Wild type and constitutively activated forms of the *Drosophila* Toll receptor have different patterns of N-linked glycosylation

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Abstract **Toll is a *Drosophila* membrane protein related in sequence to the mammalian platelet glycoprotein 1B and to the interleukin-1 receptor. It mediates a signal transduction pathway leading to the development of dorsoventral polarity in the *Drosophila* embryo. In this paper we show that a constitutively activated mutant receptor, Toll10B, is processed into a distinct isoform of slower electrophoretic mobility when compared with the wild type molecule in both cell lines and the embryo. The wild type protein can also be processed into this form if over-expressed but in the embryo is present as the smaller species. We show that the decrease in the mobility of Toll10B and over-expressed wild type receptors is caused by altered patterns of N-linked glycosylation and that both forms are secreted to the cell surface. On the basis of these results, we propose that the Toll10B receptor is unable to associate with a limiting co-factor which when bound directly or indirectly masks supplementary N-linked glycosylation sites.**

Key words: Toll receptor; *Drosophila*; Glycosylation

1. Introduction

The *Drosophila* Toll protein is a membrane receptor required during pre-cellular embryogenesis for the generation of dorsoventral polarity (for a review see [1]). The cytoplasmic region of Toll is related to that of the type 1 interleukin-1 receptor [2,3] and it is likely that the cellular signalling pathways mediated by the two receptors are very similar at the biochemical level [4]. The extracellular domain of Toll contains two blocks of leucine-rich repeats (LRRs) flanked by cysteine-rich sequences [5]. In the related receptor platelet glycopreotein 1B, the cysteine-rich sequences form two sets of disulphide bonds which are required for binding of the ligands thrombin and von Willebrand factor [6,7]. Genetic studies have identified a mutant form of Toll, Toll10B, that is constitutively activated [3]. The activating mutation changes a cysteine residue to tyrosine in the sequence flanking the second block of LRRs. Thus in the Toll10B receptor the conformation of the second cysteine-rich sequence may be disrupted in a manner which mimics the binding of an activating ligand. The extracellular domain of Toll contains eighteen consensus sites for N-linked glycosylation and it is known that at least some of these sites are modified [8,9].

Previously we reported that all the components of the signal transduction pathway are present in the embryonic cell line SL2 and that the pathway is effectively activated by inducing the expression of the Toll10B receptor [10]. We now show that the Toll10B receptor is glycosylated to a greater extent than the wild type molecule in these cells. The hyper-glycosylated form is also produced by embryos derived from mothers carrying the $Toll^{10B}$ gene and when wild type Toll protein is over-expressed in cell culture.

2. Experimental

2.1. Establishment and maintenance of cell lines 9A and SLTL10B

The culture and manipulation of SL2 cells and derivatives has been described [5]. For cell line 9A, SL2 cells were transfected with 10 μg of F9-Toll plasmid [5]. The cells were selected by culture in geneticin G418 and then cloned by limiting dilution. For SLTL10B cells, SL2 cells were transfected with 2 μg of F9-Toll10B [10] and selected with 1.5 mg·ml^{-1} of G418.

2.2. Preparation of cell and embryo protein extracts

SL2 cells were collected by centrifugation (1000 × *g*) and extracted into 'I' lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with PiC (1 mM phenyl-methyl-sulphonyl fluoride, 0.014 mg/ml pepstatin and 0.01 mg/ml leupeptin). Embryos were collected from apple juice agar, dechorionated in half-strength bleach (2 min), washed and extracted into 'I' lysis buffer supplemented with PiC. Protein content in the extract was estimated using a dye binding assay. To prepare extracts from $Toll^{10B}$/+ embryos, females that were either Sb Ser e or phenotypically normal were selected from a stock of the genotype *Tl^{10B}, mwh, e/TM3, Sb Ser; T(1;3) OR60* (Tubingen stock K317). The selected females were placed in small population cages and were sterile as no embryos hatched into larvae.

2.3. Western blotting of proteins

Protein extracts were separated by SDS-PAGE. After the electrophoresis, the gel was soaked in transfer buffer (25 mM Tris, 90 mM glycine, 20% methanol) for 10 min. The nitrocellulose membrane (Hybond-C$^+$, Amersham) and 3 MM paper (Whatman) was cut to the exact size of the gel and moistened in transfer buffer. A semi-dry blotter (ATTO) was used for transfer and a sandwich was constructed according to the manufacturer's instruction using six papers for each side. Transfer was performed at a constant voltage of 20 V overnight at 4°C or 32 V for 90 min. For blocking of non-specific binding sites, the membrane was incubated with 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 150 mM NaCl, 0.1% Tween 20 (PBTw) (0.2 ml/cm^2 of membrane) supplemented with 10% dried milk for 1 h at room temperature or overnight at 4°C. The blot was rinsed once with PBTw and incubated with 0.1 ml/cm^2 of the primary antibody solution (see [5] (1/20,000 dilution) for 2 h with agitation. The primary antibodies were diluted with PBTw supplemented with 1% dried milk. The blot was washed with PBTw three times for 5 min each and then incubated with the secondary antibody (1/1000 dilution) for 2 h. Since the *Drosophila* cell extract was found to contain several avidin binding proteins, HRP-conjugated anti-rabbit antibody (Dako) was used to obtain less background. Following three washes of 5 min in PBTw, the proteins were visualised by the ECL (enhanced chemiluminescence) system (Amersham).The blot was incubated in the substrate solution for 1 min with shaking and then the substrate solution was trapped by two sheets of 3 MM paper placed on a sheet of Dow 'Saran' film and the blot was superimposed on these papers with the blotted side up and covered with Dow 'Saran' film. By capillary action from the paper, the ECL reaction continued during

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exposure. The blot was exposed to Fuji NIF X-ray film for 5–60 s depending on the intensity of the signal. Embryonic protein extracts were prepared as previously described [11].

2.4. Deglycosylation of proteins

Cell extract was prepared from 10^7 cells and dialysed against PBS supplemented with Pic in a Centricon-10 ultra-filter. In order to reduce aggregation of the protein, 0.1% Triton X-100, was included. Samples were divided into two and endoglycosidase F (6 U/ml; Boehringer) and PiC was added to one of them. The enzyme solution used was actually a mixture of endoglycosidase F and glycopeptidase F. The deglycosylation reaction was carried out at 37°C overnight and stopped by adding SDS-PAGE sample loading buffer followed by boiling for 5 min. Polypeptides were fractionated by SDS-PAGE and identified by Western blotting.

2.5. Biotinylation of surface protein

Specific labelling of surface protein was achieved by NHS-biotinylation. In this method, cells are incubated with *n*-hydroxysuccinimide ester (NHS)-biotin which reacts covalently with the ϵ-amino group of lysine residues [12]. During labelling, endocytosis is arrested by cooling on ice and therefore only surface protein is covalently linked with biotin. Proteins are fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane. Biotinylated polypeptides are visualised by probing the transfers with HRP-conjugated streptavidin. By including an immunoprecipitation step before SDS-PAGE, surface localisation of a specific protein can be monitored. The day before the experiment, logarithmically growing cells were seeded at a concentration of 2×10^6cells/ml on a 24-well plate (1 ml/well) and incubated overnight at 26°C. In this condition, most of the SL2 cells stick to the bottom of the well. For Toll protein biotinylation, clones were heat-shocked at 37°C for 30 min and recovered at 26°C for 1 h. Cells were washed gently several times in PBS supplemented with 1.0 mM $MgCl_2$ and 0.1 mM $CaCl_2$ on ice. The biotinylation was performed by incubation in the same buffer containing 1 mg/ml of NHS-biotin on ice for 30 min. NHS-biotin can be dissolved in dimethyl sulfoxide at a concentration of 100 mg/ml and stored at −20°C. In order to quench the free NHS-biotin cells were washed with M3 media three times. Biotinylated cells were harvested by pipetting and collected by centrifugation. To prepare cells lysates, the media was aspirated and the cells solubilised in 'I' lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with PiC. Insoluble material was removed by centrifugation (14,000 × *g*, 10 min). Antibody was added (1/40th dilution) and the lysate incubated at 4°C overnight. Then 30 μl of a 50% slurry of Protein-A Sepharose was added and the solution was incubated at 4°C for a further 90 min. Finally, the immunoprecipitates were washed three times in I lysis buffer and then solubilised in SDS-PAGE sample buffer. The proteins were transferred onto nitrocellulose membranes as described above. Biotinylated proteins were visualised by probing the blot using the avidin/biotin /HRP complexes (ABC kit (Vector)).

3. Results

3.1. The Toll10B receptor is expressed as a distinct isoform of slower electrophoretic mobillity

The biogenesis of the wild type and Toll10B receptor molecules has been studied by inducing their expression in cell lines 9A and SLTL10B, respectively. Expression was induced by subjecting the cells to heat shock and the receptor molecules were detected in cell lysates by Western blot (Fig. 1). Although neither Toll transcript is translated efficiently during the period of heat shock low levels of protein can be detected. In 9A cells the wild type receptor is present as an isoform of approximately 135 kDa but by contrast the Toll10B receptor is seen mainly as a slower mobility species (ca. 150 kDa). When the cells are returned to 26°C, translation of the mRNAs is stimulated. In SLTL10B cells, Toll accumulates as a 150 kDa isoform during the recovery period. By contrast the wild type receptor continues to be expressed as the 135 kDa form but can now also be detected as a species with the same mobility as the Toll10B receptor.

3.2. The 135 and 150 kDa isoforms of Toll are generated by N-linked glycosylation

In order to determine whether the observed isoforms of Toll were a consequence of different patterns of glycosylation, extracts of induced 9A and Toll10B cells were treated with a mixture of endoglycosidase F and glycopeptidase F which together cleave all kinds of N-linked carbohydrate from protein molecules. Such treatment converts both the 135 and 150 kDa isoforms of Toll into a single species of M_r 120 kDa (Fig. 2), a mass consistent with that predicted by the cDNA sequence of Toll [8]. This result shows that the different isoforms of Toll observed in 9A and SLTL10B cells are caused by differential patterns of asparagine linked glycosylation and that the

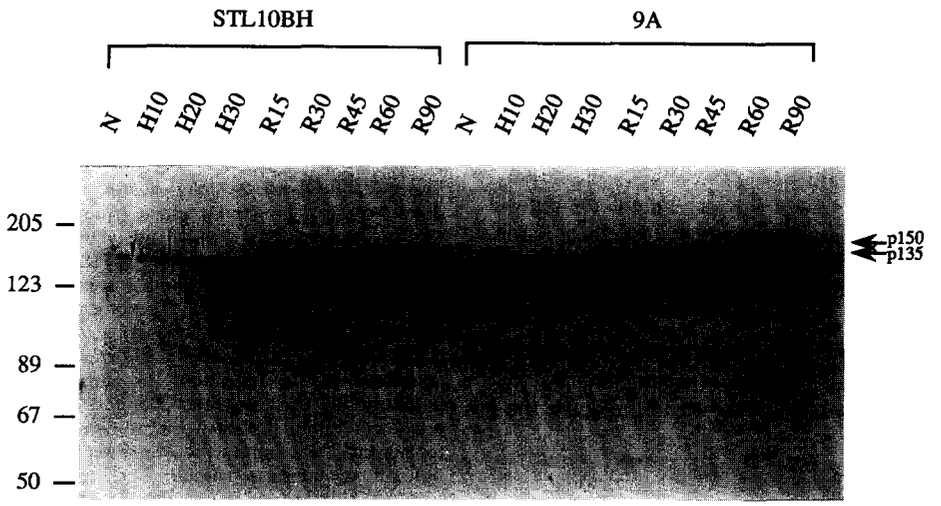

Fig. 1. Time course of Toll expression in stably transfected cells. Toll expression in SLTL10B and 9A cells was monitored during heat shock at 37°C for 30 min and during subsequent incubation at 26°C. Extracts were prepared at the indicated times and the protein detected by Western blot. The positions of molecular mass markers are shown (kDa).

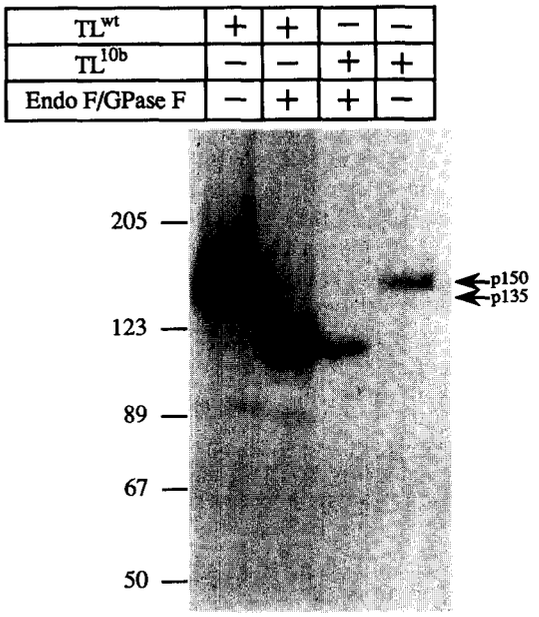

Fig. 2. Deglycosylation of Toll. Cell extracts were prepared from 9A and SLTL10B treated as described in section 2. Samples were fractionated by SDS-PAGE (7%) (2×10^6 cells/lane) and analysed by Western blot. The molecular mass of marker proteins is indicated on the left in kDa.

Toll10B mutation (^{781}Cys–Tyr) does not alter the electrophoretic mobility of the Toll protein. We also find that Toll is unaffected by treatment with phospho-mono-esterases and does not incorporate radio-labelled palmitic acid (data not shown). Thus, it is unlikely that the Toll receptor is modified either by phosphorylation or by palmitoylation.

3.3. The 135 kDa isoform of Toll predominates in the embryo

In order to determine which forms of Toll are present in the embryo, we have probed carefully staged embryonic extracts by Western blot with anti-Toll antibodies. The Toll protein is seen to accumulate in the embryo during the first two hours reaching a peak level at about 2.5 h of development, a finding that is consistent with previous reports [9,13]. It is also evident from this experiment that the 135 kDa form of Toll is predominant in the embryo (Fig. 3A). We have also examined the expression of the Toll receptor in extracts of embryos derived from mothers carrying one copy of the wild type *Toll* gene and one copy of *Toll^{10B}*. As shown in Fig. 3B, only the smaller isoform of Toll is detected in extracts of wild-type embryos. By contrast, in extract of Toll10B/+ embryos both isoforms are present.

3.4. The Toll10B receptor is secreted normally to the cell surface

It is possible that the different pattern of glycosylation displayed by the Toll10B receptor is caused by a failure of the mutant protein to be properly secreted to the cell surface. To investigate this possibility, we labelled cell surface proteins of 9A and SLTL10B cells with NHS-biotin (see section 2.4). Toll was purified by immunoprecipitation and incorporation of biotin into the receptor molecules was assayed in a Western blot procedure. As shown in Fig. 4, biotin was incorporated into the 150 kDa protein on SLTL10B cells and into both Toll isoforms in 9A with a ratio comparable with that observed in direct Western blot experiments (see Figs. 1,2). These results show that both forms of Toll are exported to the cell surface and that the differences observed are not the result of retention in the secretory pathway.

4. Discussion

In this paper we show that an activated form of the *Drosophila* Toll receptor is processed into an isoform of slower electrophoretic mobility compared with the wild type protein. The different isoforms are generated by N-linked glycosylation because after treatment with enzymes specific for *N*-glycosides both forms are converted to a single species of the same electrophoretic mobility. In *Drosophila* two types of glycosylation are known; N-linked and O-linked (see [14]). In N-linked glycosylation, carbohydrates are linked to the amino nitrogen of asparagine, whereas in *O*-glycosylation carbohydrates are linked to the hydroxyl oxygen of serine, threonine, or hydroxyl lysine. *N*-glycosides are more commonly found in mammalian glycoproteins than *O*-glycosides, but a single glycoprotein may have multiple chains, some of which are *O*-glycosides and some of which are *N*-glycosides. For *N*-glycosylation, an oligosaccharide composed of 3 glucoses, 9 mannoses and 2 *N*-acetylglucosamines derived from a dolichol pyrophosphate precursor is added to an asparagine residue in a consensus sequence of

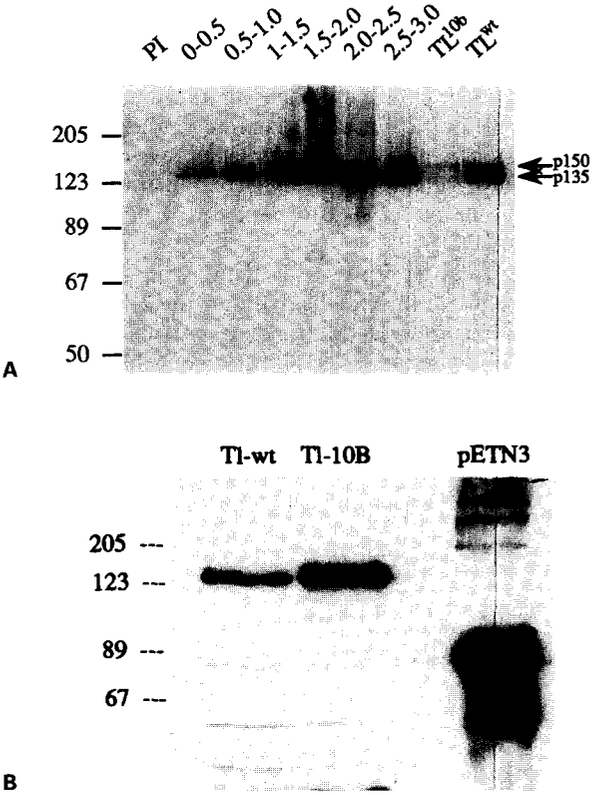

Fig. 3. (A) Toll protein in staged embryo extracts. Extracts from staged wild type embryos, 9A and SLTL10B were separated by 10% SDS-PAGE (100 μg/lane) and analysed by Western blot. The age of the extracts is indicated in hours. As a control, extract from 0–3 h embryos was probed with pre-immune serum. The apparent molecular mass of the marker proteins is indicated in kDa. (B) Toll protein in *Toll10B*/+ embryos. Extract was prepared from 0–4 h embryos as described in section 2. As a control, *E. coli* pETN3 protein (residues 18–806 of Toll (see [13]) was run (150 ng).

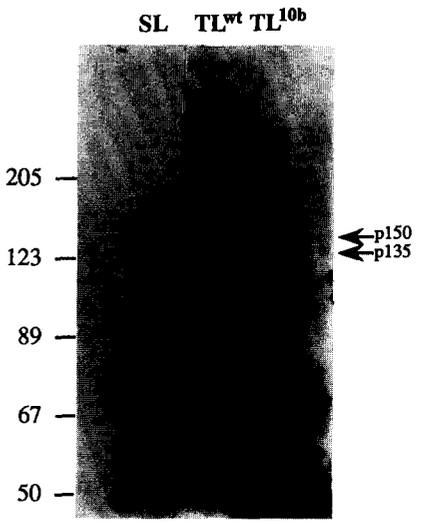

Fig. 4. Biotinylation of cell surface Toll protein. Expression of Toll was induced in 9A and SLTL10B by a 30 min heat shock. The cells were allowed to recover for 1 h and then labelled with NHS-biotin as described in section 2. Toll protein was purified by immunoprecipitation and separated by SDS-PAGE (7%). Biotinylated Toll was detected in a Western blot procedure. The apparent molecular mass of the marker proteins is indicated in kDa.

Asn-X-Ser/Thr where X is any amino acid residue except proline or hydroxyproline [15,16]. Glycosylation takes place normally as the protein is being synthesised at the luminal side of the endoplasmic reticulum (ER). Mannose residues are gradually trimmed off in the ER and the Golgi apparatus as the protein is conveyed through the secretory pathway. At the Golgi apparatus sialic acids can be added to the processed glycosides moiety.

Our findings can be explained in terms of a failure of the trimming reaction in the Golgi apparatus. For example overexpression might saturate the trimming enzymes and allow unprocessed receptor to be secreted. However, this seems unlikely as the Toll10B receptor is over glycosylated even when expressed at low levels and forms into a completely distinct isoform. Alternatively, the Toll10B receptor might adopt a distinct conformation that makes available additional glycosylation sites that are not accessible in the wild type receptor. However, the wild type receptor can be glycosylated to the same extent as Toll10B when overexpressed. This finding suggests either that the additional sites are masked by a titratable co-factor or alternatively that binding of a limiting co-factor induces a conformational change in the receptor which makes a subset of glycosylation sites inaccessible to the modifying enzymes. Such a model predicts that Toll10B is unable to associate with this co-factor and that association of the receptor with the co-factor occurs during biogenesis and secretion. The wild type Toll receptor is known to function as a heterotypic cell adhesion molecule in SL2 cells [5] and thus it is likely that these cells produce ligands that can bind specifically to the extracellular domain of the receptor. It is also possible that specific factors required for the intra-cellular signalling pathway bind to the cytoplasmic sequence of Toll and cause a structural change in the extracellular domain. A candidate for such a factor is the dorsal group protein tube which may provide a link between the receptor and activation pelle, a protein kinase of the raf family [17].

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