Development 111, 1021–1028 (1991)
Printed in Great Britain © The Company of Biologists Limited 1991

Plasma membrane localization of the *Toll* protein in the syncytial *Drosophila* embryo: importance of transmembrane signaling for dorsal–ventral pattern formation

CARL HASHIMOTO*, SUZANNE GERTTULA and KATHRYN V. ANDERSON

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA

*Present address: Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510, USA

Summary

Formation of the *Drosophila* embryo's dorsal–ventral pattern requires the maternal product of the *Toll* gene. DNA sequence and genetic analyses together suggested that the *Toll* gene product is a transmembrane protein which communicates information from an extracytoplasmic compartment to the cytoplasm. Using antibodies as probes, we show that the *Toll* protein is a 135×10^3 M_r glycoprotein which is tightly associated with embryonic membranes. During the syncytial stage when dorsal–ventral polarity is established, the maternal *Toll* protein is associated with the plasma membrane around the entire embryo. During later embryonic stages, the *Toll* protein is expressed zygotically on many cell surfaces, possibly to promote cell adhesion. The plasma membrane localization of the *Toll* protein in the syncytial embryo suggests that transmembrane signaling from the extracellular perivitelline space to the cytoplasm is required for establishment of the embryonic dorsal–ventral pattern.

Key words: *Drosophila* embryo, dorsal–ventral pattern, *Toll* protein, plasma membrane.

Introduction

The dorsal–ventral pattern of the *Drosophila* embryo's anatomy begins to unfold with the first morphogenetic movement of gastrulation, when the most ventral cells of the cellular blastoderm invaginate to form the mesoderm and lateral and dorsal cells become the ectoderm. From these germ layers will arise the structures – muscle, nerve cord, ventral and dorsal epidermis – that characterize the dorsal–ventral pattern (Campos-Ortega and Hartenstein, 1985). Formation of this pattern requires the functioning of a defined set of maternal gene products (Anderson, 1987, 1989). An embryo missing any of 11 maternal gene products known as the dorsal-group fails to produce ventral or lateral structures; only dorsal structures are made, because all cells of the mutant embryo follow the developmental path of dorsal cells of the wild-type embryo (Anderson and Nüsslein-Volhard, 1986). The similar dorsalized phenotype resulting from the absence of any dorsal-group gene product suggests that these molecules function in a common process to organize embryonic dorsal–ventral polarity.

By genetic criteria, the last to act in this process is the *dorsal* gene product (Anderson *et al.* 1985*b*). DNA sequence analyses reveal that the *dorsal* gene product is structurally similar to the product of the avian proto-oncogene *c-rel* and the protein NF-κB, a DNA-binding protein that regulates gene transcription (Steward, 1987; Ghosh *et al.* 1990; Kieran *et al.* 1990). In the syncytial blastoderm, the *dorsal* protein appears initially in the cytoplasm, uniformly distributed throughout dorsal–ventral regions, and then in nuclei. Its nuclear concentration is graded, however, with the highest amount in ventral nuclei (Roth *et al.* 1989; Rushlow *et al.* 1989; Steward, 1989). This gradient presumably leads to region-specific expression of zygotic gene products required by cells to specify the dorsal–ventral pattern (Ferguson and Anderson, 1991). The nuclear localization and gradient of the *dorsal* protein require the activities of all other dorsal-group gene products. By identifying the biochemical functions of these molecules, it should be possible to define the cell biological pathway used to organize the *dorsal* gradient and ultimately the embryo's dorsal–ventral pattern.

Two observations suggest that one of the dorsal-group gene products, *Toll*, has a unique role in organizing embryonic dorsal–ventral polarity. First, the *Toll* gene is unusual because it is readily mutable to both recessive loss-of-function and dominant gain-of-function alleles (Anderson *et al.* 1985*b*). Mothers homozygous for a recessive allele produce dorsalized embryos, whereas mothers carrying a dominant allele

produce ventralized embryos. The opposing phenotypes suggest that the *Toll* gene product is required in the embryo not only to make ventral structures but also to distribute them correctly in space. Second, in experiments in which mutant embryos are rescued by the injection of wild-type cytoplasm, where *Toll* rescuing activity is placed defines the region from which ventral structures arise (Anderson *et al.* 1985*a*). Paradoxically, the rescuing activity that induces ventral structures is not localized ventrally but distributed uniformly in the wild-type embryo. This paradox implies that the *Toll* gene product is normally present throughout the embryo but its activity is somehow restricted to ventral regions. For example, translation of the uniformly distributed *Toll* mRNA (Gerttula *et al.* 1988) might be regulated to produce more *Toll* protein on the embryo's ventral side.

From its DNA sequence, the *Toll* gene product appears to be a transmembrane protein with a cytoplasmic domain of 269 amino acids and an extracytoplasmic domain of 803 amino acids including the N-terminal signal sequence (Hashimoto *et al.* 1988). The *Toll* protein's cytoplasmic domain is similar in primary structure to the cytoplasmic domain of the interleukin 1 receptor (Schneider *et al.* 1991). *Toll*'s extracytoplasmic domain contains multiple repeats of a leucine-rich sequence found in many proteins with diverse biological functions in several organisms (Hashimoto *et al.* 1988; also see references in Krantz and Zipursky, 1990). One possible function of the leucine-rich repeats is to promote protein–protein interaction (Takahashi *et al.* 1985; Krantz and Zipursky, 1990).

The probable transmembrane configuration of the *Toll* protein and the order of dorsal-group gene activities deduced by genetic analyses (Anderson *et al.* 1985*b*) suggest that transmembrane signaling is required to establish embryonic dorsal–ventral polarity (Hashimoto *et al.* 1988). Two gene products, *easter* and *snake*, which act genetically upstream of *Toll*, appear to be secreted serine proteases like those involved in blood clotting and complement fixation (DeLotto and Spierer, 1986; Chasan and Anderson, 1989; Jin and Anderson, 1990), so they are likely to function in the same compartment as *Toll*'s extracytoplasmic domain. What extracytoplasmic compartment is involved, however, depends on which membrane of the young embryo contains the *Toll* protein. Because the *Toll* activity for defining dorsal–ventral polarity appears to be required before cellularization of the embryo (Anderson *et al.* 1985*a*; Anderson and Nüsslein-Volhard, 1986), the protein could be present in either the plasma membrane facing the outside of the embryo or membranes of internal vesicles and organelles. Distinguishing between these possibilities is important for defining the function of the *Toll* protein and the cellular mechanism used in organizing embryonic dorsal–ventral polarity. If the *Toll* protein is in the plasma membrane, it could function as a receptor for a signal within the extracellular perivitelline space. But if the protein is in internal membranes, then its extracytoplasmic domain is not accessible to a diffusible signal from other parts of the embryo.

In the work reported here, we used antibodies as probes to characterize the basic structure and distribution of the *Toll* protein in embryos. We find that the *Toll* protein is a glycosylated polypeptide of 135×10^3 M_r which is tightly associated with embryonic membranes. In the syncytial embryo, the maternally encoded *Toll* protein is associated with the plasma membrane, with no apparent dorsal–ventral asymmetry in its concentration. In the multicellular embryo, after dorsal–ventral polarity is established, the zygotically expressed protein is found on many cell surfaces. The plasma membrane localization of the maternal *Toll* protein supports the idea that transmembrane communication of an extracellular signal is involved in establishing dorsal–ventral polarity of the *Drosophila* embryo.

Materials and methods

Bacterial fusion proteins and antibodies

Two chimeric proteins with different segments of the *Toll* protein fused to bacterial proteins were made (Fig. 1A). One fusion protein contains 75 amino acids near the *Toll* protein's N terminus, beginning with amino acid 25 of the primary translation product, fused to the C terminus of protein A from *S. aureus* (Nilsson *et al.* 1985). The plasmid encoding this fusion protein was created by inserting a 225 bp fragment from a *Toll* cDNA (Hashimoto *et al.* 1988) into the plasmid pRIT2T (Pharmacia). The fusion protein was purified from bacterial homogenates by IgG affinity chromatography (Nilsson *et al.* 1985; Pharmacia). The second fusion protein contains the *E. coli* trp E protein and the 269 amino acids of *Toll*'s entire cytoplasmic domain from the first amino acid after the proposed transmembrane segment to the C terminus. To make the plasmid encoding this fusion protein, first a *Bam*HI site was created by oligonucleotide-directed mutagenesis (D. Schneider, unpublished work) in the cDNA adjacent to the region encoding the transmembrane segment and then a *Bam*HI–*Hin*dIII fragment of 1163 bp containing the *Toll* termination codon was inserted into the plasmid pATH3 (Dieckmann and Tzagoloff, 1985; Harlow and Lane, 1988). This fusion protein was purified by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) of insoluble protein pellets isolated from bacterial lysates (Kleid *et al.* 1981). Each fusion protein (about 200 μg) was mixed with adjuvant (RIBI Immunochem Research) before being injected into a rabbit. After 4 weeks, the rabbit was injected twice more with 100–150 μg of fusion protein and then bled weekly (Harlow and Lane, 1988).

Antibodies in rabbit sera specific for *Toll* protein sequences were purified by affinity chromatography with affigel 10/15 matrices (Bio-Rad) containing the appropriate trp E fusion proteins (Driever and Nüsslein-Volhard, 1988). For this purpose, a trp E fusion protein was made containing the same 75 amino acids near *Toll*'s N terminus used in the protein A fusion protein described above. Non-specific antibodies were removed by chromatography with similar columns containing trp E protein or total bacterial proteins and in some cases total proteins from *Toll*$^-$ embryos. These embryos with no detectable *Toll* mRNA were from mothers with the genotype $Df(3R)Tl^{9QRX}/Df(3R)ro^{XB3}$ (Hashimoto *et al.* 1988).

Embryo injection, immunoprecipitation, membrane isolation and protein blotting

Eggs were collected over a 1 h period from flies at 22 °C and then injected with [^{35}S]methionine (50 mCi ml^{-1}, Amersham). Injected embryos were incubated for 1 h until most had reached the syncytial blastoderm stage, 1.5 to 2.5 h post-fertilization (stage 4 of Campos-Ortega and Hartenstein, 1985), before being homogenized in lysis buffer (50 mM Hepes/NaOH pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% NP-40, 0.2% Triton X-100; Wilcox, 1986) containing protease inhibitors (0.125 μg ml^{-1} each of antipain, chymostatin, leupeptin, pepstatin; 200 units ml^{-1} aprotinin; 0.4 mM phenylmethylsulfonyl fluoride [PMSF]; Sigma).

To immunoprecipitate the *Toll* protein, embryo homogenates were centrifuged for 5 min at 4 °C and 15 000 *g* and the supernatant was mixed at 4 °C first with 2.5 mg protein A–Sepharose (Pharmacia) for 30 min and then with affinity-purified antibodies coupled to 2.5 mg protein A–Sepharose plus a 10-fold excess of similarly prepared homogenate of *Toll*$^{-}$ embryos (see above) for 2 h (Harlow and Lane, 1988). Protein A–Sepharose beads were washed four times with 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.05% NP-40 and once with 5 mM Tris–HCl pH 7.5 before being boiled for 5 min in sample buffer (Laemmli, 1970). Proteins released from the beads were electrophoresed in SDS–polyacrylamide gels (0.75×70×80 mm) containing a linear gradient of 5–15% acrylamide (Matsudaira and Burgess, 1978). Gels were treated with 1 M sodium salicylate for 30 min (Chamberlain, 1979) and dried before exposing to X-ray film at −70 °C. For deglycosylation, immunoprecipitated proteins were released from protein A–Sepharose beads by boiling in 0.5% SDS and 1% β-mercaptoethanol. A sample of released proteins was mixed with a 2-fold greater volume of 2% NP-40, 30 mM EDTA, 0.75 mM PMSF, 0.3 M potassium phosphate pH 7.5 and incubated at 37 °C for 18 h with 15 units ml^{-1} of peptide: N-glycosidase F (Tarentino *et al.* 1985; N-glycanase, Genzyme). The reaction mixture was boiled with a 2-fold greater volume of 2× sample buffer prior to electrophoresis.

Total embryonic membranes were isolated from embryos (0–4 h after oviposition at 25 °C) homogenized in hypotonic buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) containing the protease inhibitors described above (Patel *et al.* 1987). To release soluble and peripherally associated proteins from membrane vesicles, membranes were diluted and incubated in a 100-fold greater volume of 100 mM Na_2CO_3 pH 11.5 at 4 °C for 1 h (Fujiki *et al.* 1982). Membranes were then pelleted by centrifugation for 1 h at 4 °C and 100 000 *g*, and proteins in the supernatant were precipitated with 10% trichloroacetic acid. Proteins in both fractions were resolubilized in sample buffer prior to electrophoresis.

To detect the *Toll* protein in membranes by blotting (Towbin *et al.* 1979), total membrane proteins on a nitrocellulose filter (Schleicher and Schull) were probed with affinity-purified antibodies. Antibody–antigen complexes were detected with alkaline phosphatase coupled to goat anti-rabbit IgG (Jackson) and a staining reaction including nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

Antibody staining of embryos – whole mounts and sections

Embryos at different developmental stages were collected from flies at 22 °C. Dechorionated embryos were fixed, devitellinized and incubated with affinity-purified antibodies using the procedure of Patel *et al.* (1989). Antibody–antigen complexes were detected with horseradish peroxidase coupled to goat anti-rabbit IgG (Jackson) and the diaminobenzidine (DAB) staining reaction, in some cases using $CoCl_2$ to increase the stain intensity (Hsu and Soban, 1982). Embryos were dehydrated in ethanol and either cleared in methyl salicylate for observation of whole mounts or transferred to propylene oxide and embedded in Durcupan (Fluka) for sectioning. Sections of 4 μm were cut with a dry glass knife on a Porter-Blum MT-2 ultra-microtome, dried on a glass slide coated with gelatin and covered with Permount (Fisher). Whole mounts and sections of embryos were examined and photographed using a Zeiss photomicroscope with Nomarski optics.

Results

Identification and biochemical characterization of the embryonic Toll *protein*

Rabbit polyclonal antibodies were raised against two bacterial fusion proteins containing different regions of the *Toll* protein's primary structure as predicted by the cDNA sequence. One fusion protein contained 75 amino acids near the N terminus of the extracytoplasmic domain, while the second contained all 269 amino acids of the C-terminal cytoplasmic domain (Fig. 1A). Antibodies were affinity-purified before being used as probes to identify the embryonic *Toll* protein.

To check whether the antibodies reacted specifically with the *Toll* protein, we used an immunoprecipitation procedure capable of detecting small amounts of the protein among total radiolabeled proteins in embryonic extracts. Embryos injected and incubated with [^{35}S] methionine were homogenized in buffer containing non-ionic detergents to solubilize membrane proteins (see Materials and methods). From an extract of wild-type (Oregon R) embryos, antibodies against the *Toll* protein's predicted cytoplasmic domain precipitated one major polypeptide of 135×10^3 M_r (Fig. 1B, lane 2). Antibodies against the *Toll* protein's N-terminal region also precipitated a 135×10^3 M_r polypeptide, but only when the embryonic extract was first boiled in 1% SDS to denature proteins (data not shown). Because of their reactivity with denatured protein, antibodies against the N-terminal region were useful in protein blotting experiments (see below and Fig. 2). The 135×10^3 M_r polypeptide was not detectable in extracts of embryos that lack detectable *Toll* mRNA (Fig. 1B, lane 1; see Materials and methods). Since both antibodies recognize a 135×10^3 M_r polypeptide in wild-type but not *Toll*$^{-}$ embryos, we conclude that this polypeptide is the *Toll* protein.

The *Toll* protein's extracytoplasmic domain contains 17 potential N-glycosylation sites (Fig. 1A), so the protein was expected to be glycosylated. Both antibodies described above reacted with a 135×10^3 M_r polypeptide in protein blots of total embryonic glycoproteins isolated by lentil lectin chromatography (data not shown; Wilcox, 1986). To test further whether the immunoprecipitated 135×10^3 M_r polypeptide was glycosylated, we treated it with the enzyme peptide:N-glycosidase F, which removes N-linked carbohydrate from glycoproteins (Tarentino *et al.* 1985). Treatment

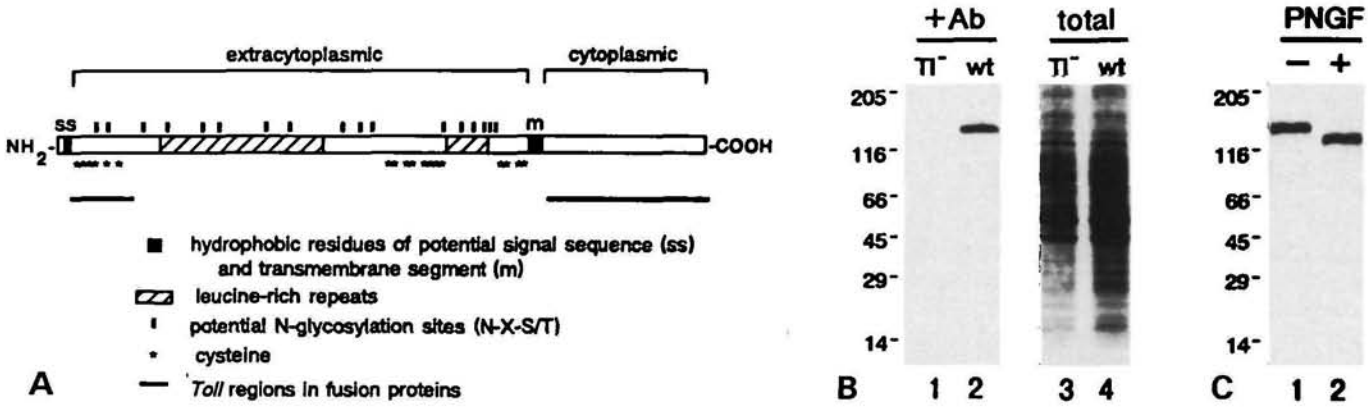

Fig. 1. *Toll* protein is a 135×10^3 M_r glycosylated polypeptide. (A) Salient features of the *Toll* protein's primary structure as revealed by DNA sequence analysis (Hashimoto *et al.* 1988). Shown also are *Toll* regions in the fusion proteins used to raise antibodies. (B) About 100 embryos (0–1 h after oviposition at 22°C) were injected with [^{35}S]methionine and incubated for 1 h at room temperature before being homogenized in buffer containing non-ionic detergents. Extracts of *Toll*$^-$ and wild-type (Oregon R) embryos (lanes 1 and 2, respectively) were subjected to immunoprecipitation analysis with antibodies against the *Toll* protein's cytoplasmic domain. Lanes 3 and 4 show total radiolabeled proteins from *Toll*$^-$ and wild-type embryos, respectively. Proteins were electrophoresed in SDS–polyacrylamide gels containing a linear gradient of 5–15 % acrylamide and visualized by fluorography. Indicated on the left are molecular weights ($\times10^{-3}$) of protein standards. (C) The 135×10^3 M_r polypeptide precipitated by anti-*Toll* antibodies as in B was mock-treated (lane 1) or treated with the deglycosylating enzyme peptide:N-glycosidase F (PNGF; lane 2).

with this enzyme converted the 135×10^3 M_r polypeptide to a smaller form of 120×10^3 M_r (Fig. 1C, cf. lanes 1 and 2). The latter is about the expected size of the primary translation product (125×10^3 M_r) of the *Toll* mRNA after removal of the N-terminal signal sequence.

The extracytoplasmic domain contains 18 cysteines distributed in 3 clusters (Fig. 1A). These cysteines could form disulfide bonds, either intra- or intermolecularly, or both. Since the immunoprecipitations shown in Fig. 1B were done under non-denaturing conditions without sulfhydryl reducing agents, the absence in the immunoprecipitates of a polypeptide distinct from the 135×10^3 M_r polypeptide suggested that the latter was not disulfide bonded to another radiolabeled polypeptide. To test, however, if the 135×10^3 M_r polypeptide forms a disulfide-linked dimer, is disulfide bonded to a non-radiolabeled polypeptide, or is only intramolecularly disulfide bonded, it was electrophoresed without prior exposure to β-mercaptoethanol. Under these conditions, the immunoprecipitated polypeptide migrated slightly faster in the SDS–polyacrylamide gel than when it had been treated similarly but under reducing conditions (data not shown). Faster electrophoretic migration under non-reducing conditions is consistent with the protein being held in a more compact conformation by intramolecular disulfide bonds (Wilcox, 1986). Thus, it appears that most of the embryonic *Toll* protein is not covalently bound to another protein and that the cysteines participate in intramolecular disulfide bonds.

Because it contains a segment of 25 hydrophobic amino acids (Fig. 1A), the *Toll* protein was expected to

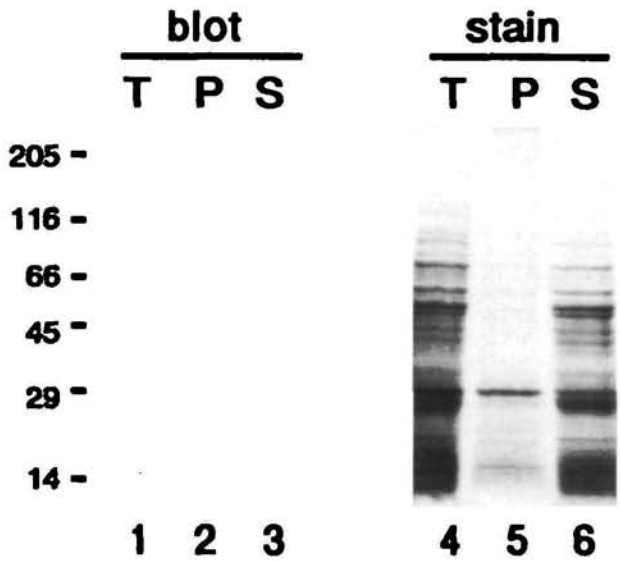

Fig. 2. *Toll* protein is tightly associated with embryonic membranes. After total membranes from wild-type embryos (0–4 h after oviposition at 25°C) were incubated in sodium carbonate pH 11.5 for 1 h, membranes were pelleted by centrifugation, while solubilized proteins were precipitated with 10 % trichloroacetic acid. Proteins in total membranes (T, lanes 1 and 4) and in membrane pellet (P, lanes 2 and 5) and soluble fraction (S, lanes 3 and 6) from the same amount of total membranes were electrophoresed in SDS–polyacrylamide gels. On the left (lanes 1–3) is a blot of these proteins probed with antibodies against the *Toll* protein's N-terminal region. On the right (lanes 4–6) is a gel of these proteins stained with Coomassie blue. Faint bands above and below the most intense band at 135×10^3 M_r might be altered forms of the *Toll* protein. Such structural heterogeneity is not apparent in immunoprecipitation analysis (Fig. 1B and C), but is detectable with antibodies against both the N-terminal region and cytoplasmic domain when proteins from embryos of varying developmental ages are examined by protein blotting – e.g. total glycoproteins from 0–4 h embryos (data not shown).

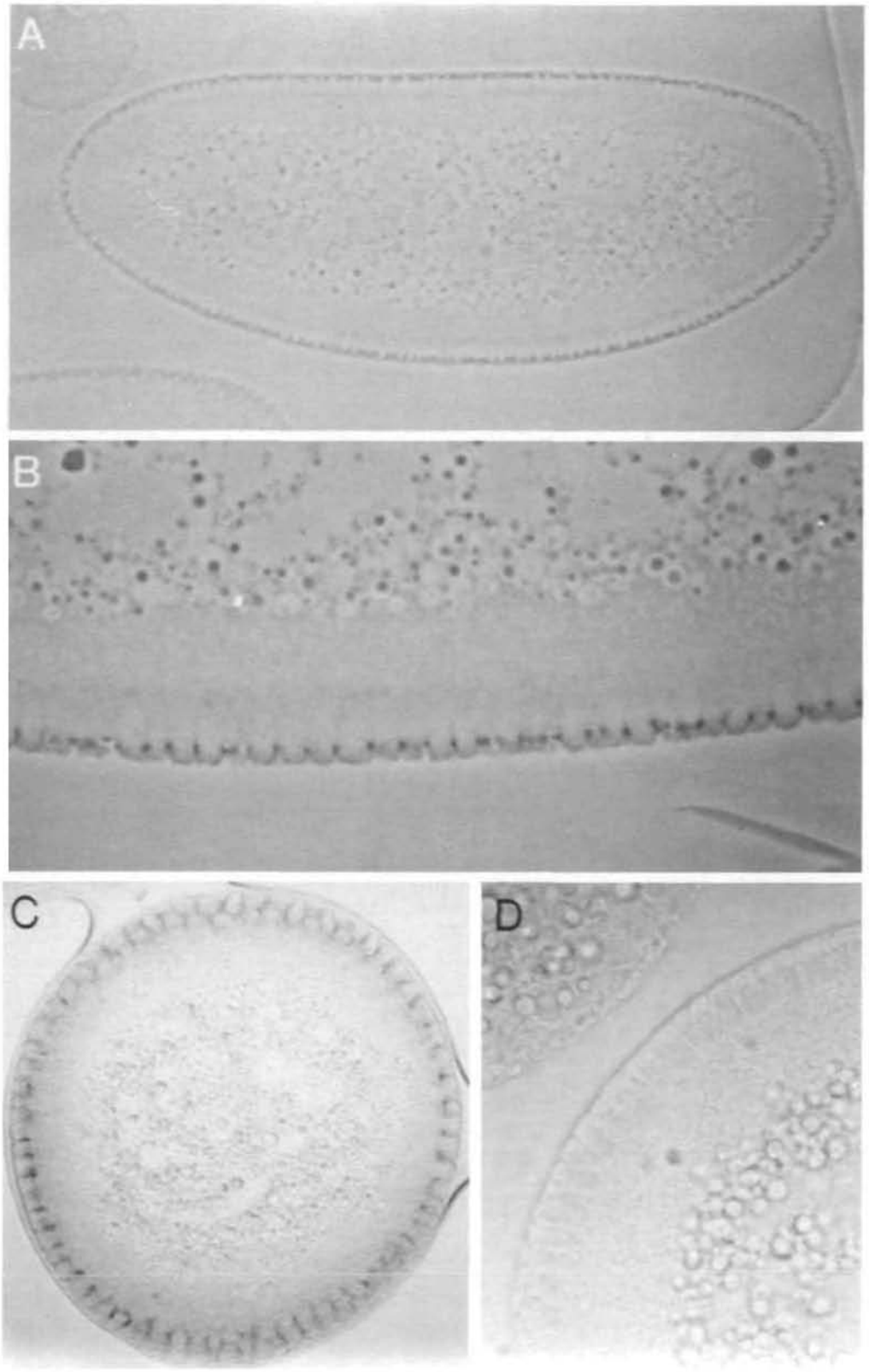

Fig. 3. Plasma membrane localization of maternal *Toll* protein in syncytial embryo. Sections of wild-type (A–C) and *Toll*$^-$ (D) embryos at syncytial blastoderm stage that were incubated with antibodies against the *Toll* protein's cytoplasmic domain (A and B) or N-terminal region (C and D). (A) Sagittal section of embryo at beginning of cellularization shows that *Toll* protein is predominantly associated with the plasma membrane all around embryonic surface (except pole cell membranes). Antibody stain here is greyish blue halo around periphery of embryo punctutated by dark blue dots at cleavage furrows. Anterior of embryo is to the left, dorsal is up. (B) Higher magnification of ventral surface shows concentration of *Toll* protein (dark blue dots) in cleavage furrows. (C) A cross-section of a slightly younger embryo shows *Toll* protein (here antibody stain is brown) all around embryonic circumference. Lattice-like pattern reflects heavy staining at pseudo-cleavage furrows. (D) Part of a cross-section of a *Toll*$^-$ embryo at a similar stage as embryos in A–C does not show any detectable antibody stain. [In A and B, $CoCl_2$ added to the diaminobenzidine staining reaction converts brown antibody stain seen in C to dark blue].

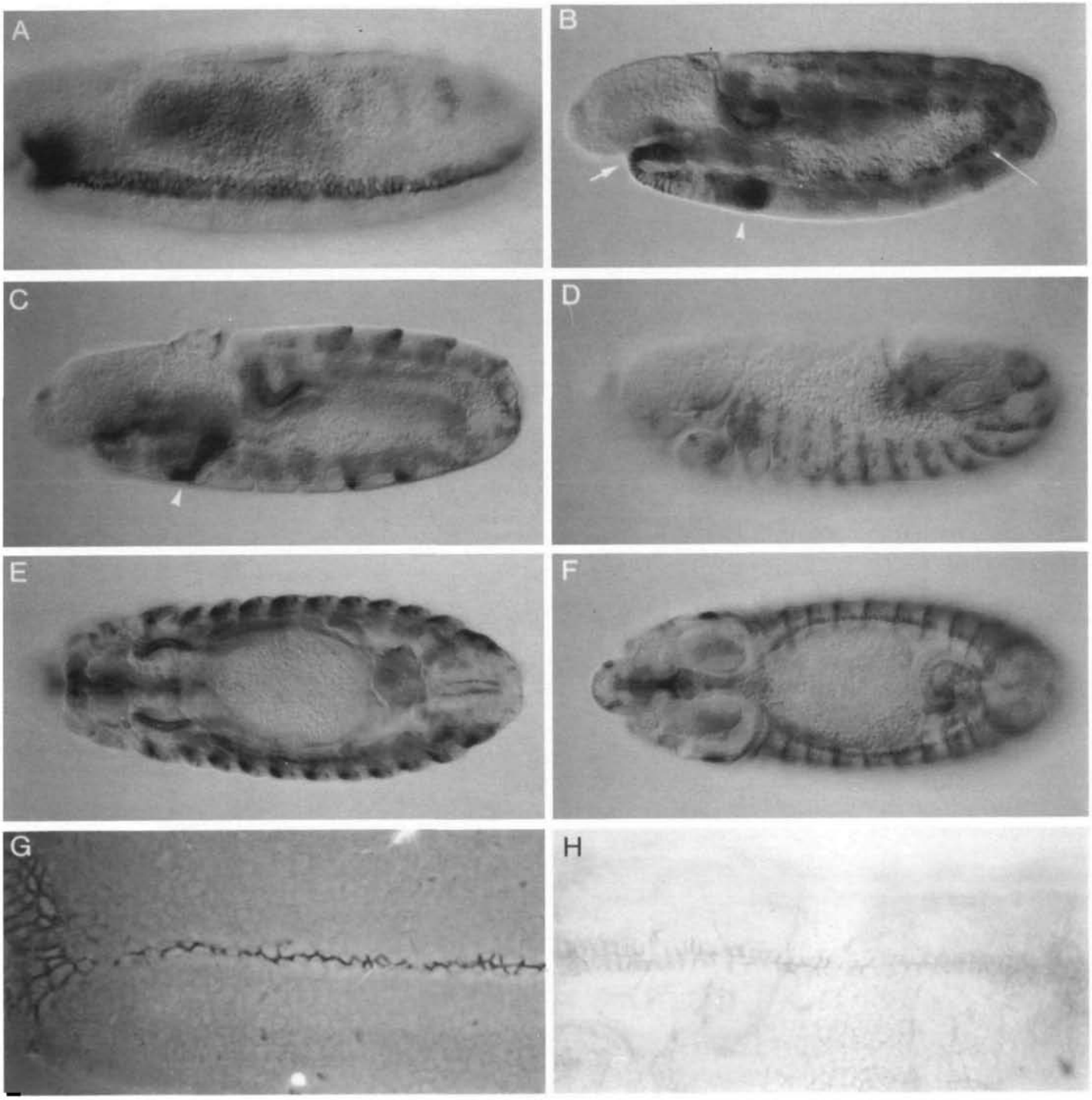

Fig. 4. Expression of zygotic *Toll* protein on cell surfaces. Whole mounts (A–F,H) and section (G) of embryos stained with antibodies against *Toll*'s cytoplasmic domain. (A) Stage 10 embryo showing *Toll* protein on cell surfaces in stomodeum, the invaginated region near anterior of embryo on the left, and in the stripe of mesectoderm that stretches along the midline of the extended germ band. (B) Stage 11 embryo. *Toll* protein is seen on cell surfaces in the stomodeum (arrow at left), salivary gland placode (arrowhead), the splanchnopleura (thin arrow at right), and proctodeum, seen here near posterior end of germ band just above salivary gland placode. (C) Early stage 12 embryo at beginning of germ band shortening. *Toll* protein is visible on cell surfaces in salivary gland invagination (arrowhead) and hindgut, the hook-shaped structure just above and to the right of salivary gland invagination. *Toll* protein is also visible in epidermis at intersegmental furrows. (D) Late stage 12 embryo near end of germ band shortening. *Toll* protein is apparent on cell surfaces in epidermis just posterior to each intersegmental boundary. (E and F) Stage 14 embryo during dorsal closure. Horizontal plane of focus near middle of embryo in E shows *Toll* protein in salivary glands (pair of brush-like structures near anterior of embryo on the left) and in epidermis at intersegmental furrows. Horizontal view at dorsal surface (F) shows *Toll* protein on cell surfaces at leading edge of the two epidermal sheets that are converging toward dorsal midline. Note restriction of *Toll* protein in this case to lateral cell surfaces. (G) Section of stage 10 embryo showing enrichment of *Toll* protein at the border between the two rows of mesectodermal cells. (Antibody stain here is dark blue.) (H) Surface view of stage 15 embryo as the two epidermal layers join at dorsal midline during dorsal closure. Note enrichment of *Toll* protein at newly formed contacts between the two layers.

cross the lipid bilayer of embryonic membranes. Such a tight membrane association should remain undisturbed by exposure to alkaline pH, which releases peripherally associated proteins from membranes (Fujiki *et al.* 1982). To test whether the 135×10^3 M_r polypeptide was an integral membrane protein, we diluted total membranes from embryos (0–4 h after oviposition at 25 °C) into Na_2CO_3 pH 11.5 buffer, then pelleted the treated membranes by centrifugation, and assayed by protein blotting the soluble and pellet fractions for the 135×10^3 M_r polypeptide. Although about 90 % of the initially membrane-associated proteins were solubilized by the carbonate treatment (Fig. 2, cf. lanes 4 and 6), almost 90 % of the 135×10^3 M_r polypeptide remained associated with the membrane fraction (Fig. 2, cf. lanes 1 and 2). Like other integral membrane proteins, this polypeptide was solubilized by non-ionic detergents (see Fig. 1B).

Localization of the maternal Toll *protein*

To determine in what embryonic membrane the *Toll* protein is localized, embryos up to a few hours after fertilization were histochemically stained with antibodies against the protein's extracytoplasmic N terminus or its cytoplasmic domain. We were particularly interested in examining embryos at the syncytial blastoderm stage, since the *Toll* protein must be active during this period to participate in establishing dorsal–ventral polarity. At least three observations have defined this critical period – between 1.5 and 2.5 h after fertilization but before cellularization – for *Toll* protein activity. First, embryonic dorsal–ventral polarity is not fixed until after fertilization, since embryos missing the *Toll* protein are rescued up to the late syncytial blastoderm stage by the injection of wild-type cytoplasm (Anderson *et al.* 1985*a*). Second, the temperature-sensitive period of *Toll* function extends from the beginning to end of the syncytial blastoderm stage (Anderson and Nüsslein-Volhard, 1986). And third, by the end of this stage, zygotic gene products are already restricted to dorsal–ventral regions defined by maternal information (Rushlow *et al.* 1987; St. Johnston and Gelbart, 1987; Thisse *et al.* 1987).

In whole-mount preparations, embryos were uniformly stained with the antibodies against the N terminus or the cytoplasmic domain (data not shown), so the *Toll* protein appeared to be present at all dorsal–ventral positions. To localize the *Toll* protein more precisely, sections of antibody-stained embryos were examined. Before cellularization of the embryo, the *Toll* protein was mostly associated with the plasma membrane at the blastoderm surface rather than in vesicles. At the earliest stage examined, before nuclear migration to the egg cortex, the protein was detectable in the plasma membrane, but its concentration in the membrane subsequently increased, reaching a peak within the syncytial blastoderm stage (Fig. 3A). The *Toll* protein appeared to be concentrated in the pseudocleavage furrows that form transiently between nuclei before cellularization and in the cleavage furrows during cellularization (Fig. 3B and C). At all stages before cellularization, the *Toll* protein's concentration in the plasma membrane appeared to be uniform around the entire embryo (for example, Fig. 3C).

The *Toll* protein's concentration decreased after the syncytial period in which this protein must be active for dorsal–ventral polarity to be established. During cellularization, the *Toll* protein co-localized with the base of the advancing membrane. By the time of ventral furrow formation, the first gastrulation movement, the protein was barely detectable (data not shown).

Localization of the zygotic Toll *protein*

The *Toll* protein must also function zygotically, since 95 % of *Toll*$^-$ zygotes from heterozygous mothers die as first or second instar larvae (Gerttula *et al.* 1988). *In situ* hybridization studies revealed that the *Toll* gene is transcribed zygotically in a complex pattern throughout embryogenesis (Gerttula *et al.* 1988). To localize the zygotic *Toll* protein, we used antibodies against the protein to probe older embryos during the stages, germ band extension and shortening, when complex morphological changes are occurring (Campos-Ortega and Hartenstein, 1985).

The zygotic *Toll* protein was found in all cell types known to contain the zygotic *Toll* mRNA. In each case, the protein was concentrated at the cell surface. When the germ band was fully extended (stages 10–11 of Campos-Ortega and Hartenstein, 1985), the *Toll* protein was associated with the plasma membranes of cells in the mesectoderm, stomodeum, proctodeum, anterior and posterior midguts, and splanchnopleura, the prospective visceral mesoderm (Fig. 4A,B). The protein was also found on the surfaces of cells in the salivary gland placode (Fig. 4B) and adjacent to the segmentally repeated tracheal placodes. During and after germ band shortening (stages 12–14), the *Toll* protein was localized at cell surfaces in a number of cell types, including the salivary gland (Fig. 4C), foregut, hindgut (Fig. 4C), Malpighian tubules and the epidermis at intersegmental boundaries (Fig. 4C–F).

In many cell types, the *Toll* protein was not uniformly distributed on the cell surface. For example, the protein was highly enriched at the boundary between the two rows of mesectodermal cells that meet at the ventral midline (Fig. 4A and G). During dorsal closure (stages 14–15), when epidermal sheets stretch to cover the embryo's dorsal surface, the *Toll* protein was restricted to the lateral surfaces of cells at the leading edge of each sheet (Fig. 4F). As the two epidermal layers fused at the dorsal midline, however, the protein was also concentrated at the newly formed contacts (Fig. 4H). Enrichment of the *Toll* protein at contacts between cell layers as in these two cases would be consistent with a role for this protein in cell adhesion.

Discussion

Localization and function of the Toll *protein in the plasma membrane*

Earlier DNA sequence analysis suggested that the *Toll*

gene product is a transmembrane protein with a large extracytoplasmic domain and a smaller cytoplasmic domain (Hashimoto *et al.* 1988; Fig. 1). Here we have shown that the *Toll* protein behaves as an integral membrane protein under experimental conditions where non-integral proteins are selectively dissociated from membranes (Fig. 2). Like many membrane-associated proteins, the *Toll* protein is glycosylated (Fig. 1).

Because the *Toll* protein is localized in the plasma membrane of the syncytial blastoderm (Fig. 3) and of later embryonic cells (Fig. 4), what we had called its extracytoplasmic domain would be an extracellular domain. Thus, during the syncytial blastoderm stage, when embryonic dorsal–ventral asymmetry is first detectable, this domain should project into the perivitelline space, the compartment bounded by the plasma membrane and the vitelline envelope, the innermost layer of the eggshell. Does its plasma membrane localization indicate that the *Toll* protein is a receptor capable of converting an extracellular signal into intracellular ones? A receptor function is supported by the recent finding of similarities in primary structure between the cytoplasmic domains of the *Toll* protein and the mammalian receptor for interleukin 1, a polypeptide hormone that regulates cellular activities involved in the immune response and inflammatory reactions (26% identity; Schneider *et al.* 1991). What signaling molecule or event the *Toll* protein's extracellular domain detects is not evident from simple examination of this domain's amino acid sequence. The extracellular domain contains multiple repeats of a leucine-rich sequence found in many proteins with diverse biological functions (Hashimoto *et al.* 1988). This diversity precludes the assignment of a specific biological function to the leucine-rich repeats, but suggests instead that these repeats have a specialized biochemical property adaptable to many biological reactions – for example, the ability to promote protein–protein interaction (Takahashi *et al.* 1985; Krantz and Zipursky, 1990).

The *Toll* protein is found all around the syncytial blastoderm's surface (Fig. 3) when the dorsal–ventral gradient of nuclear *dorsal* protein is being established (Roth *et al.* 1989; Rushlow *et al.* 1989; Steward, 1989). Thus, asymmetric *Toll* activity, as defined in cytoplasmic injection experiments, cannot be caused by localized expression of the *Toll* protein from the uniformly distributed mRNA (Gerttula *et al.* 1988). If the *Toll* protein does function as a receptor in a transmembrane signaling pathway that causes the cytoplasmic-to-nuclear translocation of the *dorsal* protein, then how could the apparently uniform distribution of this receptor lead to the highest concentration of *dorsal* protein in ventral nuclei? One possibility is that *Toll*'s ligand in the perivitelline space is localized to ventral regions of the embryo, so receptor activation is spatially restricted.

Possible function of the Toll *protein in cell adhesion*

For a clue to the biochemical function of the *Toll* protein, we have examined the complex distribution pattern of the zygotically expressed protein in embryos. The maternal and zygotic proteins appear to have the same biochemical activities, since embryos missing the maternal RNA are rescued by the injection of the zygotically synthesized RNA (Gerttula *et al.* 1988). Both proteins are associated with the plasma membrane (Figs 3 and 4). Perhaps the most interesting localization pattern is the enrichment of the zygotic *Toll* protein between cells that express the protein, as in the two rows of mesectodermal cells or the epidermal cells that meet during dorsal closure (Fig. 4). Chaoptin is another *Drosophila* protein localized to closely apposed membranes. This protein, required for photoreceptor cell morphogenesis, is composed almost entirely of leucine-rich repeats (Van Vactor *et al.* 1988; Reinke *et al.* 1988). Transfected cultured cells expressing chaoptin on their surfaces have been found to adhere specifically to one another, probably because of the affinity between leucine-rich repeats (Krantz and Zipursky, 1990). Thus, the *Toll* protein whose extracellular domain contains these repeats could function analogously as a homophilic adhesion molecule. This role for the zygotic protein may also involve intracellular signaling. Whether the maternal protein functions as a homophilic adhesion molecule is unclear, since it is localized in the plasma membrane of the syncytial blastoderm which faces the pervitelline space and the vitelline envelope, not another cell surface. It is possible that two *Toll* polypeptides in the same membrane associate non-covalently, and this dimerization is important for the maternal protein's function as a receptor.

Importance of information in the perivitelline space for organizing embryonic axes

The importance of the extracellular perivitelline space for defining embryonic dorsal–ventral polarity is increasingly clear. Normally the dorsal–ventral polarities of the embryo and the surrounding eggshell are coupled, so communication across an extracellular compartment at some developmental stage was thought to be important for the formation of embryonic pattern. Part of this communication occurs during oogenesis, requiring the functioning of maternal molecules that are distinct from the dorsal-group gene products required only for embryonic polarity (Schüpbach, 1987; Manseau and Schüpbach, 1989). Some of these molecules are synthesized by the nurse cells or oocyte (germ line cells), while others are synthesized by the surrounding follicle cells (somatic cells), which also secrete components of the eggshell (King, 1970; Mahowald and Kambysellis, 1980). It is not yet understood how intercellular communication during oogenesis requiring these molecules is mechanistically related to the post-fertilization process that requires *Toll*. The germ line cells may provide information that guides the behavior and activities of somatic cells, which in turn establish spatial information outside the oocyte that is interpreted after fertilization in a transmembrane signaling process by the embryo to define its dorsal–ventral polarity.

Transmembrane signaling between the perivitelline space and the cytoplasm appears to be an essential step in cellular mechanisms used to organize axial pattern in the *Drosophila* embryo. Organization of the terminal regions of the embryo's anterior–posterior pattern (Nüsslein-Volhard *et al.* 1987; Klingler *et al.* 1988; Strecker *et al.* 1989) also requires the functioning of a transmembrane protein, in this case *torso*. *torso*'s cytoplasmic domain appears to have tyrosine kinase activity capable of affecting intracellular reactions (Sprenger *et al.* 1989). Although *torso* activity is required only in the terminal regions, the protein is uniformly distributed in the plasma membrane of the oocyte and syncytial blastoderm (Casanova and Struhl, 1989). Localized *torso* activity could result, however, from the spatially restricted distribution of *torso*'s ligand, produced by a sub-population of the somatic follicle cells that surround the oocyte (Stevens *et al.* 1990). It will be interesting to see if an extracellular signaling component involved in organizing the dorsal–ventral pattern is also localized by a similar mechanism.

We thank Nipam Patel, Michael Hortsch, David Bowtell, and Mike Simon for helpful advice on experimental procedures, and Becky Chasan, Donald Morisato, David Schneider, and Sandy Wolin for careful reading of the manuscript. This work was supported by grants from the NIH (GM 35437), NSF (DCB 8452030), and Cancer Research Coordinating Committee of the University of California to K.V.A.

References

ANDERSON, K. V. (1987). Dorsal–ventral embryonic pattern genes of *Drosophila*. *Trends Genet.* **3**, 91–97.

ANDERSON, K. V. (1989). *Drosophila*: the maternal contribution. In *Genes and Embryos* (ed. D. M. Glover and B. D. Hames), pp. 1–37. Oxford: IRL.

ANDERSON, K. V., BOKLA, L. AND NÜSSLEIN-VOLHARD, C. (1985*a*). Establishment of dorsal–ventral polarity in the *Drosophila* embryo: the induction of polarity by the *Toll* gene product. *Cell* **42**, 791–798.

ANDERSON, K. V., JÜRGENS, G. AND NÜSSLEIN-VOLHARD, C. (1985*b*). Establishment of dorsal–ventral polarity in the *Drosophila* embryo: genetic studies on the role of the *Toll* gene product. *Cell* **42**, 779–789.

ANDERSON, K. V. AND NÜSSLEIN-VOLHARD, C. (1986). Dorsal-group genes of *Drosophila*. In *Gametogenesis and the Early Embryo*. (ed. J. Gall), pp. 177–194. New York: Alan R. Liss.

CAMPOS-ORTEGA, J. A. AND HARTENSTEIN, V. (1985). *The Embryonic Development of* Drosophila melanogaster. Berlin: Springer-Verlag.

CASANOVA, J. AND STRUHL, G. (1989). Localized surface activity of *torso*, a receptor tyrosine kinase, specifies terminal body pattern in *Drosophila*. *Genes Dev.* **3**, 2025–2038.

CHAMBERLAIN, J. P. (1979). Fluorographic detection of radioactivity in polacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**, 132–135.

CHASAN, R. AND ANDERSON, K. V. (1989). The role of *easter*, an apparent serine protease, in organizing the dorsal–ventral pattern of the *Drosophila* embryo. *Cell* **56**, 391–400.

DELOTTO, R. AND SPIERER, P. (1986). A gene required for the specification of dorsal–ventral pattern in *Drosophila* appears to encode a serine protease. *Nature* **323**, 688–692.

DIECKMANN, C. L. AND TZAGOLOFF, A. (1985). Assembly of the mitochondrial membrane system. *CBP6*, a yeast nuclear gene necessary for synthesis of cytochrome *b*. *J. biol. Chem.* **260**, 1513–1520.

DRIEVER, W. AND NÜSSLEIN-VOLHARD, C. (1988). A gradient of *bicoid* protein in *Drosophila* embryos. *Cell* **54**, 83–93.

FERGUSON, E. L. AND ANDERSON, K. V. (1991). Dorsal–ventral pattern formation in the *Drosophila* embryo: the role of zygotically active genes. In *Current Topics in Developmental Biology* (ed. H. Bode), (in press).

FUJIKI, Y., HUBBARD, A. L., FOWLER, S. AND LAZAROW, P. B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* **93**, 97–102.

GERTTULA, S., JIN, Y. AND ANDERSON, K. V. (1988). Zygotic expression and activity of the *Drosophila Toll* gene, a gene required maternally for embryonic dorsal–ventral pattern formation. *Genetics* **119**, 123–133.

GHOSH, S., GIFFORD, A. M., RIVIERE, L. R., TEMPST, P., NOLAN, G. P. AND BALTIMORE, D. (1990). Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. *Cell* **62**, 1019–1029.

HARLOW, E. AND LANE, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory.

HASHIMOTO, C., HUDSON, K. L. AND ANDERSON, K. V. (1988). The *Toll* gene of *Drosophila*, required for dorsal–ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52**, 269–279.

HSU, S.-M. AND SOBAN, E. (1982). Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. *J. Histochem. Cytochem.* **30**, 1079–1082.

JIN, Y. AND ANDERSON, K. V. (1990). Dominant and recessive alleles of the *Drosophila easter* gene are point mutations at conserved sites in the serine protease catalytic domain. *Cell* **60**, 873–881.

KIERAN, M., BLANK, V., LOGEAT, F., VANDEKERCKHOVE, J., LOTTSPEICH, F., LE BAIL, O., URBAN, M. B., KOURILSKY, P., BAEUERLE, P. A. AND ISRAËL, A. (1990). The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell* **62**, 1007–1018.

KING, R. C. (1970). *Ovarian development in* Drosophila melanogaster. New York: Academic.

KLEID, D. G., YANSURA, D., SMALL, B., DOWBENKO, D., MOORE, D. M., GRUBMAN, M. J., MCKERCHER, P. D., MORGAN, D. O., ROBERTSON, B. H. AND BACHRACH, H. L. (1981). Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. *Science* **214**, 1125–1129.

KLINGLER, M., ERDÉLYI, M., SZABAD, J. AND NÜSSLEIN-VOLHARD, C. (1988). Function of *torso* in determining the terminal anlagen of the *Drosophila* embryo. *Nature* **335**, 275–277.

KRANTZ, D. E. AND ZIPURSKY, S. L. (1990). *Drosophila* chaoptin, a member of the leucine-rich repeat family, is a photoreceptor cell-specific adhesion molecule. *EMBO J.* **9**, 1969–1977.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

MAHOWALD, A. P. AND KAMBYSELLIS, M. P. (1980). Oogenesis. In *The Genetics and Biology of* Drosophila, vol. 2 (ed. M. Ashburner and T. R. F. Wright), pp. 141–224. London: Academic.

MANSEAU, L. J. AND SCHÜPBACH, T. (1989). *cappuccino* and *spire*: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the *Drosophila* embryo. *Genes Dev.* **3**, 1437–1452.

MATSUDAIRA, P. T. AND BURGESS, D. R. (1978). SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* **87**, 386–396.

NILSSON, B., ABRAHMSÉN, L. AND UHLÉN, M. (1985). Immobilization and purification of enzymes with staphylococcal protein A gene fusion vectors. *EMBO J.* **4**, 1075–1080.

NÜSSLEIN-VOLHARD, C., FROHNHÖFER, H. G. AND LEHMANN, R. (1987). Determination of anteroposterior polarity in *Drosophila*. *Science* **238**, 1675–1681.

PATEL, N. H., MARTIN-BLANCO, E., COLEMAN, K. G., POOLE, S. J., ELLIS, M. C., KORNBERG, T. B. AND GOODMAN, C. S. (1989).

Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* **58**, 955–968.

PATEL, N. H., SNOW, P. M. AND GOODMAN, C. S. (1987). Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* **48**, 975–988.

REINKE, R., KRANTZ, D. E., YEN, D. AND ZIPURSKY, S. L. (1988). Chaoptin, a cell surface glycoprotein required for *Drosophila* photoreceptor cell morphogenesis, contains a repeat motif found in yeast and human. *Cell* **52**, 291–301.

ROTH, S., STEIN, D. AND NÜSSLEIN-VOLHARD, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59**, 1189–1202.

RUSHLOW, C., FRASCH, M., DOYLE, H. AND LEVINE, M. (1987). Maternal regulation of *zerknüllt*: a homoeobox gene controlling differentiation of dorsal tissues in *Drosophila*. *Nature* **330**, 583–586.

RUSHLOW, C. A., HAN, K., MANLEY, J. AND LEVINE, M. (1989). The graded distribution of the *dorsal* morphogen is inititiated by selective nuclear transport in *Drosophila*. *Cell* **59**, 1165–1177.

SCHNEIDER, D. S., HUDSON, K. L., LIN, T.-Y. AND ANDERSON, K. V. (1991). Dominant and recessive mutations define functional domains of *Toll*, a transmembrane protein required for dorsal–ventral polarity in the *Drosophila* embryo. *Genes Dev.* (in press).

SCHÜPBACH, T. (1987). Germ line and soma cooperate during oogenesis to establish the dorsoventral patterns of egg shell and embryo in *Drosophila melanogaster*. *Cell* **49**, 699–707.

SPRENGER, F., STEVENS, L. M. AND NÜSSLEIN-VOLHARD, C. (1989). The *Drosophila* gene *torso* encodes a putative receptor tyrosine kinase. *Nature* **338**, 478–483.

STEVENS, L. M., FROHNHÖFER, H. G., KLINGLER, M. AND NÜSSLEIN-VOLHARD, C. (1990). Localized requirement for *torso-like* expression in follicle cells for development of terminal anlagen of the *Drosophila* embryo. *Nature* **346**, 660–663.

STEWARD, R. (1987). *Dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, c-*rel*. *Science* **238**, 692–694.

STEWARD, R. (1989). Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. *Cell* **59**, 1179–1188.

ST JOHNSTON, R. D. AND GELBART, W. M. (1987). *Decapentaplegic* transcripts are localized along the dorsal–ventral axis of the *Drosophila* embryo. *EMBO J.* **6**, 2785–2791.

STRECKER, T. R., HALSELL, S. R., FISHER, W. W. AND LIPSHITZ, H. D. (1989). Reciprocal effects of hyper- and hypoactivity mutations in the *Drosophila* pattern gene *torso*. *Science* **243**, 1062–1066.

TAKAHASHI, N., TAKAHASHI, Y. AND PUTNAM, F. W. (1985). Periodicity of leucine and tandem repetition of a 24-amino acid segment in the primary structure of leucine-rich α_2-glycoprotein of human serum. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1906–1910.

TARENTINO, A. L., GOMEZ, C. M. AND PLUMMER, JR, T. H. (1985). Deglycosylation of asparagine-linked glycans by peptide:N-glycosidase F. *Biochem.* **24**, 4665–4671.

THISSE, B., STOETZEL, C., EL MESSAL, M. AND PERRIN-SCHMITT, F. (1987). Genes of the *Drosophila* maternal dorsal group control the specific expression of the zygotic gene *twist* in presumptive mesodermal cells. *Genes Dev.* **1**, 709–715.

TOWBIN, H., STAEHELIN, T. AND GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350–4354.

VAN VACTOR, JR, D., KRANTZ, D. E., REINKE, R. AND ZIPURSKY, S. L. (1988). Analysis of mutants in chaoptin, a photoreceptor cell-specific glycoprotein in *Drosophila*, reveals its role in cellular morphogenesis. *Cell* **52**, 281–290.

WILCOX, M. (1986). Cell Surface Antigens. In Drosophila: *a practical approach* (ed. D. B. Roberts), pp. 243–274. Oxford: IRL.

(*Accepted 11 January 1991*)