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# The *Drosophila* membrane receptor Toll can function to promote cellular adhesion

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**The product of the Toll gene is a membrane protein required for the formation of dorso-ventral polarity during early embryogenesis in *Drosophila melanogaster*. It acts together with the other dorsal group gene products to specify a nuclear gradient of *dorsal* morphogen in the syncitial blastoderm stage embryo. Here we report the presence in Toll protein of additional sequences held in common with the human membrane receptor platelet glycoprotein 1b (Gp1b). We propose that these sequences in Toll form disulphide linked extracellular domains that are important for the binding of ligands in the perivitelline space of the embryo. In addition, we show that expression of Toll protein induced in a non-adhesive cell line promotes cellular adhesion, a property held in common with the related *Drosophila* glycoprotein chaoptin. Toll protein in such aggregates accumulates at sites of cell-cell interaction, a characteristic displayed by other cellular adhesion molecules. Taken together these findings suggest that the biochemical function of Toll protein is more closely analogous to that of Gp1b than previously thought.**

*Key words:* cellular adhesion/*Drosophila*/Toll protein/receptor

## Introduction

During early development, the embryo of *Drosphila melanogaster* responds to distinct pattern forming cues that result in anterior-posterior (Nüsslein-Volhard *et al.*, 1987) and dorso-ventral differentiation. Genetic studies have identified a group of 11 maternal effect genes null mutations of which result in the embryo being unable to generate normal dorsal-ventral polarity: instead, all the cells of the blastoderm embryo differentiate along a path normally taken by the most dorsal cells (Anderson and Nüsslein-Volhard, 1984; Roth *et al.*, 1989). By contrast null mutations of the maternal effect gene *cactus* cause partial ventralization of the embryo (Schüpbach and Wieschaus, 1989). Thus the products of these genes acting together appear to constitute a system of dorso-ventral positional information.

The primary morphogen of dorso-ventral polarity is the product of the *dorsal* gene, a *Drosophila* homologue of the dominant transforming oncogene v-*rel* (Steward, 1987; Rushlow *et al.*, 1989) that has the properties of a transcription factor (Gelinas and Temin, 1988). The *dorsal* transcript is uniformly distributed in the egg and early embryo but dorsal protein is observed to accumulate in nuclei in a dorso-ventral gradient by the syncytial blastoderm stage of embryogenesis (Steward *et al.*, 1988). The observed nuclear gradient of dorsal protein is now known to arise by differential nuclear localization of protein that is uniformly distributed in the cytoplasm during oogenesis and early embryonic development (Steward, 1989; Roth *et al.*, 1989; Rushlow *et al.*, 1989). Furthermore, unmodified dorsal protein synthesized in culture cells remains in the cytoplasm but truncated proteins are able to localize to the nucleus and activate expression of the known downstream gene *zen* (Rushlow *et al.*, 1989). The dorsalized phenotype observed in all other dorsal group mutants arises from a failure of the dorsal protein to localize in the nucleus (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989) and thus these genes act together to form a gradient of nuclear localization in the embryo.

Of the 10 remaining dorsal group genes, three have been subject to molecular analysis. The *snake* and *easter* genes encode proteins related in sequence to serine proteinases (Delotto and Spierer, 1986; Chasan and Anderson, 1989). It is unlikely that snake and easter proteins act to process dorsal for nuclear localization as they have been shown to act upstream of *Toll* (see below), but they may be involved in activation of Toll (Anderson, 1989). Several lines of evidence suggest that the spatially restricted activation of *Toll* is central to appropriate dorso-ventral embryonic pattern formation. *Toll* acts upstream of *dorsal* (Anderson, 1989) and dominant alleles cause an embryo to form in which blastoderm cell fates are shifted ventrally (Anderson *et al.*, 1985a) a phenotype that correlates with uptake of dorsal protein by dorsal and lateral nuclei (Roth *et al.*, 1989; Steward, 1989). In addition, rescue experiments in which $Toll^{+}$ cytoplasm or mRNA is injected into $Toll^{-}$ embryos results in site of injection rescue of ventral structures, whereas in similar experiments using embryos lacking other dorsal group products, ventral structure is always rescued on the curved presumptive ventral surface of the egg shell (Anderson *et al.*, 1985b). Taken together, these results suggest that Toll protein function directly influences nuclear localization of dorsal protein. The Toll protein sequence deduced from cDNA sequences is predicted to be transmembrane (Hashimoto *et al.*, 1988). An N-terminal extracytoplasmic domain consists mainly of blocks of 22-26 amino acid long leucine rich repeat sequences that are also found in a number of other membrane proteins. This domain is followed by a hydrophobic sequence that could form a transmembrane $\alpha$-helix and a cytoplasmic domain of 267 amino acids.

The *Toll* gene is also expressed zygotically and transcripts accumulate at sites in the embryo undergoing active invagination. However, the only phenotype associated with zygotic *Toll* expression is a reduction in larval viability to ~5% of normal (Gertulla *et al.*, 1988).

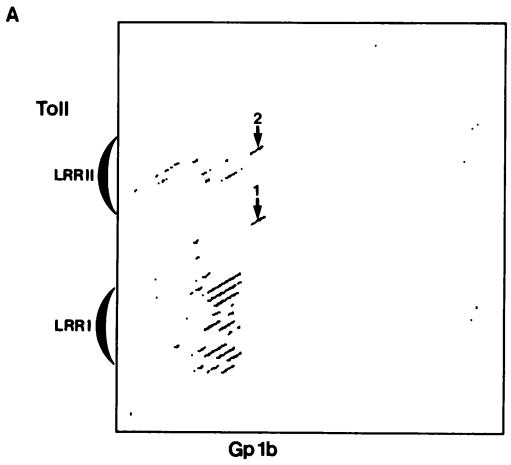
In this paper we report the presence in Toll protein of two disulphide bond forming sequences found also in the membrane receptor and adhesion factor platelet glycoprotein 1b. The construction of a derivative of the *Drosophila* cell

line Schneider 2 in which *Toll* expression can be induced by heat shock is also described. Expression of *Toll* causes cellular aggregation and Toll protein is seen to accumulate at sites of cell−cell interaction.

## Results

### *A conserved disulphide forming sequence found in Toll protein and platelet glycoprotein 1b*

It has been reported previously that the Toll protein is related to the human membrane receptor platelet glycoprotein 1b (Gp1b) by virtue of their common leucine rich repeat sequences (LRRs) (Hashimoto *et al.*, 1988). A comparison between Toll protein and platelet glycoprotein 1b (Gp1b) (Titani *et al.*, 1987; Lopez *et al.*, 1987) made using the DIAGON program (Staden, 1982) reveals that in addition to the homology due to the presence of LRRs, a conserved 20 amino acid sequence located immediately C-terminal to the two blocks of LRRs in Toll and the single block in Gp1b is present (Figure 1). The significance of the matches between these sequences is extremely high, having a double matching probability of $3 \times 10^{-8}$ and thus their conservation is likely to be of structural or functional significance. The sequence is also conserved in human serum leucine rich glycoprotein (LRG) (Takahashi *et al.*, 1985) and the $\beta$ chain of Gp1b (Gp1b$\beta$) (Lopez *et al.*, 1988) but not in the other five known LRR containing proteins. An alignment of the two copies found in Toll and those of Gp1b$\alpha$, Gp1b$\beta$ and LRG is shown in Figure 1B. In Gp1b the two conserved cysteine residues form disulphide bonds with cysteines at positions 264 and 278 while LRG in which only one cysteine is conserved forms a single disulphide bridge between residues 268 and 294 (Titani *et al.*, 1987; Takahashi *et al.*, 1985). In Toll protein, similarly disposed and spaced cysteines are found at positions 597 and 618 and 781 and 799. Thus it is very probable that these sequences in Toll adopt a looped disulphide linked structure similar to that of Gp1b and LRG.




B

| | | |
|---|---|---|
| Toll 1 | (558-577) | LNDNPLVCDCTILWFIQLVR |
| Toll 2 | (748-767) | LSGNPWMCDCTAKPLLLFTQ |
| Gp 1bα | (202-221) | LHGNPWLCNCEILYFRRWLQ |
| Gp 1bβ | (61-80) | LGANPWRCDCRLVPLRAWLA |
| LRG | (261-280) | ISGNPWICDQNLSDLYRWLQ |
| Consensus | | L-GNPW*CDC-*--L--WLQ |

**Fig. 1.** Homology between Toll protein and human platelet glycoprotein Ib (Gp1b). (**A**) Comparison of Toll and Gp1b using DIAGON (Staden, 1982). A span of 25 and a score of 285 were used. The positions in the Toll protein of the two blocks of leucine rich repeats (LRR I and LRR II) are indicated. The two matches in Toll protein to the sequence flanking the repeat block in Gp1b are indicated (1) and (2). (**B**) Alignment of cysteine containing sequences. Alignment of GpIbα, GpIbβ (Lopez *et al.*, 1988), Toll and LRG (Takahashi *et al.*, 1985) is shown. A consensus is indicated when 60% of the sequences have the same residue. * = hydrophobic residues.

### *Synthesis of Toll protein is induced by heat shock in the cell line SL2-F9-Toll*

The ability of a Schneider 2 cell line transformed with F9-Toll (SL2-F9-Toll) (see Materials and methods and Figure 8) to synthesize Toll protein in response to heat shock was tested by metabolic labelling with [$^{35}$S]methionine and specific immunoprecipitation with anti-Toll antibody. A Schneider 2 cell line transformed with F9 plasmid alone was used as a control (SL2-F9). In the absence of heat shock, no specific protein species corresponding either to heat shock proteins or to Toll can be detected in either cell line (Figure 2a and c). However, upon exposure to heat shock two specific bands of $M_r$ ~70 and 95 kd, probably corresponding to heat shock proteins, are induced in both SL2-F9 and SL2-F9-Toll cells (Figure 2b and d). In SL2-F9-Toll cells bands of $M_r$ ~150 and 165 kd are also seen (Figure 2d). Specific anti-Toll antibodies (see Figure 3) used in immunoprecipitation experiments do not purify any labelled protein species from extracts of uninduced SL2-F9 and SL2-F9-Toll or from heat shock treated SL2-F9 cells (Figure 2e, f and g). By contrast, the $M_r$ 150 and 165 kd species are purified from extracts of heat shock induced SL2-F9-Toll cells (Figure 2h). We have determined the sequence of a 1.8 kb genomic DNA fragment reported previously to contain the entire 5′ region of the Toll transcript. The sequence determined is the same as the cDNA of Toll (Hashimoto *et al.*, 1988), including the presumed translational start site, from nucleotide 555 but upstream of this no identity with the cDNA is detected. The sequences surrounding nucleotide 555 closely match the consensus for 3′ splice junctions and thus it seems that the rest of the transcript is encoded in an exon or exons located 5′ to this genomic DNA fragment. However, it appears that Toll protein can be synthesized under heat shock regulation in SL2-F9-Toll cells despite the presence of 788 bases of 5′ untranslated intron sequence between the heat shock promoter and the *Toll* translational initiation codon.

The estimated molecular masses of the Toll protein species are somewhat greater than that predicted from the cDNA sequence (125 kd) (Hashimoto *et al.*, 1988). However it is likely that Toll is modified by glycosylation and such processing is known to reduce mobility in SDS−PAGE. The purification of two distinct species in immunoprecipitation experiments provides evidence of differential post-translational processing by glycosylation, proteolysis or phosphorylation.

### *Expression of Toll protein in SL2-F9-Toll causes cellular adhesion*

To test whether Toll protein is able to mediate cell−cell interaction of SL2-F9-Toll cells an aggregation assay was

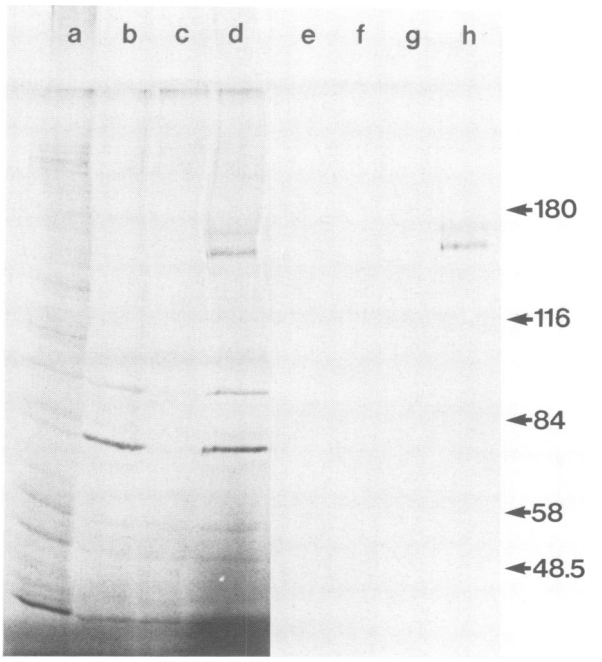



**Fig. 2.** Inducible expression of Toll protein in SL2-F9-Toll cells. Proteins were separated on a 9% SDS−polyacrylamide gel (Laemmli, 1970). The gel was dried and autoradiographed for 5 days. Cells were induced by incubation at 37°C for 30 mins and allowed to recover 150 min. (**a**) Uninduced SL2-F9 cells; (**b**) induced SL2-F9 cells; (**c**) uninduced SL2-F9-Toll cells; (**d**) induced SL2-F9-Toll cells; (**e−h**) as (**a−d**) but immunoprecipitated with specific anti-Toll antibody. The positions of mol. wt markers are indicated.

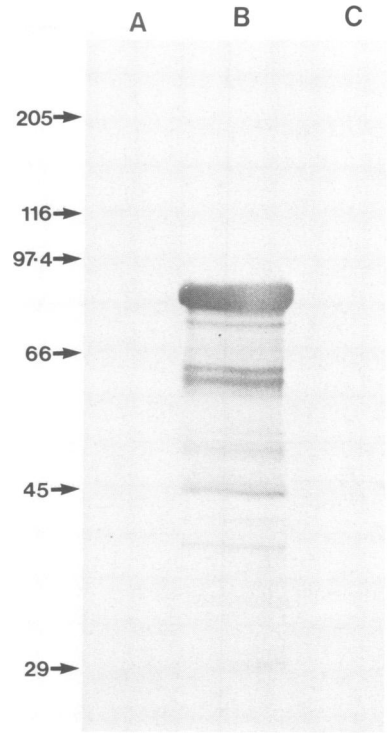



**Fig. 3.** Specificity of anti-Toll antibodies. Proteins were separated by SDS−PAGE (9%) and transferred to nitrocellulose paper. The Western blot was incubated with anti-Toll antibody (1:50). (**A**) Molecular mass markers (myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase) (10 μg) (**B**) A protein consisting of residues 18−804 of Toll (5 μg) (Hashimoto *et al.*, 1988) which had been produced in *Escherichia coli* (N.J.Gay, unpublished results). The identity of this protein has been confirmed by direct protein sequence analysis. (**C**) *Drosophila* nuclear protein extract (~50 μg). The antibody binds to the Toll protein and degradation products but not to the markers or the nuclear protein extract.

performed. Schneider 2 cell line derivatives are ideal for this purpose as they grow normally as single unclumped cells with low adhesivity and they do not aggregate when cell surface glycoproteins known not to function in cell adhesion are overexpressed (Snow *et al.*, 1989). In such assays, uninduced SL2-F9, SL2-F9-Toll and heat shock induced SL2-F9 cells do not aggregate together [Figure 4(i)A−C] whereas induced SL2-F9-Toll cells form together in large aggregates consisting of tens to hundreds of cells [Figure 4(i) D]. The aggregation is also manifest at a macroscopic level, as induced SL2-F9-Toll cell cultures adopt a characteristic granular appearance [Figure 4(ii)]. Quantification of Toll mediated aggregation (Figure 5) shows it to be comparable in extent and time-scale to that induced by other adhesion molecules such as chaoptin and fasciclin 111 (Krantz and Zipursky, 1990; Snow *et al.*, 1989). Furthermore, the elimination of $Ca^{2+}$ by the addition of EGTA to the adhesion assay has no effect on the aggregation (result not shown). Adhesion promoted by Fasciclin 111, fasciclin 1 and chaoptin is also calcium independent (Snow *et al.*, 1989; Elkins *et al.*, 1990; Krantz and Zipursky, 1990) but that mediated by cadherins is calcium dependent (Nagafuchi *et al.*, 1987).

Purified antibody preparations specific either for residues 88−403 or 572−1097 of Toll (Hashimoto *et al.*, 1988) do not have a detectable effect on Toll mediated aggregation (result not shown). However, it should be noted that such a negative result does not rule out a direct involvement of Toll in adhesion. In the case of fasciclin 1 which is known from other evidence to participate directly in cell adhesion, specific antibody preparations also fail to inhibit aggregation (Elkins *et al.*, 1990). Indeed, the results of such antibody inhibition assays ought to be treated with caution as the bivalent nature of non-Fab antibody preparations should expedite rather than inhibit aggregation. Furthermore, antibody inhibition even when achieved with Fab preparations could result from the blocking of an activating ligand binding site and thus would still not be proof of a direct involvement of the antigen in adhesion.

### *Toll mediated adhesion is heterotypic*

In order to characterize the nature of Toll promoted aggregation further we have examined whether it is necessary for both cells to express *Toll* for adhesion to occur. This was studied using mixing experiments between heat shock treated SL2-F9-Toll cells and control SL2-F9 cells which had been labelled with the lipophilic fluorescent dye DiI (see Materials and methods). Both SL2-F9 (Figure 6A and B) and SL2-F9-Toll (Figure 6C and D) cells are seen to attach to performed SL2-F9-Toll aggregates and SL2-F9 cells also form into aggregates when added to the assay at the time of induction (result not shown). A statistical analysis of such aggregate formation by SL2-F9-Toll and SL2-F9 cells (Materials and methods and Table I) shows that the difference between the means is insignificant at the 5% level. We conclude that adhesion mediated by Toll is heterotypic and

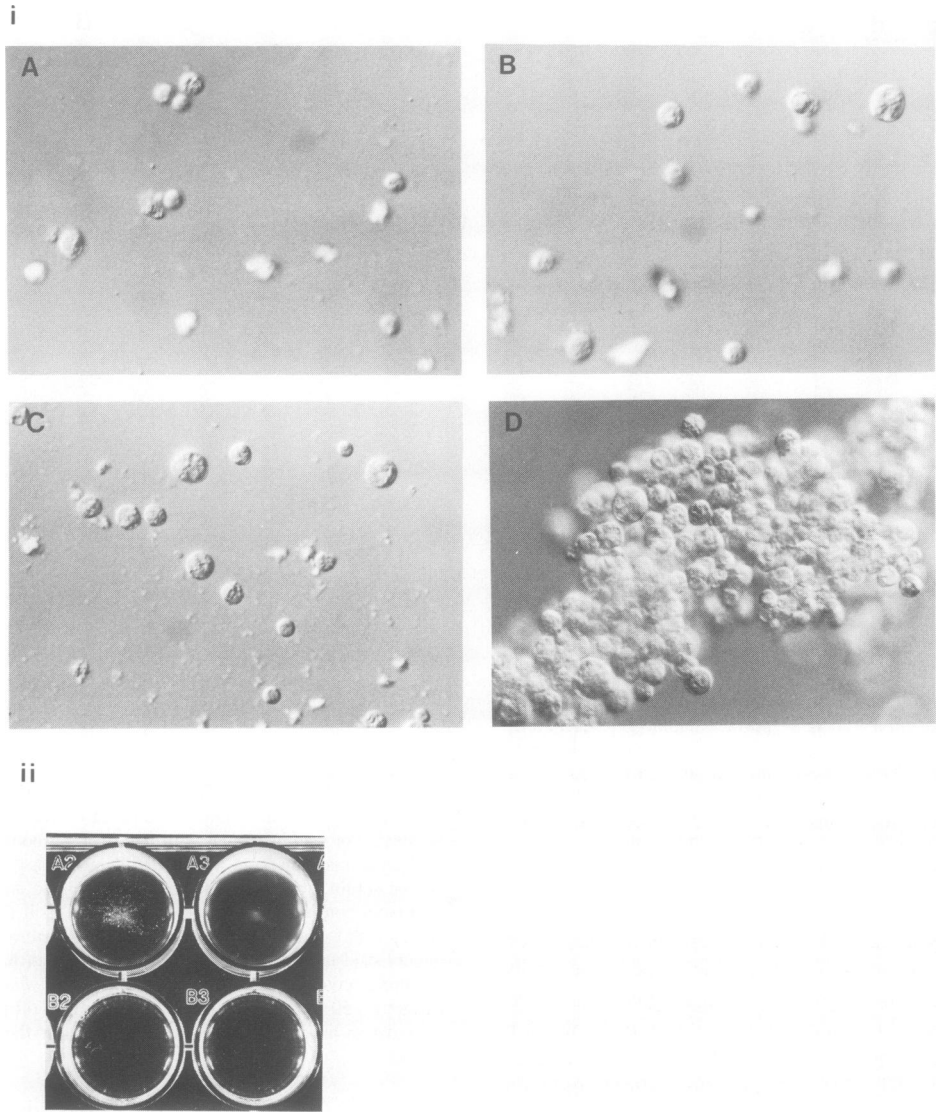



**Fig. 4.** Cellular aggregation assays. Aggregation assays were performed as described (Snow *et al.*, 1989) and the cultures were viewed using Nomarski interference optics. Heat shock induction was as described in the legend to Figure 2. (**i**) (**A**) uninduced SL2-F9 cells; (**B**) induced SL2-F9 cells; (**C**) uninduced SL2-F9-Toll cells; (**D**) induced SL2-F9-Toll cells; (**ii**) macroscopic appearance of the aggregated cultures: (**A2**) induced SL2-F9-Toll cells; (**B2**) uninduced SL2-F9-Toll cells; (**A3**) induced SL2-F9 cells; (**B3**) uninduced SL2-F9 cells.

therefore distinct from that mediated by other cellular adhesion molecules, such as cadherins, fasciclin 111 and chaoptin which have been examined by this criterion (Nose *et al.*, 1990; Snow *et al.*, 1989; Krantz and Zipursky, 1990). It is possible that Schneider 2 cells express Toll protein at a low level and that adhesion by SL2-F9 cells is in fact homotypic. However, this seems unlikely as the SL2-F9 cells do not self associate in the absence of induced *Toll* expression and Toll protein cannot be detected in these cells either in metabolic labelling immunoprecipitation experiments (see Figure 2) or in Western blots.

### *Subcellular localization of Toll protein in induced SL2-F9-Toll cells*

We have used immunofluorescent microscopy of cells stained with specific anti-Toll antibodies and a rhodamine conjugated secondary antibody to determine the subcellular localization of Toll protein in SL2-F9-Toll cells. In such experiments,

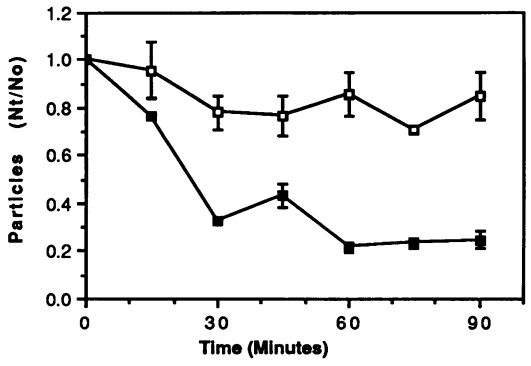



**Fig. 5.** Kinetics of intercellular aggregation by cells expressing Toll. Aggregation is expressed by the ratio $N_t/N_0$ where $N_0$ is the number of particles present at time 0 and $N_t$ is the number of particles (cellular aggregates and single cells) present at time $t$. □ = SL2-F9 cells; ■ = SL2-F9-Toll cells.

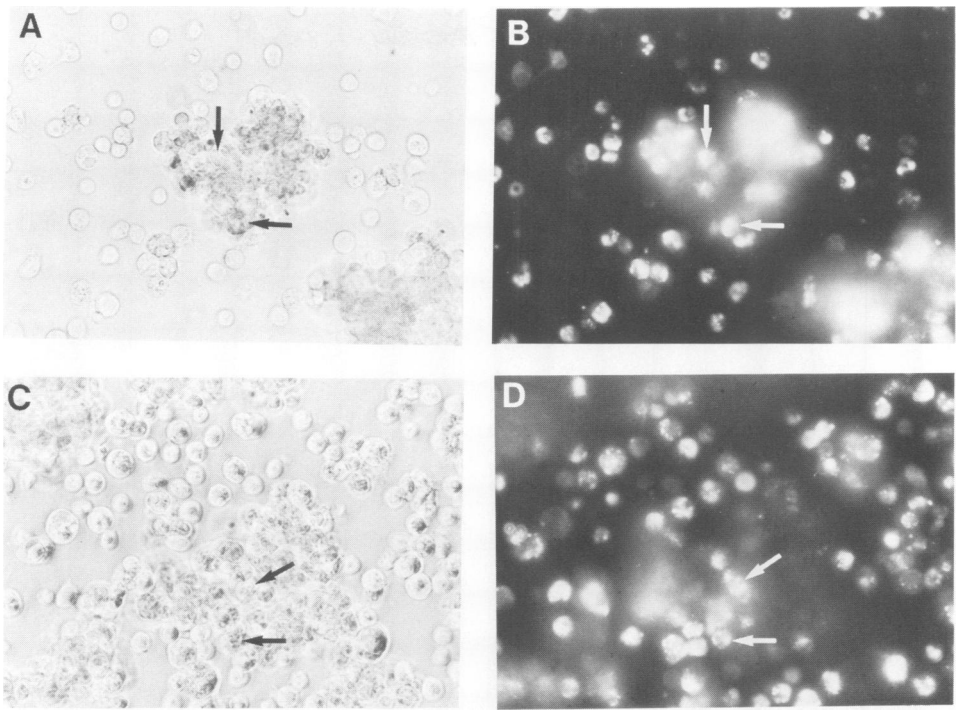



**Fig. 6.** Cell aggregation is mediated by Toll in a heterotypic manner. SL2-F9-Toll cells were induced, allowed to recover for 1.5 h and aggregates formed by gentle agitation. To these either heat shock treated DiI-labelled SL2-F9-Toll (**A** and **B**) or SL2-F9 cells (**C** and **D**) were added. Agitation was then continued for 1.5 h and cultures examined by phase contrast (**A** and **C**) or fluorescence (**B** and **D**) microscopy. Arrows indicate DiI-labelled cells associated with unlabelled pre-formed aggregates of SL2-F9 cells.

uninduced SL2-F9,SL2-F9-Toll and induced SL2-F9 cells show only faint background staining (for example Figure 7D) but by contrast SL2-F9-Toll cells have a characteristic pattern of staining. Uniform cytoplasmic staining which is excluded from nuclei and bright punctate cytoplasmic particles indicative of accumulation of the protein into multivesicular bodies, can be seen clearly (Figure 7A and C). In addition, Toll protein accumulates in the plasma membrane of aggregates at sites of cell−cell interaction. Although some unaggregated and aggregate border cells have discernible peripheral membrane staining, the accumulation observed at sites of cell−cell interaction is very pronounced. It should be noted that the level to which Toll protein is expressed varies between individual cells. This is because SL2-F9-Toll is not clonal and probably represents a mixture of a number of independently transformed lines.

## Discussion

### *Homology between Toll and a human receptor mediating cellular adhesion*

The finding in Toll of sequences held in common with the von Willebrand factor (vWf) binding domain of platelet glycoprotein 1b (Gp1b) strengthens the structural and functional analogy between these two membrane proteins. Gp1b is a membrane receptor for vWf glycoprotein complex and binding results in the adhesion of platelets to subendothelial connective tissue at sites of endothelial damage (Lopez *et al.*, 1987). The binding site of Gp1b for vWf has been mapped to the disulphide forming sequences and reduction of the disulphide bonds abolishes binding (Vicente *et al.*, 1990). Gp1b also has low and high affinity

**Table I.** Statistical quantification of homo and heterotypic aggregation

| DiI-labelled cell line | Mean % of dil-labelled cells in mixed aggregates |
|---|---|
| SL2-F9-Toll | 55 ± 13 |
| SL2-F9 | 50 ± 15 |

binding sites for the secreted serine proteinase thrombin which lie within the LRR and disulphide bond forming sequences (Harmon and Jamieson, 1986). Thrombin binding results in a decreased affinity for Gp1b of several monoclonal antibodies, a finding that correlates with a marked clustering of Gp1b molecules on the platelet surface (Michelson and Bernard, 1987) and also causes an increase in cytoplasmic free $Ca^{2+}$, probably mediated by a G-protein pathway (Crouch and Lapetina, 1988). Increased cytoplasmic calcium levels cause activation of a calcium dependent neutral protease and the activated protease degrades an actin binding protein by which Gp1b is anchored to the actin cytoskeleton (Okita *et al.*, 1985; Fox *et al.*, 1983; Ezzell *et al.*, 1988).

The presence in Toll of sequences known to participate in ligand binding to Gp1b, strongly suggests that the Toll protein also functions as a membrane receptor. In the syncytial embryo, Toll protein is localized in the egg plasma membrane (Anderson, 1989; N.J.Gay unpublished data) and it is likely that ligands interact with the extracytoplasmic portion of Toll in the peri-vitelline space. Gp1b has a single block of LRRs and a single disulphide sequence, but in Toll protein there are two such sequences which may constitute independent ligand binding domains. The identity of the Toll ligands is currently unknown but two other dorsal group genes, *snake* and *easter* (DeLotto and Spierer, 1985; Chasan and Anderson, 1989), encode secreted serine protease

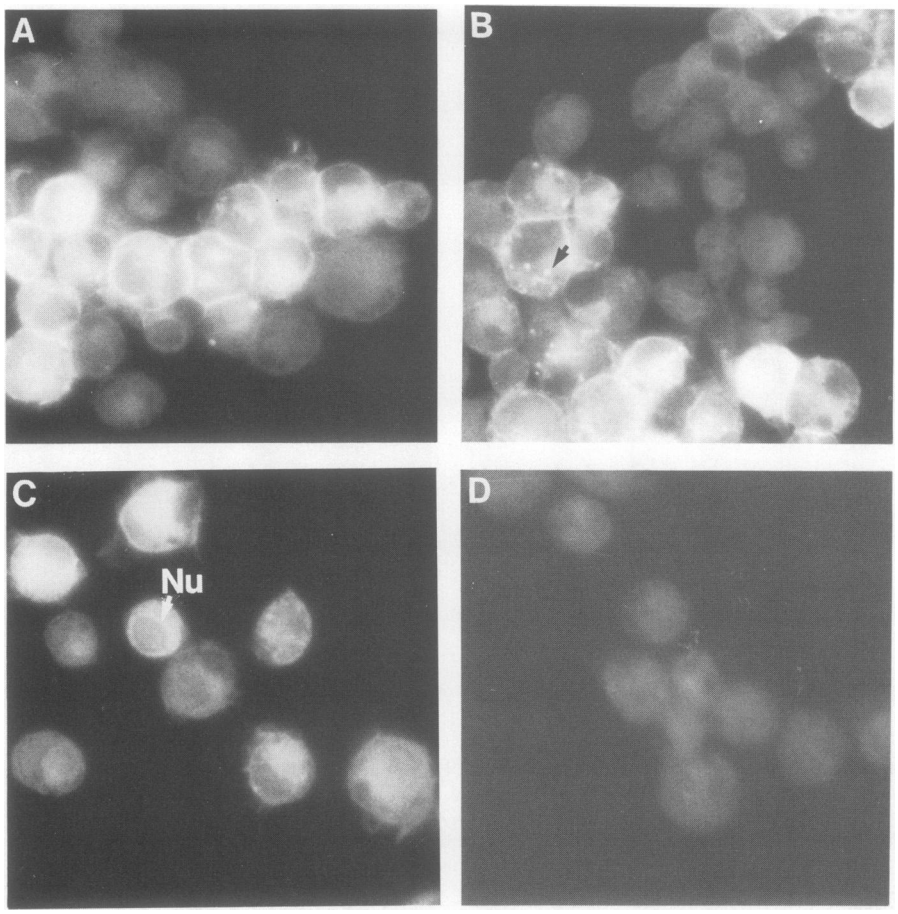



**Fig. 7.** Subcellular localization of Toll protein in SL2-F9-Toll cells. SL2-F9-Toll cells were induced by heat shock and allowed to recover for 3 h. The cells were fixed and stained for immunofluorescence microscopy as described in Materials and methods. (**A**) and (**B**). Aggregated cells showing accumulation of Toll protein at sites of cell–cell interaction, multivesicular bodies (arrowed) and exclusion of protein from nuclei. (One such nucleus is labelled Nu). (**C**) Unaggregated cells; (**D**) control staining of uninduced SL2-F9-Toll cells. Note that this photograph was exposed to 25% of the light level of (**A**)–(**C**) but for three times as long.

homologues of thrombin and these are obvious candidate ligands for Toll.

The question remains as to why *Toll* should be activated specifically on the ventral side of the embryo. An answer to this may be that activating ligands are specifically localized ventrally. Alternatively, the initial binding of ligands to Toll could cause a conformational change and consequent self association of Toll protein molecules within the membrane in a manner similar to self association of Gp1b in response to thrombin. Such a conformational change might cause alterations in the affinity of adjacent Toll molecules for heterologous ligands and their ability to transduce a transmembrane signal.

### *Role of Toll protein in cell adhesion*

The results reported here also show that Toll protein can function directly or indirectly in cell adhesion. If cellular adhesion is mediated directly by Toll it probably involves the extracytoplasmic domain of the protein which is comprised mainly of LRRs. Thus, Toll may function in a manner similar to chaoptin (Reinke *et al.*, 1988; Van Vactor *et al.*, 1988; Krantz and Zipursky, 1990). Chaoptin is a photoreceptor cell specific intrinsic membrane glycoprotein that consists largely of 41 copies of LRRs. Recent experiments of a similar nature to those reported here demonstrated that chaoptin is able to mediate cellular adhesion when expression of it was induced by Schneider 2 cell line derivatives. Evidence was also presented suggesting that the interactions were homotypic in nature and thus to some extent distinct from those mediated by Toll. (Krantz and Zipursky, 1990). However, it should be cautioned that the evidence for homotypic interaction was not conclusive and a statistical evaluation of the data was not presented.

Our results leave open the possibility that Toll may promote cell adhesion indirectly by transducing an extracellular signal that, in turn, causes activation of other adhesion molecules on the surface of the Schneider 2 cells such as the PS integrins (Leptin *et al.*, 1989). Indeed, thrombin induced platelet aggregation is not mediated directly by Gp1b but by the integrin related glycoprotein complex GpIIb–GpIIIa (Phillips *et al.*, 1988), which is indirectly activated by thrombin binding to Gp1b. The heterotypic nature of Toll induced interactions and our inability to inhibit aggregation with specific antibodies are consistent with but do not prove such a mechanism. The promotion of cell adhesion by Toll is unlikely to be important in generating dorso-ventral polarity but it may be relevant to the zygotic Toll phenotype of reduced larval viability. Zygotic expression of *Toll* is localized in regions of the embryo undergoing active invagination, a process that presumably involves cell–cell interaction (Gertulla *et al.*, 1988).

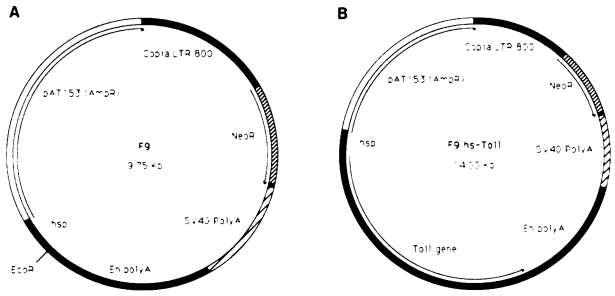



**Fig. 8.** Structures of F9 and F9-Toll plasmids.

Accumulation of protein at sites of cell−cell interaction is characteristic of cell adhesion molecules being observed also in the *Drosophila* adhesion molecule fasciclin III (Snow *et al.*, 1989) and also in vertebrate E-cadherin mediated aggregation (Nagafuchi *et al.*, 1987).

## Materials and methods

### *Molecular biology techniques*

Routine techniques are given in Maniatis *et al.* (1982).

### *Construction of F9-Toll*

The salient features of both F9 and F9-Toll are shown in Figure 8. F9 was constructed by Dr Stephen Poole and is a derivation of pCopNeo (Rio and Rubin, 1985; Gay *et al.*, 1988a). To construct F9-Toll a λ-recombinant clone (λ-Toll) containing the two 3′ exons of the *Toll* gene was isolated from a library of *Drosophila* genomic DNA (Maniatis *et al.*, 1978). λ-Toll DNA was purified as described (Huynh *et al.*, 1985) and a 4.2 kb *Nsi*I−*Hin*dIII fragment which contains the whole of the translated portion of the *Toll* gene was flush-ended and cloned into pUC18 (*Sma*I site) (Yanisch-Perron *et al.*, 1985) to form p18NH.

Digestion of p18NH with *Sac*I and *Hin*dIII releases the *Nsi*I−*Hin*dIII fragment. This fragment was flush-ended and ligated with the F9 plasmid which had been cut with *Eco*RI and the ends filled in to produce F9-Toll (see Figure 8B).

### *Production and purification of anti-Toll antibody*

p18NH was digested with *Pvu*II and a 1.4 kb fragment (nucleotides 836−2240 in Hashimoto *et al.*, 1988) was cloned into pET3c (Rosenberg *et al.*, 1987) which had been digested with *Bam*HI and the ends filled out. This plasmid (pETP14) directs the expression of a fusion protein of ~35 kd which is terminated on an exon−intron, junction which lies at nucleotide 1793 of Hashimoto *et al.* (1988). It was found that this plasmid is unstable in the T7 polymerase strains BL21 DE3 and BL21 DE3 (pLysS) (Studier and Moffat, 1986). Thus, pETP14 is propagated in a genetic background lacking T7 polymerase. To express the fusion protein pETP14 plasmid DNA is used to transform BL21 DE3 (pLysS) and plate cultures of transformed colonies are used directly to grow and induce large scale (4 l) cultures. Induced cultures were harvested by centrifugation (13 000 *g*) and resuspended in 1/50 volume of 20% glycerol 0.1% NP40, 20 mM Tris pH 7.6, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM PMSF (phenylmethylsulphonyl fluoride). Cells were broken by two passages through a French Press at 15 000 p.s.i. and insoluble material collected by centrifugation at 100 000 *g*. The fusion protein forms inclusion bodies which sediment as a tight white pellet overlaid by membranes and this inclusion body pellet is essentially pure protein. The fusion protein in the inclusion body pellet was either purified further by preparative SDS−PAGE (Walker *et al.*, 1982) or used directly after refolding from 6 M Urea, 10 mM Tris pH 7.6, 1 mM DTT, 0.1 mM EDTA. For antibody production rabbits were inoculated subcutaneously with 0.5 mg of protein in Freunds complete adjuvant, boosted at 4 weeks (incomplete Freunds adjuvant) and bled at 6 weeks. Serum was precipitated by the addition of 40% ammonium sulphate, and redissolved in, and dialysed against, PBS (7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$, 150 mM NaCl). Specific anti-Toll antibodies were purified by affinity chromatography. The pETP14 fusion protein (3 mg/ml) was refolded by dialysis into 0.1 M $NaHCO_3$, 0.5 M NaCl. To solubilize the protein 0.1% SDS was added and it was then coupled to CNBr activated Sepharose (Pharmacia) according to the manufacturer's instructions. Antibody was bound to the affinity column in PBS (7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$, 150 mM NaCl) and eluted with 0.1 M glycine pH 2.5. Western blot analysis was performed as previously described (Gay *et al.*, 1988b) except that SLR labelled Protein A was used as secondary (0.5 μCi/ml) (Amersham International).

### *Cell culture techniques*

Schneider line 2 (SL2) (Schneider, 1972) was cultured in M3 media (Shields and Sang, 1977) at 25°C as described (DiNocera and Dawid, 1983). Large scale preparation of plasmids F9 and F9-Toll was by caesium chloride centrifugation (Maniatis, 1982; Miller, 1987) and spermine precipitation (Hoopes and McClure, 1981).

Transformation of SL2 was carried out using calcium phosphate precipitates (DiNocera and Dawid, 1983). Metabolic labelling of cells with [$^{35}$S]methionine was carried out according to Rio *et al.* (1986) and specific immunoprecipitation reactions as described (Gay *et al.*, 1988a).

Cell aggregation and cell mixing experiments were performed after labelling cells with fluorescent lipophilic dye DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethyl indocarbocyanine perchlorate) (Molecular Probes). The kinetics of cellular aggregation were quantitated as described (Snow *et al.*, 1989). To quantify the heterotypic aggregate formation aliquots of cell cultures (0.025 ml) were spotted onto slides and aggregates flattened to a monolayer. The percentages of DiI-labelled cells within individual aggregates were then calculated.

### *Immunofluorescent microscopy*

This was performed as described (Gay *et al.*, 1988b) except that cells were cytocentrifuged onto coverslips (50×*g* 5 min) prior to fixation.

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## Note added in proof

The binding site in Gp1b for thrombin has now been tightly localized to residues 216–274 (Figure 1b). For details see Katagiri *et al.* (1990) *Thromb. Haemost.*, **63**, 122–126.