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, 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 dorsализed 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. 1985b). 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. 1985b). Mothers homozygous for a recessive allele produce dorsализed 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. 1985a). 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. 1985b) 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. 1985a; 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 \(135 \times 10^3\) M, 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 BamHI 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 BamHI–HindIII 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 \(\mu\)g) was mixed with adjuvant (RIBI Immunocen Research) before being injected into a rabbit. After 4 weeks, the rabbit was injected twice more with 100–150 \(\mu\)g 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)Tol9QRX/Df(3R)roX1 (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 [35S]methionine (50 mM Ci m−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 MgCl2, 1 mM CaCl2, 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 mM 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 mM 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 (Pate1 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 Na2CO3 pH 11.5 at 4°C for 1 h (Fujiki et al., 1982). Membranes were then pelleted by centrifugation at 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 CoCl2 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 [35S]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 135×103 M₀ (Fig. 1B, lane 2). Antibodies against the Toll protein’s N-terminal region also precipitated a 135×103 M₀ 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 135×103 M₀ 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 135×103 M₀ 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 135×103 M₀ polypeptide in protein blots of total embryonic glycoproteins isolated by lentil lectin chromatography (data not shown; Wilcox, 1986). To test further whether the immunoprecipitated 135×103 M₀ 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 Mr 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 [35S]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 (×10^-3) of protein standards. (C) The 135×10^3 Mr 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 Mr polypeptide to a smaller form of 120×10^3 Mr (Fig. 1C, cf. lanes 1 and 2). The latter is about the expected size of the primary translation product (125×10^3 Mr) 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 immunoprecipitates 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 Mr polypeptide suggested that the latter was not disulfide bonded to another radiolabeled polypeptide. To test, however, if the 135×10^3 Mr polypeptide forms a disulfide-linked dimer, 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. 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 punctuated 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, CoCl₂ 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 joint 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 135×10^6 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 135×10^6 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 135×10^6 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. 1985a). 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; Thissel 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 pseudo cleavage 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, 1988).

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, proctodaeum, 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 reactions 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 perivitelline 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.

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