time embryos complete gastrulation, pairs of cells along the ventral midline begin to express Shark. The staining of these cells continues throughout germ-band extension and retraction. Prior to gastrulation, these cells are far apart on the ventral surface of the embryo, but following gastrulation and the invagination of the mesodermal and endodermal primordia, these two lines of cells come together. Thus, these cells, staining intensely with Shark antiserum, demarcate the invagination of nonectodermal cells (Fig. 3*C*).

Like most higher metazoans, *Drosophila* embryos develop an alimentary canal that fundamentally is a tube running the length of the fly. After gastrulation the endoderm is located exclusively within the embryo and organizes itself into an elongated cavity. Because the endoderm is entirely within the embryo, it does not communicate with the outside of the organism in the early stages of its development. To complete this communication and create the alimentary tube, ectodermally derived cells must invaginate from both ends of the embryo. In later stages, these invaginations, the stomodeum rostrally and the proctodeum caudally, fuse with the endodermal cavity giving rise to the hind- and foregut of the first-instar larva (27).

The proctodeum begins to form during germ-band extension at the same time that the pole cells are carried into the embryo (27). As the proctodeum first becomes discernible, its epithelial cells express Shark apically (Fig. 3*D*). Strikingly, as the cells on the anterior surface of the embryo begin the analogous process rostrally, they invaginate to form the stomodeum, and they too express Shark at high levels (Fig. 3*D*). With progressing development of the fore- and hindgut, these ectodermal structures continue to express Shark. A notable exception is the failure of ectodermally derived epithelial Malpighian tubules to express Shark (Fig. 3 *F* and *G*).

At this time, other cells of the ectoderm are simultaneously invaginating, including the tracheal placodes. Such invaginations first appear at stage ten and give rise to the mature respiratory system of a larva (28). They begin as small "pits" in the outer surface of the embryo (Fig. 3 *H* and *I*). As the embryo develops, the pits branch and form a fine treelike network within the embryo. Similar to the ectodermal gut and epidermis, the tracheal system expresses Shark luminally throughout its development (Fig. 3*J*).

Another ectodermally derived tissue, the stomatogastric nervous system, is unusual among neural tissues. Unlike most neural tissue, the stomatogastric neurons derive from epithelial invaginations in the roof of the stomodeum. These neurons only maintain their epithelial character during their early development, and by late embryogenesis they have delaminated and reorganized into neural tissue with no epithelial surface (2). However, during the time that they maintain their epithelial character, stomatogastric cells express Shark (Fig. 3*K*). In a pattern that mimics Crumbs expression precisely, these cells cease expressing Shark as they begin to delaminate (4).

Among the organs and tissues of the head, many derive from invaginations and buds that begin to form after gastrulation. There are three gnathal buds that form on each side of the embryonic head, the labial, mandibular, and maxillary. Together with the clypeolabrum, a protuberance that forms at the most anterior portion of the developing head, and the stomodeum, the gnathal buds give rise to the most rostral structures of the foregut, including the pharynx, the frontal sac, and the atrium. All are originally composed of epithelial sheets of ectoderm and maintain their epithelial structure throughout development (29). Gnathal structures all express Shark from the time they are first visible as distinct protuberances (Fig. 3*I*). In the same region of the head and at the same time, the salivary gland primordium begins to invaginate. It, too, is derived from ectodermal cells and maintains a distinct tubular, epithelial structure and ultimately opens into the foregut. As might be expected, the cells that line the salivary gland express Shark throughout their development (Fig. 3*L*).

Physical Mapping of the shark Gene. To begin the genetic analysis of Shark, its gene was physically mapped by *in situ* hybridization to third-instar larval salivary gland polytene chromosomes using genomic clone TK17 as a probe (30). The shark clone hybridized to a region just centromeric to a slight

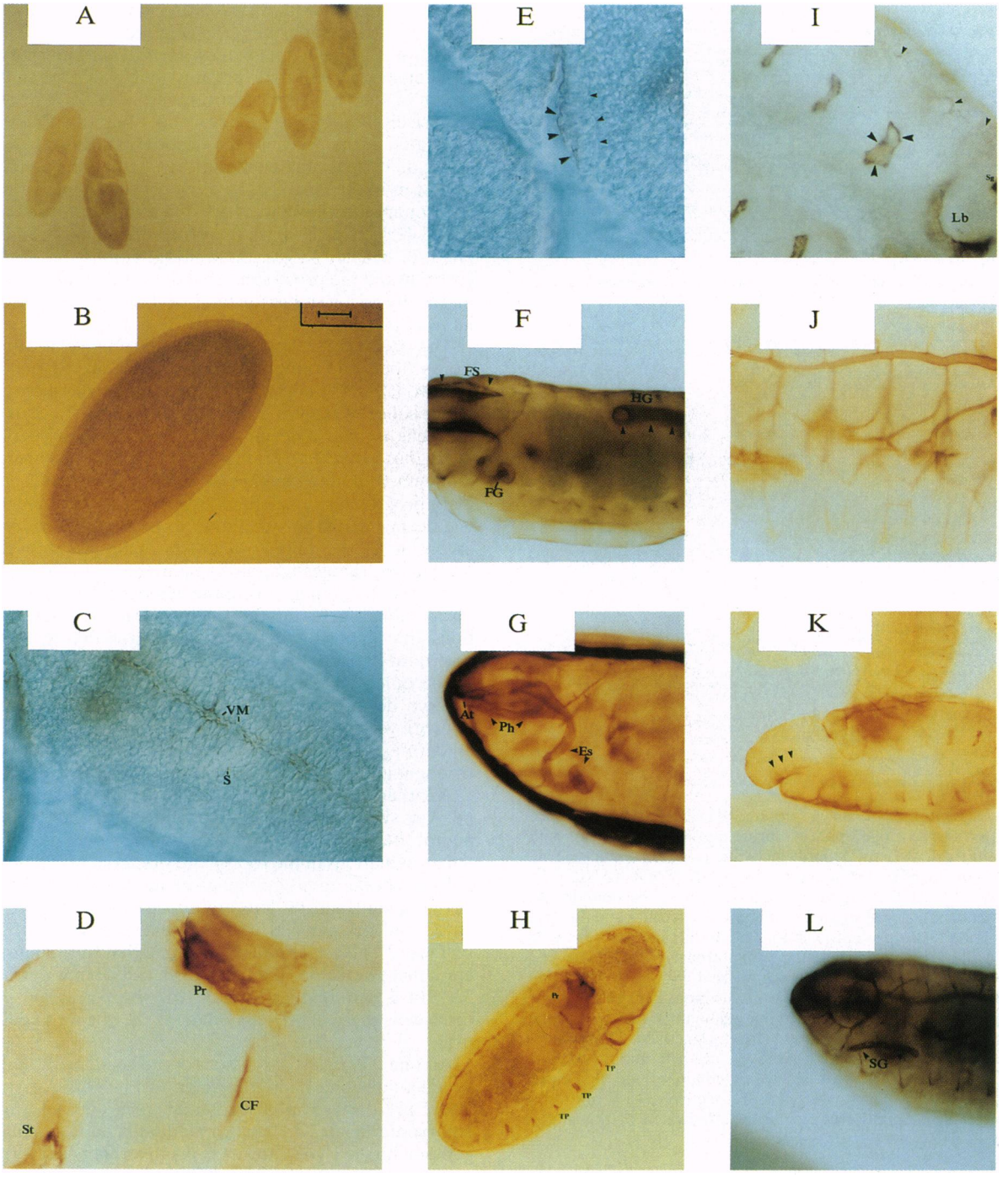

FIG. 3. The expression of Shark in *Drosophila* during embryogenesis. Three peptides derived from the predicted sequence were produced and used to generate a polyclonal anti-Shark antiserum. To delineate Shark expression, we stained whole-mount embryos with affinity-purified anti-Shark antibodies. (*A*) Embryos at all stages that were stained after their preincubation with the peptide antigens (5 mM of each peptide in PBS/0.1% Triton X-100). Identical, negative results were obtained when embryos were stained with preimmune serum (data not shown). (*B*) Pregastrulation embryos. Prior to gastrulation, there is no detectable expression of Shark. Bar = 100 μm. (*C*) Gastrulation embryos. Ectodermal cell staining is particularly intense along the cephalic furrow and ventral midline (VM). Sporadic staining is also seen among epidermal cells (S). In postgastrulation embryos (*D–L*), groups of ectodermally derived cells invaginate to form many nonneuronal structures. The proctodeum (Pr) and stomodeum (St) begin to express Shark intensely as soon as they are distinguishable during stages 8 and 9, respectively. In a stage 9 embryo, both can be easily visualized together with the remnants of the cephalic furrow (CF) (*D*). Apical expression of Shark can be appreciated in the cephalic furrow, where staining is limited to the luminal surface (large arrowheads) and absent from the basal surface (small arrowheads) (*E*). As the stomodeum develops, it gives rise to much of the foregut (FG) including the atrium (At), pharynx (Ph), and esophagus (Es), all of which continue to express Shark through late embryogenesis (*F* and *G*). Similarly, the proctodeum gives rise to the hindgut (HG), and it too expresses Shark throughout its development (*F*). Another ectodermally derived structure, the frontal sac (FS), also expresses Shark (*F*). The developing tracheal system also expresses Shark. It develops from 20 invaginations called tracheal pits (TP), first discernible by staining at stage 11 (*H*). Shark is expressed exclusively on the lumenal surface of the tracheal cells (arrowheads) (*I*), and the staining of late-stage embryos delineates the luminal surface of the entire respiratory system (*J*). The cells of the stomatogastric nervous system express Shark during the period (stages 10–11) when they transiently form a set of invaginations in the stomodeum (arrowheads) (*K*). Even late in embryonic development ectodermally derived epithelial structures, such as the salivary gland (Sg, SG), express Shark (*I*, *K*, *L*). Lb, labium.

constriction at 53A, on the right arm of chromosome two (data not shown). While no known tyrosine kinase genes map to this region, several embryonic lethal mutations do (31). One such mutant, shotgun, possesses a phenotype very similar to crumbs. However, embryos mutant for two different shotgun alleles express Shark protein (A.W.F. and E.R.S., unpublished observations).

Possible Function of Shark and Analysis of Its Action. The expression and predicted structure of Shark suggest that it transduces a signal received on the apical surface of ectodermal epithelia. Because its expression overlaps so closely with that of Crumbs, an attractive hypothesis is that Shark serves to transduce intracellularly the Crumbs intercellular signal. If this hypothesis is correct, then tissues that do not express Shark should be minimally affected in crumbs mutants. In fact, there are several tissues that normally express Crumbs but not Shark. These include two nonepithelial tissues, the external sensory organs and the chordotonal organs of the peripheral nervous system, and the Malpighian tubules (4). Consistent with our hypothesis, in crumbs null mutants, these neural tissues are normal and the Malpighian tubules only minimally affected (4, 5).

While both tyrosine kinases and ANK repeats have been shown to transduce developmental signals, and SH2 domains are known to participate intimately in tyrosine kinase signaling, no pathways have yet been identified that involve both ANK and tyrosine kinase signaling. Shark, however, appears to lie at the intersection of two such pathways. Whether this convergence proves to be of general importance or a unique feature of fly ectodermal epithelial development remains to be shown. However, several points argue for its more general significance. ANK repeat signaling through a kinase has precedent: signaling by the Notch ANK repeat protein requires the serine/threonine shaggy kinase (32). Second, the recent identification of a partial cDNA that encodes the hydra protein HTK16 (13) with the same overall structure as Shark suggests that Shark belongs to a family of signaling proteins and that the signaling mechanisms employed by Shark in flies will also be found in developing systems of other organisms.

Furthermore, if Shark does indeed help to regulate the polarity of ectodermal epithelia, especially as they invaginate, then it would be participating in a process generally required in the development of higher eukaryotes. Specifically, the generation of stomodea and proctodea, which ultimately connect the endodermal gut to the exterior of an animal, are found in metazoans ranging from cnidarians to primates.

A detailed characterization of the signal that Shark transduces awaits isolation of mutations in the shark gene. However, there exist non-crumbs mutants that possess phenotypes similar to crumbs and that specifically affect Shark-expressing cells. The existence of these mutants will facilitate the rapid further functional characterization of Shark.

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