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A leucine-rich repeat peptide derived from the *Drosophila* Toll receptor forms extended filaments with a β-sheet structure

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Leucine-rich repeats (LRRs) are 22–28 amino acid-long sequence motifs found in a family of cytoplasmic, membrane and extracellular proteins. There is evidence that LRRs function in signal transduction, cellular adhesion and protein–protein interactions. Here we report unusual properties of a synthetic LRR peptide derived from the sequence of the *Drosophila* membrane receptor Toll. In neutral solution the peptide forms a gel revealed by electron microscopy to consist of extended filaments approximately 8 nm in thickness. As the gel forms, the circular dichroism spectrum of the peptide solution changes from one characteristic of random coil to one associated with β-sheet structures. Molecular modelling suggests that the peptides form an amphipathic structure with a predominantly apolar and charged surface. Based on these results, models for the gross structure of the peptide filaments and a possible molecular mechanism for cellular adhesion are proposed.

Drosophila Toll receptor; Leucine-rich repeat peptide; Circular dichroism spectroscopy; Secondary structure

1. INTRODUCTION

The product of the *Drosophila Toll* gene is a membrane receptor which functions during early embryogenesis in the generation of dorso-ventral polarity (for a review see [1]). The primary structure of the Toll protein shows it to consist of extracellular, transmembrane and cytoplasmic domains [2]. The C-terminal, cytoplasmic portion of the protein is homologous to that of the interleukin 1 receptor [3,4] while the extracellular domain is related to a family of membrane, extracellular matrix and cytoplasmic proteins distinguished by the presence of leucine-rich repeat sequences (LRRs). To date this family consists of yeast adenylate cyclase [5], the *Drosophila* adhesion molecule chaoptin [6], mammalian proteoglycans PG1 and PG11 [7], the human lutropin-gonadotropin receptor [8], human ribonuclease/angiogenin inhibitor [9] and the human platelet receptor glycoprotein 1b (α,β) complex (Gp1b) [10,11]. Toll protein has additional sequences in common with Gp1b which may constitute ligand binding sites [12]. LRRs are 22–28 amino acids long and tend to occur within proteins in tandemly repeated blocks of between 1 and 26 copies. Apart from an N-terminal proline residue, a series of 6 conserved leucines and a single asparagine, LRR sequences are highly divergent.

In the case of adenylate cyclase, LRRs have a functional role, as site directed mutations located within LRRs cause disruption of the normal G-protein mediated signalling pathway [13]. The LRRs are also implicated in cell–cell interactions as chaoptin, which consists almost entirely of LRRs, and also Toll mediates cellular adhesion between apposed cell membranes [6,12]. In addition, the evidence suggests that the LRRs found in various proteoglycans and in ribonuclease/angiogenin inhibitor are involved in specific protein–protein interactions [8,14].

At present little if anything is known about the three-dimensional structure that LRRs adopt although it has been proposed that they form amphipathic structures with hydrophobic surfaces capable of interacting with membranes [15]. However, projection of LRRs into an α-helical structure does not result in such amphiphilicity and it is now clear that LRRs are not intrinsic to the membrane [6]. In this paper we describe the unusual properties of a peptide constituting a single LRR unit from the *Drosophila* Toll receptor. It adopts a β-sheet structure in neutral solution and forms into extended filaments. These findings support the idea that LRRs mediate protein–protein interactions and cellular adhesion.

2. EXPERIMENTAL

2.1. *Peptide synthesis*

The peptide NH_2-PANLLTDMRNLSHLELRANIEEM-COOH (TL-LRR1) corresponding to residues 166–188 of the *Drosophila melanogaster* Toll protein [2] was synthesised using solid phase Fmoc (fluorenylmethoxycarbonyl) chemistry [17] on a Pharmacia/LKB Biolynx Automated Peptide Synthesiser. The peptide chain was assembled on N_2- Fmoc-methionine linked to 1.1 g of Ultrasyn A resin (substitution 70.8 μmol/g). Fmoc-protected amino acids (0.5 mmol) used in the synthesis were all *O*-pentafluorophenyl (-OPfp) esters, coupled in the

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presence of 1-hydroxybenzatriazole (HOBt) catalyst, with the exception of serine, threonine and arginine, which were present in the *O*-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (-ODhbt) ester form. Side chain protection groups were *t*-butyl (Ser,Thr), *t*-butoxy (Asp, Glu), trityl (His) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl, Mtr-, (Arg). The synthesis was performed at room temperature using coupling times, in dimethyl formamide, of 45–90 min; double couplings were performed at positions P_1, L_4, T_6, M_8 and S_{12}. Deprotection reactions were performed for 10 min in 20% piperidine in dimethylformamide.

The efficiency of chain cleavage from the resin and removal of the Mtr side-chain-protecting group from the peptide by various cleavage reagents was monitored using analytical reverse phase HPLC (Brownlee Aquapore RP300, 2.1 × 30 mm in 0.1% trifluoroacetic acid/acetonitrile). The best combination of reagents was determined as trifluroacetic acid/thioanisole/ethanedithiol/anisole, 90:5:3:2 (by vol.) for 20 h at room temperature. After removal of the acid by rotary evaporation, the crude peptide was precipitated with diethyl ether, washed in the same solvent and freeze-dried from a solution of 10% acetic acid.

Purification of the peptide was performed by reverse phase HPLC on a YMC-pack column (C_{18}), 25 mm × 25 cm, in 0.1% TFA with a gradient (30–43% over 37 min at 3 ml/min) of acetonitrile containing 0.1% TFA. The sample was dissolved in 98–100% AR formic acid and diluted out with water to 5% acid before application to the column. The principal component from each run was collected and freeze-dried. The quality of the purified material was checked by (i) sequence analysis on a ABI 477A Pulsed Liquid Protein Sequencer; (ii) analytical HPLC on a Rainin Dynamax C_8 column, 4.6 × 250 mm in 0.1% TFA/acetonitrile; (iii) electrospray mass spectrometry in 1% acetic acid/50% methanol on a VG BioQ spectrometer; (iv) amino acid compositional analysis, after acid hydrolysis (24 h) in 6 M HCl containing 0.1% 2-mercaptoethanol at 110°C, on an LKB 4400 Amino Acid Analyser using ninhydrin detection.

The yield of the peptide was 18 μmol, representing a synthesis yield of 24%.

2.2. *Circular dichroism spectroscopy*

The purified peptide was dissolved in 50 mM Tris-HCl, pH 8.2, and then diluted to 260 μg ml^{-1} in 10 mM sodium phosphate buffer pH 7.8. Spectra of such peptide solutions were recorded between 190 and 240 nm using a Jovin Yvon CD6 instrument and then subtracted from appropriate buffer blank spectra. The concentration of the peptide solutions was calculated by reference to amino acid analysis of the purified product and the CD is expressed as mean residue ellipticity (see [17]).

2.3. *Electron microscopy*

Samples of peptide solution were diluted in 10 mM sodium phosphate, pH 7.8, applied to EM grids and then stained for 1 min with a 0.5% solution of uranyl acetate. The grids were examined with a Philips EM300 instrument.

2.3. *Molecular modelling*

Molecular models were generated using the program Macromodel [18].

3. RESULTS

3.1. *Purity of peptide TL-LLR1*

As described above, TL-LRR1 peptide was purified by reverse phase high pressure liquid chromatography (HPLC) using a preparative C18 column. The product purified by this procedure elutes from an analytical C8 HPLC column as a single peak with a minor shoulder (Fig. 1). Protein sequence analysis indicates that at least 98% of this product is of the desired amino acid sequence with 0.1% being 1 residue shorter. The predicted molecular mass of the peptide is 2681.1 Da and, in close agreement with this prediction, analysis of the product using electrospray mass spectrometry indicates a mass of 2680.74 Da +0.74. No truncated or modified peptides were detected at the 2% level.

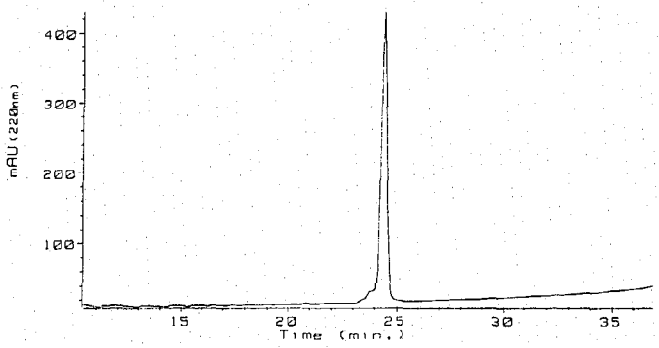

Fig. 1. Purity of TL-LRR1 monitored by reverse phase HPLC. A sample (approx. 1 nmol) of TL-LRR1 was dissolved in water and applied to an analytical C8 column as described in section 2. The peptide was eluted with a gradient of 0–64% acetonitrile containing 0.1% TFA over 30 min at a flow of 0.7 ml/min (Hewlett Packard 1090M liquid chromatograph). Detection was at 220 nm and diode array analysis suggested the major peak to be essentially homogeneous.

3.2. *Properties of TL-LRR1 in solution*

Purified TL-LRR1 peptide dissolves readily at neutral pH and is observed over a period of time to form a clear, viscous gel, with no evidence of a precipitate. The formation of this gelatinous solution can be monitored by circular dichroism spectroscopy. Initially, a strong negative CD band is observed at 200 nm (Fig. 2A) but as the peptide solution becomes more viscous, the 200 nm band diminishes (Fig. 2B–D) and finally is replaced by a spectrum displaying a positive band at approximately 195 nm and a negative band at 216.5 nm, (Fig. 2E). This data shows that the gel formation by this peptide is accompanied by a change in structure from a disordered form (negative band at 200 nm) to a β-sheet structure (see [17]). The interactions giving rise to the β-sheet structures appear to be intermolecular as the initial rate of formation of the β-sheet CD (as judged by the change in mean residue ellipticity at 200 nm) is concentration dependent (Fig. 3). When dissolved at an acidic pH (5.5) the peptide is significantly less soluble and does not form into a gel. The CD spectra show that at this pH it remains predominantly in a disordered conformation (result not shown). Titration of the gel to acidic pH causes it to form a precipitate.

3.3. *The TL-LRR1 gel consists of extended filaments*

In order to investigate the nature of the gel formed by TL-LRR1, samples were negatively stained and examined in the electron microscope. This reveals that the gel consists of extended filaments or fibres (Fig. 4). The thickness of the filaments is between 7 and 9 nm

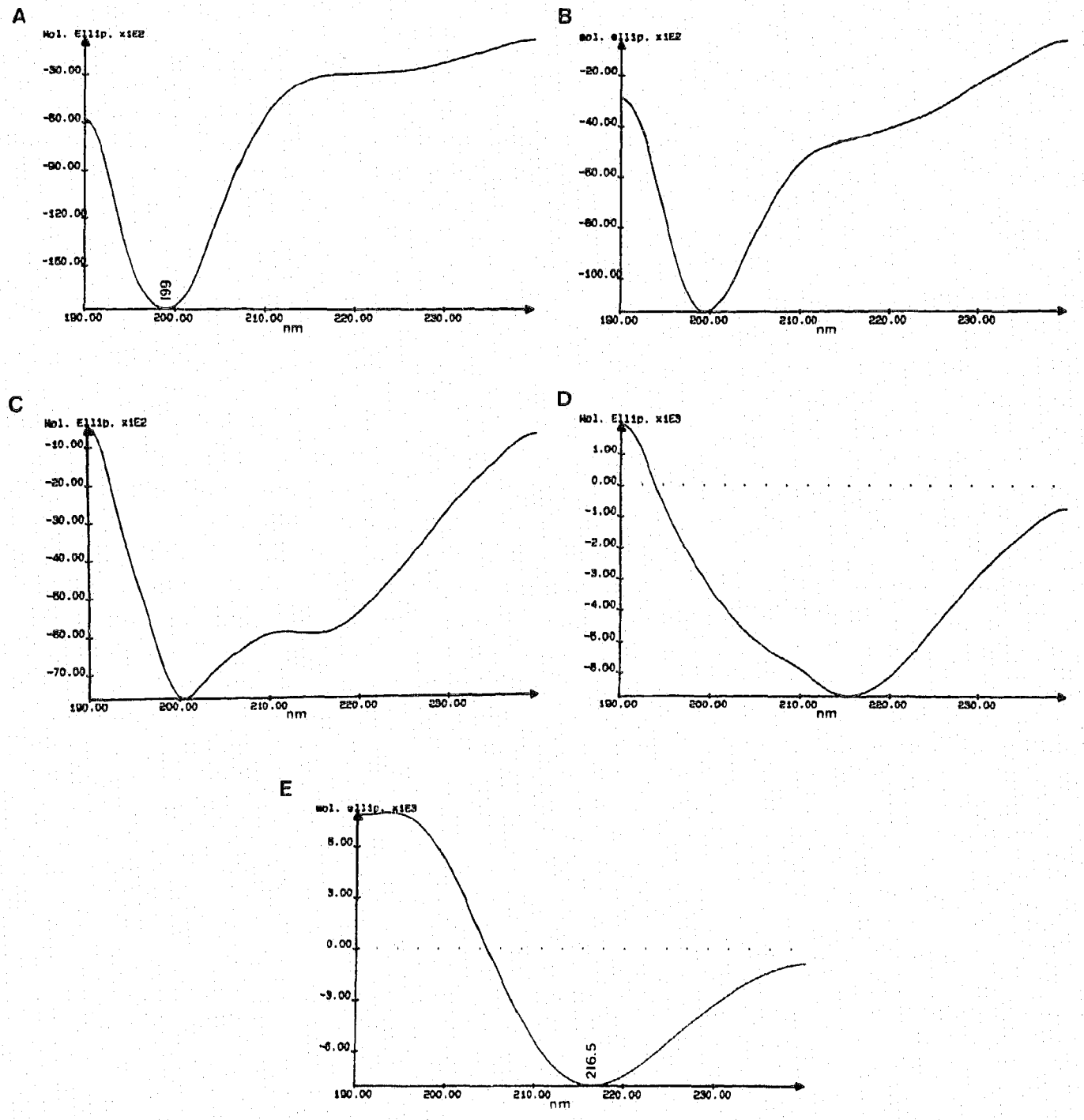

Fig. 2. Circular dichroism spectra of peptide TL-LRR1 solutions. TL-LRR peptide was dissolved at 2.6 mg·ml^{-1} as described in section 2. Samples were taken at various time points and the CD spectra measured. The spectra shown are composed of 3 scans measured in 0.5 nm stept for 0.5 s each. (A) 0 min, (B) 15 min, (C) 30 min, (D) 45 min, (E) 75 min. The positions of the CD band minima are indicated in (A) and (E).

and they are seen to extend to at least several micrometres in length. This finding is in accord with the expected properties of a gel.

3.4. *The TL-LRR1 peptides in β conformation would be amphipathic in nature*

The structure of TL-LRR1 filaments has been investigated using the molecular modelling program Macromodel [18]. Figure 5 shows the TL-LRR1 peptide modelled as a parallel β-sheet structure. The model indicates that the side chains of the peptides will form a predominantly apolar surface including 4 of the 6 conserved hydrophobic residues and a hydrophilic surface with 6 of the 7 charged side chains in the peptide se-

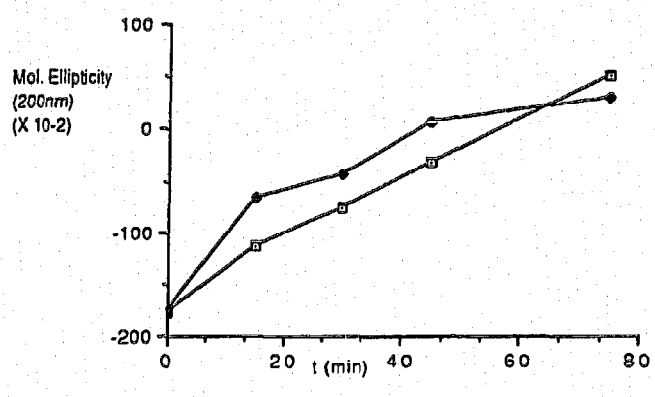

Fig. 3. The initial rate of formation of β structure is concentration dependent. CD spectra were collected as in Fig. 2 for TL-LRR1 peptide dissolved at either 2.6 mg·ml^{-1} (□–□) or 5.2 mg·ml^{-1} (•–•). The rate of formation β structure is expressed as the change in the CD at 200 nm, i.e. the disappearance of peptide in the random coil or disordered form.

quence. An antiparallel β-structure would segregate the side chains in the same amphipathic fashion. It should be noted that although the CD spectra indicate a predominance of β sheet structure, it remains possible that other structures such as β bends and β bulges could be present and be important for the formation of the filaments.

4. DISCUSSION

Although the LRRs of the chaoptin have been proposed to form into amphipathic β structures [6] the results reported above provide the first direct measurements of LRR secondary structure. Furthermore, it would appear that β structure is common to LRR sequences as other peptides derived from Toll and chaoptin can also form β sheets (N.J. Gay, unpublished results), [6]. The data presented here do not determine whether TL-LRR1 peptides form into anti-parallel or parallel β-sheet structures [17]. However a molecular model of the peptides indicates that they would form an amphipathic structure with the conserved hydrophobic residues clustering on one surface.

A basic subunit structure such as that illustrated in Fig. 5 leaves open several possibilities as to the gross composition of the filaments. Firstly, the peptide molecules could form into very long arrays of β-sheets. Such a structure accords with the observed thickness of the filaments as the length of the peptides in β conformation measured from the molecular model is 7.6 nm. Pleated sheets consisting of up to 10 strands have been observed in known structures [19]. A second possibility is that the filaments are stabilised by specific interactions between the amino acid sidechains. These could involve the formation of salt bridges between side chains on the char-

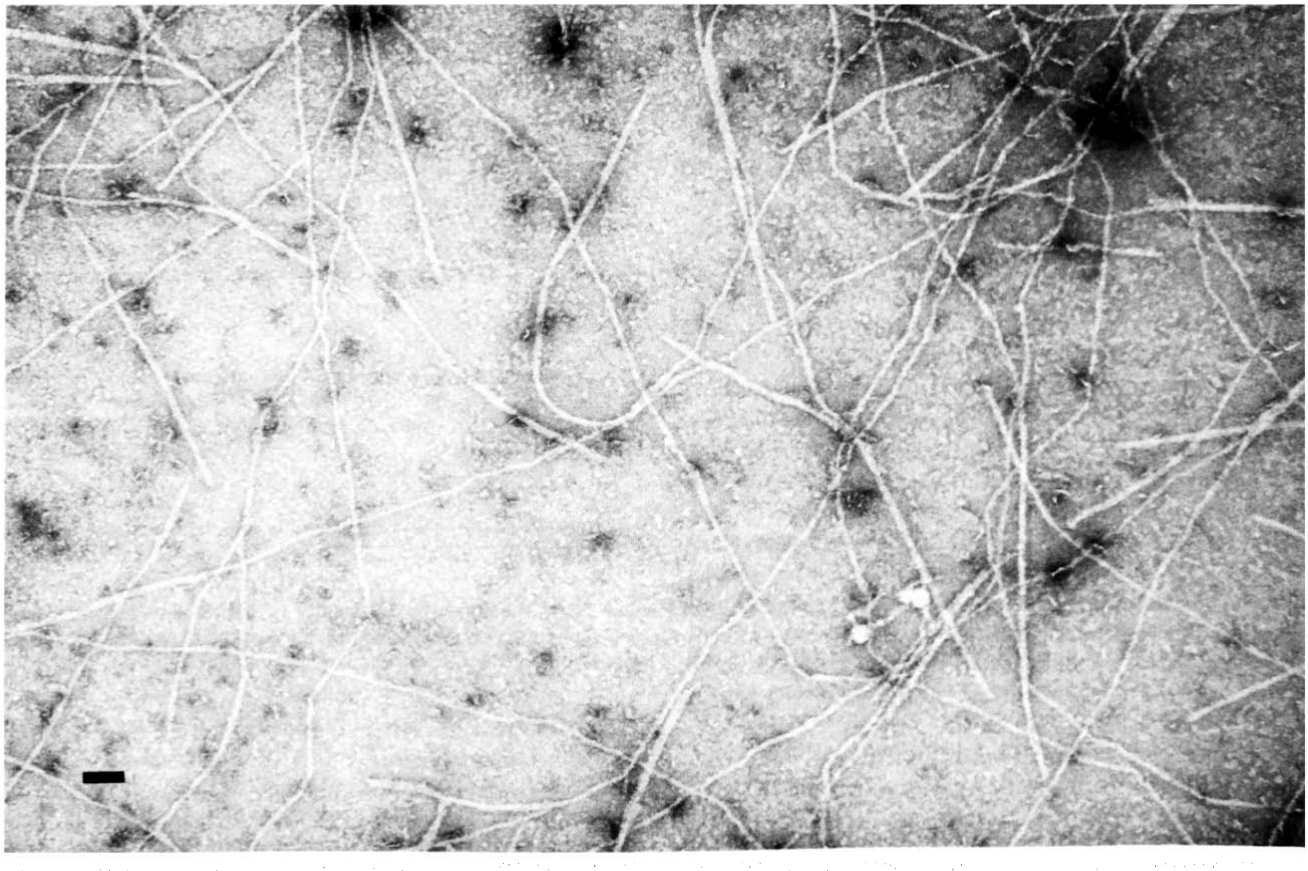

Fig. 4. The gel formed by TL-LRR1 consists of extended filaments. Samples were prepared as described in experimental procedures. The scale bar indicates 100 nm.

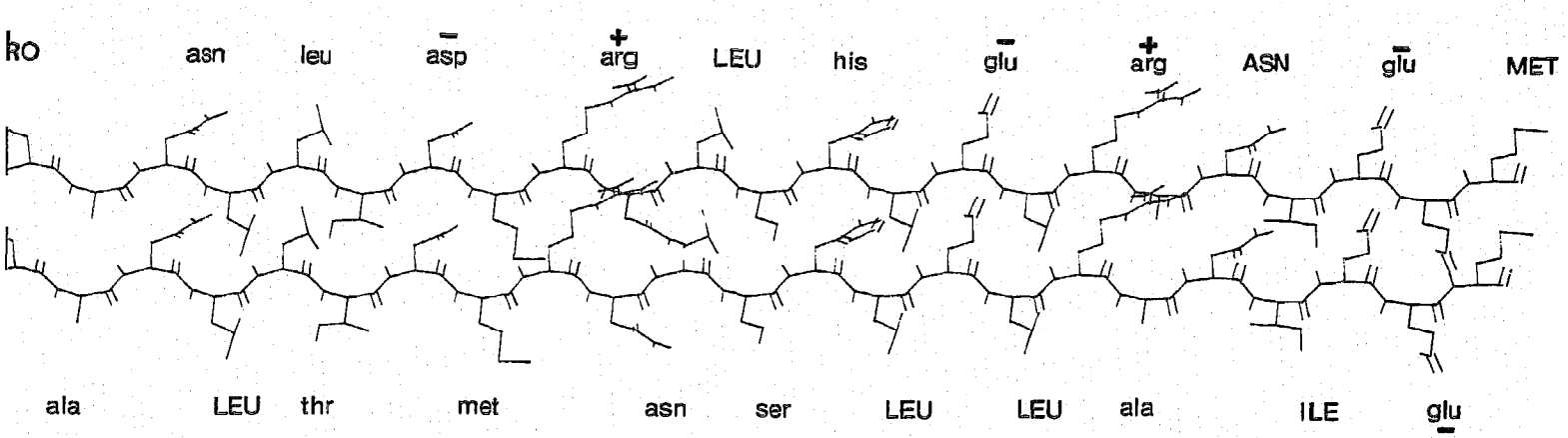

Fig. 5. Molecular model of TL-LRR1 peptide in a parallel β-sheet conformation. The diagram is annotated in the three-letter code to indicate the composition of the two faces of the proposed structure. Residues conserved in all or most LRRs are in upper case letters and divergent amino acids in lower case. Side chains that carry a net charge at neutral pH are indicated + or −.

ged surfaces of 2 peptide units and hydrophobic and Van der Waals interactions between side chains on the largely apolar surface. Again, such a structure would be in accord with the measured thickness of the filaments. A further possibility is that the peptide units are aligned along the axis of the filament in a staggered array, and again this might involve ionic interactions between charged sidechains. However, such filaments would be expected to have a thickness not greater than about 2 nm, significantly thinner than actually observed. In order to distinguish definitely between these and other possibilities, further structural studies are required.

The finding that TL-LRR1 forms intermolecular β-sheet structures supports the view that LRRs can participate in protein–protein interactions and homotypic cellular adhesion. It could be that LRRs expressed on the cell surface are initially of disordered structure and that interaction with similarly disordered LRRs on an adjacent cell causes the formation of an extended and stable intermolecular β structure of the type formed by TL-LRR1. Such a mechanism could provide a molecular basis for cellular adhesion mediated by LRRs, such as that observed with chaoptin. Furthermore, it has been proposed that the dominant ventralising properties of truncated Toll molecules could be caused by their interaction with full length transmembrane molecules [4] and the closely related Gp1b receptor complex may undergo ligand induced multimerisation [20]. It seems possible that such phenomena could also involve interactions between LRRs.

The peculiar properties of TL-LRR1 described here may find practical applications. The gel of TL-LRR1 is analogous to that formed by polymerised acrylamide molecules and by uncrosslinked fibrin polymers. It seems possible that this peptide might form the basis of novel separation media and may find use in affinity or biosensor applications.

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