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Ventralization of the *Drosophila* Embryo by Deletion of Extracellular Leucine-rich Repeats in the Toll Protein

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Dorsoventral polarity of the *Drosophila* embryo is established by a signal transduction pathway in which the maternal transmembrane protein Toll appears to function as the receptor for a ventrally localized extracellular ligand. Certain dominant *Toll* alleles encode proteins that behave as partially ligand-independent receptors, causing embryos containing these proteins to become ventralized. In extracts of embryos derived from mothers carrying these dominant alleles, we detected a polypeptide of ~35 kDa in addition to full-length Toll polypeptides with antibodies to Toll. Our biochemical analyses suggest that the smaller polypeptide is a truncated form of Toll lacking extracellular domain sequences. To assay the biological activity of such a shortened form of Toll, we synthesized RNA encoding a mutant polypeptide lacking the leucine-rich repeats that comprise most of Toll's extracellular domain and injected this RNA into embryos. The truncated Toll protein elicited the most ventral cell fate independently of the wild-type Toll protein and its ligand. These results support the view that Toll is a receptor whose extracellular domain regulates the intrinsic signaling activity of its cytoplasmic domain.

INTRODUCTION

Dorsoventral polarity of the *Drosophila* embryo is established shortly after fertilization, when the embryo is still a syncytium of many nuclei, with the formation of a nuclear concentration gradient of the dorsal protein. Initially, the maternally encoded dorsal protein is uniformly distributed in the cytoplasm, but then is directed to the nucleus in ventral regions of the embryo (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989). The dorsal protein is a member of the *rel* proto-oncogene family of DNA-binding proteins found in vertebrate and invertebrate organisms (Govind and Steward, 1991). It activates and represses the transcription of some of the earliest zygotic genes to be expressed during embryonic development. The dorsal protein's graded nuclear concentration thus directs regional expression of zygotic gene products necessary for dorsoventral patterning of the embryo (Ferguson and Anderson, 1991).

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Nuclear localization of the dorsal protein is the last step in a signal transduction pathway that begins with the production of an extracellular signal in the perivitelline space, the compartment between the innermost eggshell layer and the embryonic plasma membrane (reviewed in Chasan and Anderson, 1993). Twelve maternal gene products function in this pathway, including the transmembrane Toll protein, which is required to transmit the extracellular signal to the interior of the embryo (Hashimoto *et al.*, 1988, 1991). The extracellular signal appears to be the ligand for the Toll protein (Stein *et al.*, 1991). Two genes that act genetically upstream of *Toll* encode extracellular serine proteases necessary for processing of the spätzle protein, the likely precursor of Toll's ligand (Anderson *et al.*, 1985b; DeLotto and Spierer, 1986; Chasan and Anderson, 1989; Morisato and Anderson, 1994; Schneider *et al.*, 1994). Other genes that act genetically downstream of *Toll* encode molecules that function in the cytoplasm to interpret the signal transmitted by Toll (Letsou *et al.*, 1991; Roth *et al.*, 1991; Geisler *et al.*, 1992; Kidd, 1992; Hecht and Anderson, 1993; Shelton and Wasserman, 1993). Because Toll's

concentration in the embryonic plasma membrane is uniform (Hashimoto *et al.*, 1991) yet dorsal's nuclear concentration is graded, Toll's transmembrane signaling activity appears to be spatially regulated, perhaps by localized production of its ligand (Stein *et al.*, 1991). How this reaction is spatially restricted is still mysterious, but may involve the preferential activation of serine proteases required to produce Toll's ligand on the ventral side of the embryo (Chasan *et al.*, 1992; Smith and DeLotto, 1994).

How Toll functions biochemically in transmembrane signaling is unknown. Toll is a 135-kDa transmembrane protein with a large extracellular domain and a smaller cytoplasmic domain of 269 amino acids (Hashimoto *et al.*, 1988; see Figure 1). Thus, Toll could function as a signal transducing receptor that binds a ligand with its extracellular domain and activates intracellular signaling through its cytoplasmic domain. Toll's extracellular domain is composed mostly of a structural motif called the leucine-rich repeat that has been found in transmembrane receptors and many other proteins with different biological functions and cellular locations (Hashimoto *et al.*, 1988; see references in Kobe and Deisenhofer, 1994). The three-dimensional structure of ribonuclease inhibitor, which consists mostly of leucine-rich repeats, suggests that these repeats in Toll could form a flexible binding surface suitable for strong and specific interaction with a protein ligand (Kobe and Deisenhofer, 1993). Toll's cytoplasmic domain is structurally similar to that of the mammalian interleukin-1 (IL-1) receptor, a key participant in inflammatory reactions and the immune response (Sims *et al.*, 1988; Gay and Keith, 1991; Schneider *et al.*, 1991). The cytoplasmic domains of Toll and the IL-1 receptor do not resemble tyrosine kinases or other known enzymes, so how these two proteins function in signal transduction is not clear. However, Toll and the IL-1 receptor likely participate in similar intracellular signaling pathways, as the molecules that function downstream of these proteins have been evolutionary conserved (Wasserman, 1993).

To understand how Toll functions in transmembrane signaling, we began biochemical analyses of the proteins encoded by certain dominant gain-of-function Toll alleles. These alleles encode proteins in which one of four extracellular cysteines near the transmembrane domain is converted to a tyrosine (Schneider *et al.*, 1991; see Figure 1). In contrast to the wild-type Toll protein, these mutant proteins are active in all dorsoventral regions, regardless of the presence or absence of the wild-type protein or its putative ligand (Anderson *et al.*, 1985b; Schneider *et al.*, 1991). Thus, embryos carrying one of these mutant proteins become ventralized, as the dorsal protein is induced to enter nuclei at all dorsoventral positions and cells adopt the fates of more ventral cells in the wild-type embryo (Anderson *et al.*, 1985b; Steward, 1989; Roth *et al.*, 1989). Using antibodies to isolate the mutant Toll proteins from embryonic extracts, we detected a 35-kDa polypeptide that appears to be a truncated Toll molecule missing extracellular domain sequences. A form of Toll lacking the normal extracellular domain that we created by in vitro mutagenesis and introduced into embryos by RNA injection behaves as a dominant gain-of-function mutant, eliciting the most ventral cell fate in the presence or absence of the wild-type Toll protein or Toll's ligand. These results suggest that Toll functions as a receptor whose intracellular signaling activity is regulated by its extracellular domain.

MATERIALS AND METHODS

Embryos

Embryos were collected as follows: embryos containing normal levels of the wild-type Toll protein (wt) were collected from Oregon R females; embryos lacking the Toll protein (Tl$^-$) from Df(3R)Tl^{9QRX}/Df(3R)ro^{XB3} females (Hashimoto *et al.*, 1988); embryos containing the protein encoded by a dominant *Toll* allele from females carrying the Tl^{10b}, Tl^1, or Tl^{9Q} allele in the presence of one copy of the wild-type *Toll* gene or in trans to Df(3R)*ro80b*, a deficiency that uncovers *Toll* and fails to produce detectable Toll protein (Anderson *et al.*, 1985b; Hashimoto *et al.*, 1988; Erdélyi and Szabad, 1989); and embryos containing the protein encoded by the Tl^{10b} allele but lacking the normal activity of the *nudel* or *tube* gene product from ndl^4 Tl^{10b}/ndl^3 and $tub^{R5.6}$ Tl^{10b}/tub^1 females. The chromosomes carrying both the Tl^{10b} mutation and the ndl^4 or $tub^{R5.6}$ mutation were constructed by Kathryn Anderson (University of California, Berkeley, CA). Flies with the genotypes $spz^{D1\text{-}RPQ}$/*Df(3R)* $Tl^{84c\text{-}RXD}$ (Morisato and Anderson, 1994) and tub^1/tub^2 (Letsou *et al.*, 1991) were used to produce embryos lacking the normal activity of the *spätzle* and *tube* gene products.

Immunoprecipitation

Injection of embryos with [^{35}S]methionine (Amersham, Arlington Heights, IL), preparation of embryonic homogenates, immunoprecipitation with affinity-purified antibodies coupled to protein A-Sepharose (Pharmacia, Piscataway, NJ), visualization of proteins in 5–15% gradient polyacrylamide gels, and deglycosylation of proteins with peptide *N*-glycosidase F (*N*-glycanase; Genzyme, Cambridge, MA) have all been described previously (Hashimoto *et al.*, 1991).

In the sequential immunoprecipitation experiments shown in Figure 3A, the following procedure was used. The immunoprecipitates obtained under nondenaturing conditions as described (Hashimoto *et al.*, 1991) were boiled for 5 min in 1% sodium dodecyl sulfate (SDS) to release proteins bound to the protein A-Sepharose beads. The proteins released by this treatment were diluted into a 10-fold greater volume of NDET buffer (1% NP-40, 0.4% sodium deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4) and then subjected to a second immunoprecipitation. In this case, the protein A-Sepharose beads were washed with NDET buffer containing 0.1% SDS.

Construction of ΔLRR

The plasmid encoding ΔLRR was derived from pTl, a pGem2 plasmid (Promega, Madison, WI) containing a full-length cDNA encoding the Toll protein (Hashimoto, unpublished data; Schneider *et al.*, 1991). The following strategy was used to construct the ΔLRR plasmid. Two DNA fragments were synthesized by PCR using pTl DNA as the template and the following oligonucleotides: for fragment 1, 5′-CTGCAG**ATGCAT**CTGTAGTGG-3′ (473–493; *Nsi*I) and 5′-CCGCCG**GGATCC**GTGCAAACGGTCCAAGTG-3′ (1007–1024;

*Bam*HI); for fragment 2, 5′-CCGCCG**GGATCC**GAAAAGGGCGT-GTTCATAG-3′ (2978–2996; *Bam*HI) and 5′-TCAAGT**AGGCCT**-TCAGC-3′ (3475–3491; *Stu*I). The underlined sequences are identical or complementary to sequences in the sense strand of the full-length *Toll* cDNA at the nucleotide positions indicated in parentheses (Hashimoto *et al.*, 1988). The sequences in bold lettering refer to the restriction sites, also indicated in parentheses, which are unique in a given fragment. After fragment 1 was digested near its termini with *Nsi*I and *Bam*HI, and fragment 2 near its termini with *Bam*HI and *Stu*I, the two fragments were inserted into the gap left by digesting pTl with *Nsi*I and *Stu*I. The result of this final step was the plasmid encoding the ΔLRR protein, which has the sequence glycine-serine (encoded by the *Bam*HI site) in place of amino acids 151–801 in Toll's extracellular domain (Hashimoto *et al.*, 1988; see Figure 1). DNA sequence analyses verified that the region of the ΔLRR plasmid generated by PCR did not contain any adventitious mutation.

RNA Injection into Embryos

The plasmid encoding the ΔLRR protein was linearized with *Sma*I, and SP6 transcripts were synthesized from the linearized DNA template essentially as described earlier (Schneider *et al.*, 1991). The amount of RNA synthesized was estimated by ultraviolet spectrophotometry. Embryos aged 0–1 h were collected, dechorionated, and injected essentially as described previously (Anderson and Nüsslein-Volhard, 1984; Anderson *et al.*, 1985a; Chasan and Anderson, 1989; Shelton and Wasserman, 1993). After injection, embryos were allowed to develop for 3 h at 22°C, then were fixed and probed with a 1:5000 dilution of antibodies against the twist protein (Stein *et al.*, 1991; Ferguson and Anderson, 1992).

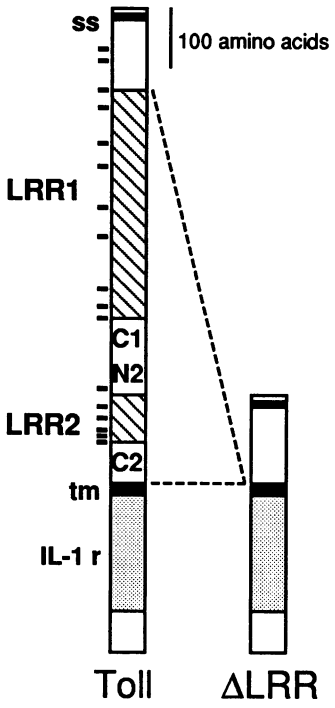

Figure 1. Schematics of Toll and the truncated protein ΔLRR. The black boxes represent hydrophobic amino acids of putative signal sequence (ss) and transmembrane domain (tm), the hatched boxes represent the two blocks of leucine-rich repeats (LRR1 and LRR2), and the shaded box represents the region of structural similarity between the cytoplasmic domains of Toll and the IL-1 receptor (IL-1 r). C1, N2, and C2 are regions with a characteristic spacing of cysteines flanking the N- or C-terminus of leucine-rich repeats in some proteins (Kobe and Deisenhofer, 1994). In each of the Tl^{10b}, Tl^{1}, and Tl^{9Q} alleles, a different one of the four cysteines in the C2 region is mutated to a tyrosine (Schneider *et al.*, 1991). Each short horizontal bar represents one of the 17 potential *N*-linked glycosylation sites in Toll. The dashed lines define the region of Toll's extracellular domain that was deleted to make the ΔLRR protein (see MATERIALS AND METHODS).

RESULTS

A 35-kDa Polypeptide Is Present in Immunoprecipitates Containing Toll Proteins Encoded by Dominant Gain-of-Function Alleles

Each of the dominant *Toll* alleles Tl^{10b}, Tl^{1}, and Tl^{9Q} encodes a protein in which a different one of four extracellular cysteines near the transmembrane domain is mutated to a tyrosine (Schneider *et al.*, 1991). Female flies heterozygous for any of these alleles produce ventralized embryos (Anderson *et al.*, 1985b; Erdélyi and Szabad, 1989). In these embryos, the mutant Toll protein appears to be active at all dorsoventral positions regardless of the presence or absence of Toll's putative ligand. Tl^{10b} produces a stronger ventralized phenotype than either Tl^{1} or Tl^{9Q}, which appear to be equal in ventralizing activity. Embryos aged 0–1 h post-fertilization from mothers carrying the Tl^{10b} allele (either Tl^{10b}/Tl^{+} or Tl^{10b}/Tl^{-}; see MATERIALS AND METHODS) were injected with [^{35}S]methionine, incubated for 1 h to allow incorporation of the radiolabel into proteins, and then homogenized in buffer containing nonionic detergent. The Toll protein in the homogenate was precipitated with antibodies directed against Toll's cytoplasmic domain (Hashimoto *et al.*, 1991).

A polypeptide of ~135 kDa, the size of the wild-type Toll protein, was seen in the immunoprecipitate from embryonic extracts containing the protein encoded by the Tl^{10b} allele (Figure 2A, cf. lanes 2 and 3). In addition, a polypeptide of ~35 kDa was detectable in the immunoprecipitate (Figure 2A, lane 3). This polypeptide was not immunoprecipitated from extracts of embryos from Tl^{-}/Tl^{-} or wild-type mothers (Figure 2A, lanes 1 and 2). Detection of the 35-kDa polypeptide did not depend on the presence of the wild-type Toll protein, as this polypeptide was immunoprecipitated from extracts of embryos from Tl^{10b}/Tl^{-} (Figure 2C, lane 3) as well as Tl^{10b}/Tl^{+} (Figure 2A, lane 3) mothers.

A 35-kDa polypeptide was detectable in immunoprecipitates containing the proteins encoded by the alleles Tl^{1} (Figure 2B, lane 2) and Tl^{9Q} (our unpublished results). However, the amount of the 35-kDa polypeptide relative to the 135-kDa Toll protein was higher in the Tl^{10b} than in the Tl^{1} or Tl^{9Q} embryos (Figure 2B, cf. lanes 2 and 3). Because the 35-kDa polypeptide appeared to be most abundant in extracts containing the protein encoded by the Tl^{10b} allele, we further analyzed this polypeptide from these extracts.

The 35-kDa polypeptide was also immunoprecipitated from extracts of embryos from mothers carrying the Tl^{10b} allele and mutant for the *nudel* gene (Figure 2C, lane 1). The *nudel* gene is required genetically upstream of *Toll* (Anderson *et al.*, 1985b), and its product appears to function in activating the serine protease cascade necessary to generate the putative li-

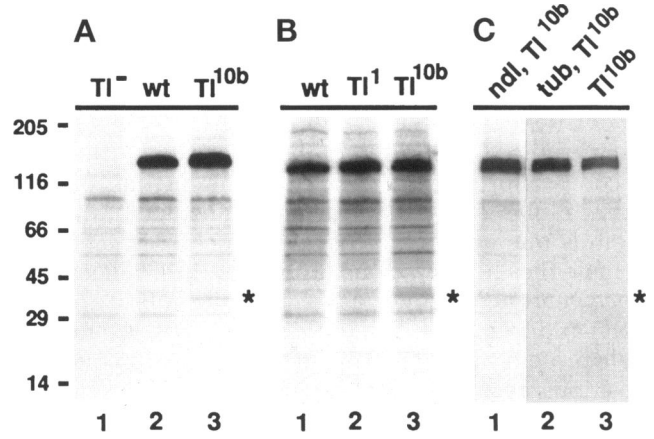

Figure 2. A 35-kDa polypeptide is present in immunoprecipitates containing dominant mutant Toll proteins. Extracts of embryonic proteins labeled in vivo with [^{35}S]methionine were incubated with antibodies to Toll's cytoplasmic domain, and immunoprecipitated proteins were fractionated in SDS-polyacrylamide gels and visualized by fluorography. The migration position of the 35-kDa polypeptide is marked with an asterisk. The numbers at the left refer to the polypeptide standards ($\times 10^{-3}$ kDa). (A) Proteins immunoprecipitated from extracts of embryos from Tl^{-}/Tl^{-} (lane 1), wild-type (lane 2), and Tl^{10b}/Tl^{+} (lane 3) mothers are shown. The full-length Toll protein of ~135 kDa is present in the Tl^{10b} embryos (lane 3). In addition, a polypeptide of ~35 kDa, not detectable in Tl^{-} or wild-type embryos (lanes 1 and 2), is visible. (B) The 35-kDa polypeptide is present in Tl^{1} embryos (lane 2). It appears to be more abundant in Tl^{10b} embryos (lane 3), but is not detectable in wild-type embryos (lane 1). (C) The 35-kDa polypeptide is detectable in extracts of embryos from mothers mutant for the *nudel* (lane 1) or the *tube* (lane 2) gene in addition to being heterozygous for the Tl^{10b} mutation, and from Tl^{10b}/Tl^{-} mothers (lane 3).

gand for the Toll protein (Stein *et al.*, 1991; Chasan *et al.*, 1992; Morisato and Anderson, 1994; Smith and DeLotto, 1994). Also, as shown in Figure 2C (lane 2), the 35-kDa polypeptide was equally present in embryos from mothers carrying the Tl^{10b} allele and mutant for the *tube* gene, which acts genetically downstream of *Toll* (Hecht and Anderson, 1993). These results show that the presence of the 35-kDa polypeptide in embryos is dependent on the presence of the protein encoded by the dominant *Toll* allele but not the wild-type Toll protein or maternal components that function genetically before and after *Toll*.

Identification of the 35-kDa Polypeptide as a Truncated Toll Molecule Containing Cytoplasmic Domain Sequences and Carbohydrate

We wondered if the 35-kDa polypeptide was immunoprecipitated because it is a truncated form of the full-length Toll polypeptide encoded by the dominant alleles and therefore is recognized by the antibodies against Toll's cytoplasmic domain, or because it is a distinct protein bound noncovalently or via a disulfide linkage to the dominant mutant Toll proteins. To distinguish between these possibilities, we performed the following sequential immunoprecipitation experiments. First, the antibodies against Toll's cytoplasmic domain were used to precipitate the 35-kDa polypeptide and the protein encoded by the Tl^{10b} allele from embryonic extracts under nondenaturing conditions as described above. Second, these proteins were dissociated from the antibodies bound to protein A-Sepharose beads by heating the beads in 1% SDS and were subjected to immunoprecipitation with the antibodies against either Toll's N-terminus or Toll's cytoplasmic domain (see MATERIALS AND METHODS). The SDS treatment should dissociate any protein noncovalently bound to the Toll protein.

As shown in Figure 3A (lane 2), both the 35-kDa polypeptide and the full-length mutant Toll protein were precipitated by antibodies against Toll's cytoplasmic domain after being subjected to SDS treatment. The 35-kDa polypeptide appeared to be less efficiently precipitated compared with the full-length Toll protein during the second immunoprecipitation

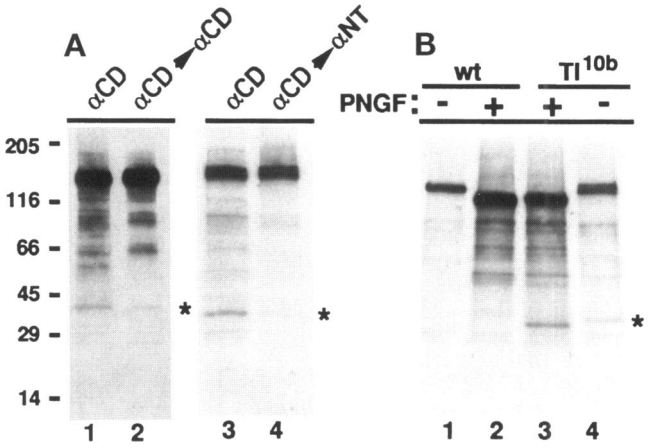

Figure 3. The 35-kDa polypeptide reacts with antibodies to Toll's cytoplasmic domain and appears to be glycosylated. (A) Proteins precipitated from Tl^{10b} extracts with antibodies to Toll's cytoplasmic domain (lanes 1 and 3) were released from the antibodies by treatment with 1% SDS, and the released proteins were incubated with either antibodies to Toll's cytoplasmic domain (lane 2) or antibodies to Toll's N-terminus (lane 4). The antibodies to Toll's cytoplasmic domain precipitate the 35-kDa polypeptide after the SDS treatment, which suggests that this polypeptide is directly recognized by the antibodies. (B) The proteins released from the antibodies by SDS treatment as in part A above were either mock treated (lane 4) or treated with peptide *N*-glycosidase F (PNGF), which removes *N*-linked carbohydrate (lane 3). The 35-kDa polypeptide appears to be converted to a faster migrating form by the enzymatic treatment, which suggests that it is glycosylated. Immunoprecipitated proteins from wild-type extracts that were subjected to the same treatments are also shown (lanes 1 and 2). The increase in intensities of the bands corresponding to the full-length Toll protein and the 35-kDa polypeptide after enzymatic treatment may partially reflect the conversion of polypeptides with slightly varying molecular weights, caused by heterogeneity in glycosylation, to forms with a uniform molecular weight.

step, but the reason for this discrepancy was not clear (Figure 3A, lanes 1 and 2). Nonetheless, the immunoprecipitation of both the 35-kDa polypeptide and the full-length mutant Toll protein after SDS treatment indicates that the 35-kDa polypeptide is unlikely to be a distinct protein bound noncovalently to the full-length mutant protein. If the 35-kDa polypeptide is a distinct protein disulfide-bonded to the full-length mutant protein, then it should also be precipitated by antibodies against Toll's N-terminus, which precipitate the full-length mutant protein. However, the 35-kDa polypeptide was not precipitated after SDS treatment by antibodies against Toll's N-terminus, although the full-length protein was precipitated under the same conditions (Figure 3B, lanes 3 and 4). The results of these sequential immunoprecipitation experiments indicate that the 35-kDa polypeptide is specifically recognized by antibodies to Toll's cytoplasmic domain. Thus, the 35-kDa polypeptide appears to be a severely truncated form of the protein encoded by the Tl^{10b} allele, lacking this protein's N-terminal extracellular sequences but containing its cytoplasmic domain sequences.

In Western blot analysis of embryonic extracts containing the Tl^{10b}-encoded protein, a 35-kDa polypeptide was detectable with antibodies to Toll's cytoplasmic domain, but not with antibodies to Toll's N-terminus (our unpublished experiments). However, Western blot analysis was much less sensitive than the immunoprecipitation method described above for detecting this polypeptide. For this reason, we used immunoprecipitation to detect and isolate the 35-kDa polypeptide for subsequent experiments.

The immunoprecipitated proteins released by SDS treatment were also treated with peptide *N*-glycosidase F, which removes all *N*-linked carbohydrate from glycoproteins (Tarentino *et al.*, 1985). As shown in Figure 3B (lanes 1 and 2 with lanes 3 and 4), treatment with this enzyme converted the full-length Toll protein encoded by the Tl^{10b} allele to a form that migrated similarly to the deglycosylated form of the wild-type protein (Hashimoto *et al.*, 1991). Interestingly, enzymatic treatment also converted the 35-kDa polypeptide to a form that migrated slightly faster in gels, behavior consistent with this molecule bearing *N*-linked carbohydrate (Figure 3B, lanes 3 and 4). This result suggests that the 35-kDa polypeptide is glycosylated and hence contains some extracellular domain sequences of Toll (see Figure 1 for potential *N*-linked glycosylation sites in Toll).

Intracellular Signaling Activity of a Truncated Toll Polypeptide Lacking Extracellular Leucine-rich Repeats

Our identification of the 35-kDa polypeptide as a truncated form of the Tl^{10b}-encoded protein raised the possibility that the truncated polypeptide is responsible for the unregulated Toll activity that ventralizes embryos, analogous to tyrosine kinase receptors whose intracellular signaling functions are activated by truncations in their extracellular domains (Ullrich and Schlessinger, 1990). To test if Toll's intracellular signaling activity could be similarly activated, we constructed a mutant protein that lacks the leucine-rich repeats and some flanking regions that comprise most of Toll's extracellular domain but retains the putative signal sequence (ΔLRR; Figure 1). We synthesized and injected SP6 RNA encoding the mutant protein into embryos missing the wild-type Toll protein. Embryos lacking the maternal Toll protein become dorsalized, because they fail to make ventral structures (Anderson *et al.*, 1985b). However, if these embryos are injected with wild-type cytoplasm or Toll RNA, they will produce ventral structures (Anderson *et al.*, 1985a; Hashimoto *et al.*, 1988; Roth, 1993; Hashimoto, unpublished data).

To score for rescue of ventral structures, we probed injected embryos with antibodies against the twist protein. Twist is normally expressed in the mesoderm, which represents the most ventral cell fate in the embryo (Thisse *et al.*, 1988); thus, twist expression is an unambiguous marker for strong rescue of injected embryos (Stein *et al.*, 1991). As shown in Figure 4, wild-type embryos express twist in the ventral-most cells, whereas embryos missing the Toll protein fail to express twist (Figure 4, panels A and B).

Embryos lacking Toll that were injected either dorsally or ventrally with RNA encoding the ΔLRR protein expressed twist at the injection site (Figure 4, C and D, and Table 1). Thus, like the wild-type Toll protein, the ΔLRR protein is capable of defining dorsoventral polarity by specifying the ventral side of the embryo (Anderson *et al.*, 1985a; Hashimoto *et al.*, 1988). ΔLRR did not elicit twist expression in embryos derived from mothers homozygous for the *tube* gene (Table 1), which acts genetically downstream of *Toll* (Hecht and Anderson, 1993). Thus, the ΔLRR protein appears to be functioning through the normal intracellular signaling pathway used to establish dorsoventral polarity. These results suggest that the intracellular signaling function of Toll does not require the presence of the leucine-rich repeats or an intact extracellular domain of the Toll protein.

Ligand-independent and Dominant Activities of the Truncated Toll Polypeptide

To test whether the ΔLRR protein activates intracellular signaling in the absence of Toll's putative ligand, we injected the ΔLRR transcript into embryos derived from mothers mutant for the *spätzle* gene (Morisato and Anderson, 1994). These embryos are dorsalized, presumably because they lack Toll's ligand produced

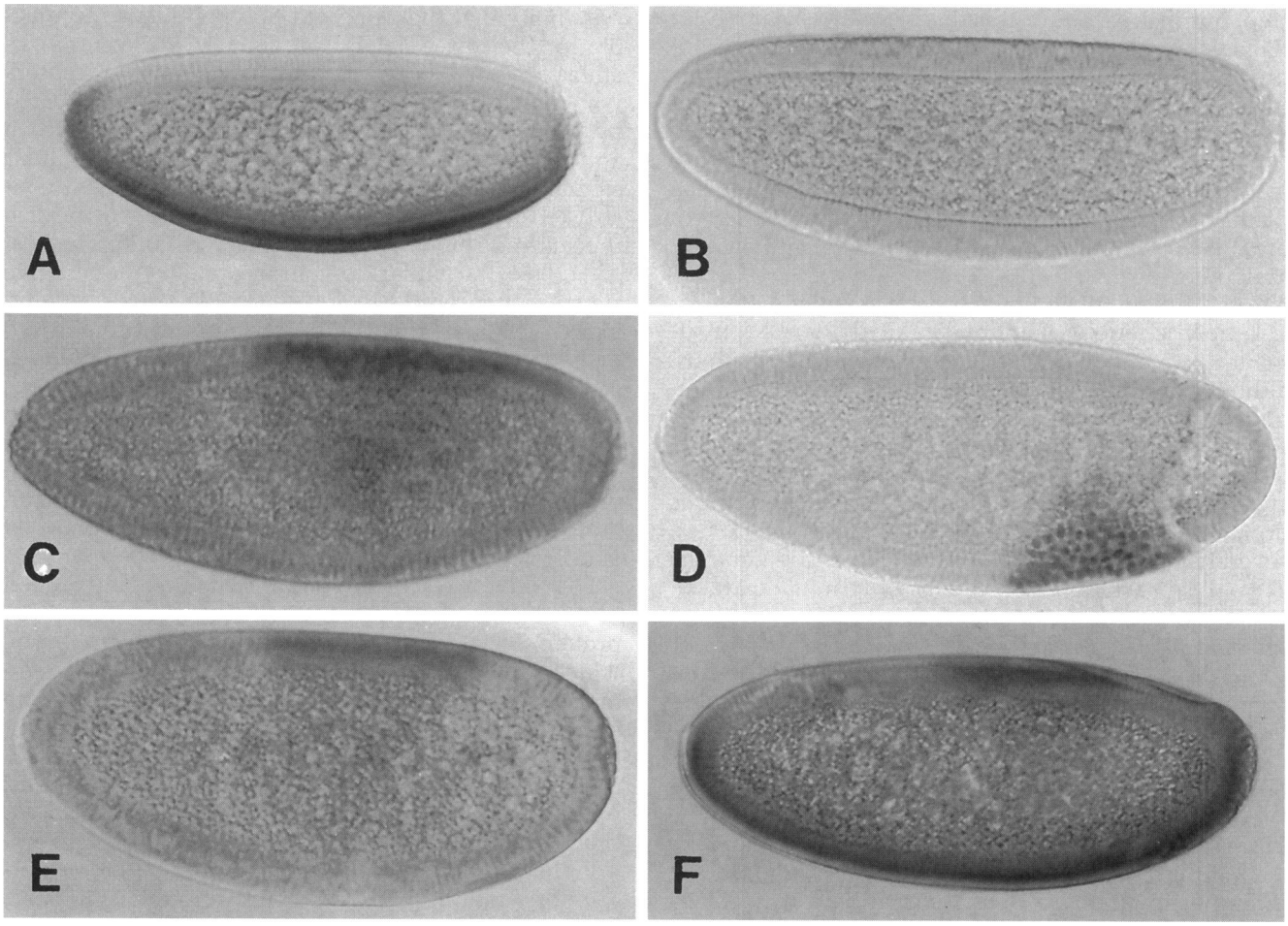

Figure 4. A truncated Toll polypeptide lacking extracellular sequences (ΔLRR) is constitutively active in embryos. SP6 RNA encoding the ΔLRR protein (see Figure 1) was injected into the dorsal or ventral side of embryos. Embryos were probed with antibodies to the twist protein, a marker for the most ventral cell fate. In all of the pictures, anterior of embryo is to the left and dorsal is up. (A) Uninjected embryo from wild-type mother shows twist expression in the ventral half. (B) Uninjected embryo missing the maternal Toll protein becomes dorsalized, so it fails to express twist. (C) Embryo lacking Toll injected dorsally expresses twist around the injection site. (D) Embryo lacking Toll injected ventrally expresses twist at the injection site. (E) Embryo lacking the spätzle protein (the putative precursor to Toll's ligand) becomes dorsalized and normally does not express twist, but does so when injected with the ΔLRR RNA as shown here. (F) Embryo from wild-type mother injected dorsally expresses twist at the injection site, and expresses twist along its ventral side as in uninjected embryo in panel A.

by proteolytic processing of the spätzle protein (Morisato and Anderson, 1994; Schneider *et al.*, 1994). Injection of the ΔLRR transcript into embryos from *spätzle*$^-$ mothers provoked twist expression at the injection site (Figure 4E and Table 1). These embryos were rescued by injection as efficiently as embryos from *Toll*$^-$ or wild-type mothers (Table 1), so the activity of the ΔLRR protein appears to be insensitive to the presence or absence of Toll's ligand.

Because the ΔLRR protein does not require ligand binding for its activity, we expected that it would be active on the dorsal side of the wild-type embryo where the Toll protein is present but apparently not activated. Localization of Toll's ligand on the ventral side of the embryo is presumed to prevent the Toll protein on the dorsal side from being active in intracellular signaling (see INTRODUCTION). Injection of ΔLRR RNA into the dorsal side of wild-type embryos, which are unperturbed by injection of wild-type Toll RNA (Schneider *et al.*, 1991), elicited twist expression at the injection site (Figure 4F and Table 1). Injection into the ventral side locally expanded the region that normally expresses twist (our unpublished results). Most of the injected embryos did not hatch as larvae, but did develop to the point of showing movement, indicative of muscle differentiation, and reduced amounts of ventrolateral and dorsolateral cuticular structures (our unpublished results). These results indicate that the ΔLRR protein behaves as a dominant mutant, capa-

Table 1. Twist expression in embryos injected with ΔLRR transcript

Recipient	Relative RNA concentration	Injection position	Twist expression (%)	Number of embryos injected
Toll$^-$	100	Ventral	96	26
	100	Dorsal	97	29
	10	Ventral	29	31
	10	Dorsal	34	41
	1	Ventral	0	22
	1	Dorsal	0	23
Spätzle$^-$	100	Ventral	96	46
	100	Dorsal	100	34
	10	Ventral	36	53
	10	Dorsal	38	64
	1	Ventral	0	31
	1	Dorsal	0	25
Wild type	100	Dorsal	97	31
Tube$^-$	100	Ventral	0	32
	100	Dorsal	0	21

Embryos injected with RNA encoding the ΔLRR protein were probed with antibodies to the twist protein, which is normally expressed in the mesoderm and therefore is an unambiguous marker for the most ventral cell fate in the embryo (Thisse *et al.*, 1988). RNA at 1600 μg/ml (= 100) or at 10- and 100-fold lower concentrations were injected either ventrally or dorsally at about 30% egg length (measured from the posterior end). The exact volume delivered in each embryo was not measured but likely did not exceed 200–300 pl (2–3% of egg volume; see MATERIALS AND METHODS for references).

ble of ventralizing the embryo in the presence of the wild-type Toll protein.

DISCUSSION

The maternally encoded Toll protein appears to play a critical role as the membrane receptor for a ventrally localized extracellular ligand in a transmembrane signaling pathway that establishes dorsoventral polarity of the *Drosophila* embryo. To understand how Toll functions in transmembrane signaling, we biochemically analyzed the proteins, encoded by dominant *Toll* alleles, that behave as partially ligand-independent receptors. In embryos containing these mutant proteins, we identified a 35-kDa polypeptide that appears to be a truncated form of the Toll protein lacking most of the extracellular domain. A similar form of Toll, created by in vitro mutagenesis and introduced into embryos, is constitutively active in generating an intracellular signal. Our results suggest that the Toll protein functions as a receptor whose intracellular signaling activity is controlled by its extracellular domain.

Relationship of the 35-kDa Polypeptide to Dominant Gain-of-Function Toll Alleles

Our immunoprecipitation analyses suggest that the 35-kDa polypeptide is a severely truncated yet membrane-bound form of the Toll protein. The 35-kDa polypeptide is recognized by antibodies against Toll's cytoplasmic domain, so it contains at least some cytoplasmic domain sequences. It is not recognized by antibodies raised against 75 amino acids near Toll's N-terminus, so it lacks at least these residues. More of the extracellular domain must be deleted, however, to produce the 35-kDa polypeptide, which is only slightly bigger than the cytoplasmic domain alone (~30 kDa). Some of the extracellular domain must also be retained, because the 35-kDa polypeptide appears to be glycosylated. Toll has 17 potential *N*-linked glycosylation sites in its extracellular domain (Figure 1; Hashimoto *et al.*, 1988). A polypeptide stretching from the glycosylation site nearest the transmembrane region to the cytoplasmic C-terminus would be about 40 kDa. Because of this slight discrepancy in size, we cannot rule out that the 35-kDa polypeptide is also missing some of Toll's cytoplasmic domain.

Because it is difficult to isolate enough of the 35-kDa polypeptide for direct peptide sequencing, we can only speculate that this polypeptide is a proteolytic degradation product of the full-length polypeptides encoded by the dominant gain-of-function *Toll* alleles. In each of these alleles, a different one of four cysteines adjacent to the transmembrane region is converted to a tyrosine (Schneider *et al.*, 1991; Figure 1). These mutations may disrupt intramolecular disulfide bonds required for proper folding of the extracellular domain. It is possible that disruption of these disulfide bonds unfolds a part of the protein, exposing certain residues to fortuitous proteolysis.

This interpretation of the 35-kDa polypeptide's origin may be consistent with the fact that the activities of the proteins encoded by the dominant alleles are not completely ligand-independent. Embryos carrying these mutant proteins, in the presence or absence of the wild-type protein, become ventralized; but if they are also lacking Toll's ligand, they become lateralized (Anderson *et al.*, 1985b). Ventralized embryos retain some dorsoventral polarity, implying that the activities of the mutant proteins are spatially asymmetric. Thus, these proteins are still responsive to Toll's ligand, which appears to be preferentially produced on the ventral side of the embryo (Stein *et al.*, 1991; Morisato and Anderson, 1994). In the absence of Toll's ligand, the intrinsic activities of the mutant proteins appear to be expressed uniformly at all dorsoventral positions to produce lateralized embryos lacking polarity. One interpretation of these observations is that the truncated 35-kDa polypeptide derived from the mutant proteins has ligand-independent Toll signaling activity, like the ΔLRR protein (Figure 4E and F, and Table 1), and is therefore responsible for the spatially uniform signaling that causes the lateralized phenotype in the absence of Toll's ligand. However, in the presence of Toll's ventrally localized ligand, the

full-length mutant proteins are also activated to produce the asymmetric Toll signaling activity that ventralizes the embryo.

Toll as Signal Transducing Receptor

The constitutive signaling activity of the ΔLRR protein (Figure 4, C–F, and Table 1) suggests that how the signaling activity of Toll's cytoplasmic domain is normally activated by an extracellular signal is analogous to the mechanism used to activate receptors with tyrosine kinase activity (reviewed in Ullrich and Schlessinger, 1990). Although Toll and receptor tyrosine kinases have structurally dissimilar cytoplasmic domains, both can be activated by large deletions in the extracellular domains. In both cases, the extracellular domain may inhibit the intrinsic signaling activity of the cytoplasmic domain, and this inhibition is relieved by ligand binding or by deletion of the extracellular ligand binding region. Because the cytoplasmic domains of Toll and the mammalian IL-1 receptor are structurally similar and likely to have similar biochemical activities, we would predict that deletion of extracellular domain sequences will activate the signaling function of the IL-1 receptor.

Ligand activation of receptor tyrosine kinases is thought to induce a conformational switch that dimerizes these receptors to bring their cytoplasmic domains together (reviewed in Ullrich and Schlessinger, 1990). The cytoplasmic domains with tyrosine kinase activity phosphorylate each other, and this phosphorylation activates the cytoplasmic domains to phosphorylate downstream signaling components. Despite the fact that Toll and receptor tyrosine kinases can both be activated by deletions in their extracellular domains, we have no direct evidence that Toll dimerizes or oligomerizes upon ligand binding. As all of the 18 cysteines in the wild-type Toll protein appear to be in intramolecular disulfide bonds (Hashimoto *et al.*, 1991), we thought initially that the unpaired cysteine in the dominant mutant proteins might form intermolecular disulfide bonds to produce homodimeric constitutively active receptors. This type of mutant has been described for the mammalian erythropoietin receptor. In this mutant protein, which is active independently of its ligand, an additional cysteine not found in the wild-type protein is used to form disulfide-linked dimers (Watowich *et al.*, 1992). However, using nonreducing gels, we have been unable to detect a disulfide-linked dimer of the protein encoded by the Tl^{10b} allele (our unpublished observations).

Although Toll may transmit an extracellular signal across the membrane similarly to receptor tyrosine kinases, it differs from these receptors by the apparent absence of enzymatic activity in its cytoplasmic domain. In this respect, Toll may function more analogously to the Notch protein, a receptor for multiple ligands during embryonic development in *Drosophila* (Rebay *et al.*, 1991). No enzymatic activity has been assigned to Notch's cytoplasmic domain, but constitutive signaling activity can be produced by deletion of Notch's extracellular domain (Lieber *et al.*, 1993; Rebay *et al.*, 1993). Toll and Notch may belong to a class of receptors whose cytoplasmic domains, rather than functioning as enzymes, serve as anchors to localize signaling molecules with enzymatic activities to the plasma membrane. For example, ligand binding could ultimately cause the unmasking of a binding site in the cytoplasmic domain necessary for recruiting a signaling molecule to the plasma membrane where it becomes activated. This mode of transmembrane signaling may be used in the case of integrins, cell surface receptors for extracellular matrix components, which have been implicated in the localization and activation of intracellular signaling molecules at cell-substrate adhesion sites (reviewed in Schwartz, 1992).

Spatial Regulation of Toll's Signaling Activity

Earlier studies suggested that Toll's extracellular domain is involved in spatially regulating the signaling activity of its cytoplasmic domain in the embryo. For example, the protein encoded by the recessive Tl^{rm9} allele contains two amino acid substitutions in the two blocks of leucine-rich repeats within the extracellular domain (Schneider *et al.*, 1991). Embryos containing this mutant protein become lateralized, as if Toll signaling occurs uniformly at all dorsoventral positions (Anderson *et al.*, 1985b). Thus, the changes in the extracellular leucine-rich repeats somehow release the mutant protein from the normal spatial regulation that restricts Toll's signaling activity to the ventral side of the embryo.

Our experiments with the ΔLRR protein also provide evidence that spatial regulation of Toll's signaling activity requires its extracellular domain. Unlike the endogenous Toll protein or the wild-type Toll protein introduced by RNA injection (Schneider *et al.*, 1991), the ΔLRR protein is capable of eliciting the most ventral cell fate on the dorsal side of the normal embryo (Figure 4F and Table 1). A similar result has been obtained by injection of RNAs encoding proteins with the Tl^{10b}, Tl^{1}, and Tl^{9Q} mutations (Schneider *et al.*, 1991). These results demonstrate that the dorsal side of the embryo containing the wild-type Toll protein is competent to respond to Toll's signaling activity and is not limiting for an intracellular component that functions downstream of Toll. Rather, an extracellular factor appears to be responsible for restricting Toll's signaling activity to the ventral side of the embryo. The extracellular factor is likely to be the ventrally enriched ligand, encoded by the spätzle gene (Morisato and Anderson, 1994), that interacts with Toll's extracellular domain to block this domain's inhibitory

function, thereby causing Toll's signaling function to be asymmetrically active in the embryo.

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