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

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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 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. 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–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 dorsalized embryos, whereas mothers carrying a dominant allele

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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 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. 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, easer 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 $135 \times 10^3 \, M_r$, 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 $225 \, 000 \, bp$ fragment from a Toll cDNA (Hashimoto et al. 1988) into the plasmid pRIT21 (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 Immunoclon 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)TolV/+/Df(3R)ro e1022 (Hashimoto et al. 1988).
Embryos were collected over a 1 h period from flies at 22°C and then injected with [35S]methionine (50 mCi/ml, Amer-sham). 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 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 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 Schuell) 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 diamino-benzidine (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 M, (Fig. 1B, lane 2). Antibodies against the Toll protein's N-terminal region also precipitated a 135×103 M 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 M 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 M 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 M 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 M 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
with this enzyme converted the $135\times10^3 M_r$ polypeptide to a smaller form of $120\times10^3 M_r$ (Fig. 1C, cf. lanes 1 and 2). The latter is about the expected size of the primary translation product ($125\times10^3 M_r$) 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 $135\times10^3 M_r$ polypeptide suggested that the latter was not disulfide bonded to another radiolabeled polypeptide. To test, however, if the $135\times10^3 M_r$ 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 $\beta$-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 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 mem-
branes 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 anti-
odies against the protein’s extracytoplasmic N ter-
minus 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 cyto-
plasm (Anderson et al. 1985a). Second, the tempera-
ture-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 uni-
formly 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 morpho-
logical 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 epider-
mis 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 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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