The Structure of the Extracellular Domain of Triggering Receptor Expressed on Myeloid Cells Like Transcript-1 and Evidence for a Naturally Occurring Soluble Fragment*

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Triggering receptor expressed on myeloid cells like transcript-1 (TLT-1) is an abundant platelet-specific, type 1 transmembrane receptor. The extracellular fragment of TLT-1 consists of a single, immunoglobulin-like domain connected to the platelet cell membrane by a linker region called the stalk. Here we present evidence that a soluble fragment of the TLT-1 extracellular domain is found in serum of humans and mice and that an isoform of similar mass is released during platelet activation with thrombin. We also report the crystal structure of the immunoglobulin domain of TLT-1 determined at the resolution of 1.19 Å. The structure of TLT-1 is similar to other immunoglobulin-like variable domains, particularly those of triggering receptor expressed on myeloid cells-1 (TREM-1), the natural killer cell-activating receptor NKP44, and the polymeric immunoglobulin receptor. Particularly interesting is a 17-amino acid segment of TLT-1, homologous to a fragment of murine TREM-1, which, in turn, showed activity in blocking the TREM-1-mediated inflammatory responses in mice. Structural similarity to TREM-1 and polymeric immunoglobulin receptor, and evidence for a naturally occurring soluble fragment of the TLT-1 extracellular domain, suggest that this immunoglobulin-like domain autonomously plays an as yet unidentified, functional role.

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§ The atomic coordinates and structure factors (code 2FRG) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The abbreviations used are: TLT-1, triggering receptor expressed on myeloid cells like transcript-1; TREM, triggering receptor expressed on myeloid cell; hTLT-1, human TLT-1; mTLT-1, murine TLT-1; YFP, yellow fluorescent protein; ESI-MS, electrospray ionization-mass spectrometry; pGfR, polymeric immunoglobulin receptor; r.m.s.d., root mean square deviation; CDR, complementarity determining region.
strong foundation for future characterization of TLT-1 function, we crystallized a recombinant fragment of the hTLT-1 immunoglobulin-like domain consisting of residues 20–125, and determined its crystal structure at the resolution of 1.19 Å.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**

Human embryonic kidney (HEK) 293T cells were maintained and transfected as described earlier (13). Expression plasmids for mTLT-1 and characterization of full-length murine TLT-1 (mTLT-1) were previously described (13). The expression plasmid for mTLT-1 was tagged at the carboxyl terminus with a yellow fluorescent protein (YFP) fragment using PCR. Primers used for amplification included: forward, gttacatcagttgtagcaagggcgcag, and reverse, cgccagatttcctcgagctgctcttg, and reverse, gctggatggagtctgattg.

**Preparation of Washed Platelets**

**Murine Platelets**—Mice were bred and maintained under specific pathogen-free conditions at NCI, National Institutes of Health (NIH), Frederick, MD. Animal care was provided following the procedures outlined in *A Guide for the Care and Use of Laboratory Animals* (14). Peripheral blood collection via cardiac puncture and platelet purification from peripheral blood were performed as described earlier (15). Platelets were then washed in modified mouse Tyrode’s buffer (10 mM HEPES, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, and 12 mM NaHCO₃, pH 7.4).

**Human Platelets**—Fresh platelet concentrate in acid-citrate-dextrose was obtained from the NIH blood bank. Platelets were isolated by centrifugation at 800 × g for 10 min and resuspended in Tyrode’s solution (2 mM MgCl₂, 137 mM NaCl, 2.68 mM KCl, 3 mM NaH₂PO₄, 0.1% glucose, 5 mM HEPES, and 0.35% albumin, pH 7.35) to a final concentration of 10⁸ cells/ml.

**Western Blot Analysis**

Washed platelets (10⁹ cells) were lysed in 1 ml of lysis buffer (50 mM Tris, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 5 mg/ml aprotinin, and 5 mM EDTA, pH 8.5) and kept for 15 min on ice. Thereafter, 30 µg of protein per lane was examined by Western blot analysis as previously described (13).

**Antibodies**

Antimurine TLT-1 was generated by immunizing rabbits with a fusion protein containing residues 20–125 of mTLT-1 and a polyhistidine tag for purification (Fusion Antibodies, Belfast, Northern Ireland). An additional antimurine TLT-1 polyclonal antibody, termed mTLT-1-c, as well as antihuman TLT-1, were generated by immunizing goats, and purified by affinity chromatography (R&D Systems, Minneapolis). Horseradish peroxidase-conjugated antibodies and controls were obtained from BD Pharsingen (San Diego, CA).

**Synthetic Gene Construction**

The cDNA sequence encoding full-length hTLT-1 was obtained from GenBank™ (BC100945) (16). The expression construct used for structure determination contains only the immunoglobulin-like domain (residues 20–125). This fragment (20–125) was chosen for structural studies based on alignment of the sequence of TLT-1 with the sequence used for structure determination of NKp44 (1HKF), and predicted secondary structure of hTLT-1 from the Jufo server (5, 17). The DNA sequence was optimized for expression in Escherichia coli using the program DNAWorks (18). The gene assembly and amplification were performed as previously described (18), and then the synthetic gene was cloned into a pET-22b plasmid vector for expression in *E. coli*.

**Expression and Purification of Recombinant Immunoglobulin-like Domain of hTLT-1**

A pET-22b vector (Novagen) containing the gene encoding residues 20–125 of hTLT-1 was transformed into the *E. coli* BL21DE3-RIL-competent cells (Invitrogen). Transformed cells were grown on Luria-Bertani (LB):agar plates supplemented with 34 µg/ml chloramphenicol and 50 µg/ml of ampicillin. Individual colonies were isolated and used to inoculate 50-ml cultures of LB with chloramphenicol and ampicillin, and incubated for 12–16 h at 37 °C. The overnight cultures were resuspended in 950 ml of LB (with chloramphenicol and ampicillin) and incubated at 37 °C until optical densities showed logarithmic growth. Expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside and 1% glucose (m/v). After incubation with isopropyl-1-thio-β-D-galactopyranoside for 3–5 h, cells were harvested by centrifugation at 5,000 × g for 20 min. The cell pellet was resuspended in 50 mM Tris, pH 8, with lysozyme (1 mg/ml), deoxyribonuclease I (1 mg/ml), and ribonuclease A (1 mg/ml). After mechanical cell lysis and centrifugation (28,000 × g for 40 min), the soluble lysate was discarded. The insoluble material containing TLT-1 was washed several times, and solubilized in 8 M urea as previously described (19). After centrifugation and filtering, the resulting solution was diluted with 50 mM Tris (pH 8) containing arginine (400 mM), reduced glutathione (5 mM), and oxidized glutathione (0.5 mM) to a final protein concentration between 0.1 and 0.01 mg/ml, and final urea concentration of 50–100 mM. After stirring for 4 days at 4 °C, the refolded and oxidized solution of hTLT-1 was concentrated (Amicon ultrafiltration cell, Millipore) and subjected to size-exclusion and ion-exchange chromatographies (Sephacryl S100 HR and DEAE-Sepharose, GE Biosciences). Purity and identity of the protein were established by SDS-PAGE, and by electrospray mass spectrometry, ESI-MS (Agilent Technologies, Hewlett-Packard).

The purified hTLT-1 sample was digested with trypsin and analyzed by ESI-MS to establish the connectivity of disulfide bonds. Sequencing-grade-modified trypsin (Roche Diagnostics Gmbh, Mannheim, Germany) was dissolved in 1 mM HCl to a concentration of 1 mg/ml and mixed in a mass-ratio of 1:50 with a 1 mg/ml solution of hTLT-1 in 0.1 M Tris, pH 8.5. After overnight digestion at room temperature, samples were analyzed by ESI-MS.

**Crystallization**

Preparations of hTLT-1 immunoglobulin-like domain were subjected to crystallization trials by hanging drop vapor diffusion. Clusters of thin crystal plates initially appeared in the droplets containing 5–10 mg/ml hTLT-1, 0.8 M sodium/potassium phosphate (pH 5.6). Subsequent optimization resulted in thicker crystals. The final crystallization medium contained 1 µl each of 10 mg/ml hTLT-1 in 20 mM Tris, pH 8, and mother liquor consisting of 15–19% (w/v) polyethylene glycol 6000, and 0.5 M sodium/potassium phosphate (pH 5.6). All crystallization experiments were completed at 15 °C.

**Data Collection and Processing**

Diffraction data were collected at 100 K (Oxford Cryosystems, Oxford, UK) after brief soaking of crystals in a mother liquor, supple-
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Table 1

| Diffraction data processing and refinement statistics | Synchrotron |
|-----------------------------------------------------|-------------|
| Wavelength (Å)                                      | 1.135       |
| Resolution range (Å)                                | 30–1.19 (1.28–1.19)* |
| Space group                                         | P2_12_12 |
| Unit cell (Å)                                        | a = 47.1, b = 80.0, c = 25.8 |
| Unique reflections                                  | 202,943     |
| Redundancy                                          | 6.4 (3.5)   |
| Completeness (%)                                    | 98.3 (87.0) |
| Rmerge                                             | 0.064 (0.314) |
| I/σ(I)                                             | 25.9 (3.2)  |

Notes:
- Values shown in parentheses correspond to the outermost resolution shell.
- Rmerge = Σ||F_o| - |F_c||/Σ|F_o|, F_o minus the average intensity of symmetry-equivalent reflections.
- Rfree = Σ||F_o| - |F_c||/Σ|F_o|.
- The value of free R factor I was calculated based on a randomly chosen 3% of reflections excluded from refinement.

RESULTS

Soluble Fragment of TLT-1—TLT-1, found in the α-granules of resting platelets, is translocated to the platelet surface following activation by thrombin (1). A time-course experiment was performed using resting and activated mouse platelets to characterize activation-dependent changes in mTLT-1 expression. Following activation, platelet suspensions were combined with an equal volume of 2× lysis buffer. The equivalent of 1.5 × 10⁷ platelets was resolved by polyacrylamide gel electrophoresis. During the 5-h period, the apparent mass of mTLT-1 shifted from almost exclusively a 40-kDa form to a nearly equal mixture of 40- and 25-kDa forms (Fig. 1A). This observation led us to test whether the smaller mTLT-1 isoform was released from platelets. In subsequent experiments, platelets were centrifuged prior to lysis to separate the cell-associated and soluble fractions. Pelleted resting platelets and the resulting supernatant showed no trace of a 25-kDa isoform (Fig. 1B, lane 1 and 2). However, following activation with thrombin, a soluble form of mTLT-1 could be detected in the supernatant after removal of platelets by centrifugation (Fig. 1B, lane 4). The 25-kDa form of mTLT-1 was detected in murine serum when probed with an antibody specific for the extracellular domain of mTLT-1 but not detected in serum using an antibody specific to the cytoplasmic domain of mTLT-1, suggesting that the species in serum includes the extracellular domain (Fig. 1C). We confirmed the identity of the 25-kDa band as TLT-1 by showing that two different antibodies directed toward the extracellular domain of mTLT-1 reacted with it, whereas a series of control antibodies did not (Fig. 1D). TLT-1 was not observed in murine plasma or in defibrinated plasma (Fig. 1D, lanes 2 and 3). To test whether mTLT-1 found in serum resulted from proteolytic cleavage of the platelet surface-associated variant, or from alternative transcription of soluble isoforms, we transfected HEK293 cells with cDNA encoding mTLT-1, or a mTLT1/YFP chimera (Fig. 1E). Similar results were obtained for both forms of recombinant mTLT-1 in that the protein is observed near the expected molecular masses in cell-associated fractions (lanes 1 and 3), and isoforms of identical size were detected in conditioned cell media, irrespective of the cDNA construct used for transfection (lanes 2 and 4). The isoform released from HEK293 cells was identical in apparent molecular mass to the form detected in murine serum (lane 5).

Although there is >70% homology between the murine and human orthologs, we repeated the experiments using human platelets, serum, and HEK293 cells transfected with hTLT-1 cDNA. Human platelets and serum probed with an antibody specific for the extracellular domain of hTLT-1 demonstrate similarities to the murine system (Fig. 2, A and B). Activated human platelets show various hTLT-1 isoforms, but human serum contains only a pair of smaller forms (Fig. 2A). In human serum, two bands corresponding to apparent masses of 12 and 14 kDa were...
may have a function independent of the intact molecule. To gain a
together, experiments examining TLT-1 from platelets and recombi-
from platelets following activation with thrombin (Fig. 2
transfected with the hTLT-1 gene (Fig. 2
(12 and 14 kDa) are clearly observed in extracts from cells, which were
with the hTLT-1 cDNA encoding the smaller of two known forms of
TLT-1, or from alternative transcription, we transfected HEK293 cells
supernatants of resting platelets and from plasma (Fig. 2
following platelet activation (Fig. 2
hTLT-1 and evaluated the cell culture media for the presence of these
with the hTLT-1 cDNA encoding the smaller of two known forms of
mTLT-1/YFP; Lanes 2,0
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observed (Fig. 2A), very similar to the doublet observed in supernatant
following platelet activation (Fig. 2B). Human TLT-1 is absent from
supernatants of resting platelets and from plasma (Fig. 2B). To test
whether hTLT-1 fragments were in fact derived from cleavage of
hTLT-1, or from alternative transcription, we transfected HEK293 cells
with the hTLT-1 cDNA encoding the smaller of two known forms of
hTLT-1 and evaluated the cell culture media for the presence of these
fragments. No hTLT-1 was detected from wild-type HEK293 cells or
conditioned culture media, but full-length (35 kDa) and smaller forms
(12 and 14 kDa) are clearly observed in extracts from cells, which were
transfected with the hTLT-1 gene (Fig. 2C). Lanes 4 and 5 of Fig. 2C
show that the isoforms of recombinant hTLT-1 released from HEK293
cells (lane 4) are identical in apparent molecular mass to those released
from platelets following activation with thrombin (lane 5). Taken
together, experiments examining TLT-1 from platelets and recombi-
nant TLT-1 from HEK293 cells, suggest that the extracellular domain
may have a function independent of the intact molecule. To gain a
deeper understanding of how this domain may function, we have crys-
tallized and determined the structure of the immunoglobulin-like
domain of human TLT-1.

The Overall Structure of hTLT-1—Human TLT-1 crystallized in the
orthorhombic space group P21212 with unit cell dimensions a = 46.9 Å,
b = 80.1 Å, and c = 25.7 Å. The final model described unique confor-
mations of all the side chains, including those in the loop regions. Figs. 3
(A and B) show the overall structure of hTLT-1 consisting of nine
β-strands connected by loops in the V-type immunoglobulin-like fold.
The loop connecting strands B and C (B–C loop), and the E–F loop form
short 310 helical segments, similar to those observed in TREM-1 (Fig.
3C). The overall structure of the immunoglobulin-like domain of
hTLT-1 is very similar to antibody variable domains with the largest
differences observed in CDR loop regions (Fig. 3D). hTLT-1 has two
disulfide bonds, homologous to those seen previously in the structures of
NKp44 and plgR. Formation of the disulfide bonds was closely moni-
tored during the purification of hTLT-1 by trypsin digestion and sub-
sequent ESI-MS. One of the disulfide bonds (Cys38–Cys106) in TLT-1 is
relatively conserved in immunoglobulin-like domains, and it is found in
murine and human TREM-1, NKp44, and plgR. The second disulfide
bond in hTLT-1, connecting Cys52 located in strand βC and Cys59
(strand βC’), is not present in murine or human TREM-1 but is found in
NKp44 and plgR.

Comparison with Known Structures—The model of hTLT-1 immu-
noglobulin-like domain was used as a probe for searching the protein
data bank (PDB) using the Vector Search Alignment Tool from NCBI
(29). The search identified 53 non-redundant protein structures with
significant structural similarity to hTLT-1. The closest structural rela-
tives to hTLT-1 are cell-surface receptors, including plgR, TREM-1,
NKp44, and the mouse myeloid cell receptor, Clm-1 (PDB code: 1ZOX).
The immunoglobulin-like domain of KTLT-1 also shares significant
structural homology with T-cell receptors, cell adhesion molecules,
and other immune receptors. The root-mean-square deviation (r.m.s.d.)
between the structurally equivalent Cα atoms in hTLT-1 and a T-cell
receptor, Vε11 (PDB code: 1H5B chain A) is 1.7 Å, whereas the value
calculated for the human junctional adhesion molecule type 1 (PDB

FIGURE 2. Western analysis of human platelets, serum, and the recombinant
HEK293 cells. All samples were separated by SDS-PAGE under reducing conditions and
probed with an antibody specific for the extracellular domain. B, resting or activated platelets (1.5 × 109) in Tyrode’s buffer were pelleted by centrifugation. Lanes 1 and 3 represent 30 µg of total protein from the pellet. Lanes 2 and 4 represent 20 µg of the supernatant. In C: lane 1, 30 µg of total protein from pelleted resting murine platelets; lane 2, activated platelets; lane 3, 5 µl of supernatant. Samples were probed with an antibody specific for either the extracellular domain of murine TLT-1 (left panel) or intracellular domain of human TLT-1 (right), which cross-reacts with the intracellular domain of
mTLT-1. In D: lane 1, 5 µl of murine serum; lane 2, 5 µl of murine plasma; lane 3, 5 µl of defibrinated murine plasma. Samples were probed with various antibodies. From top to bottom: two antibodies (mTLT-1 and mTLT-C), commercial) specific to the extracellular domain of mTLT-1, antibodies specific for TREM-1, the secondary anti-Rabbit Fc, (α-Rab) alone, anti-goat F, alone (α-Goat), and hTLT-1-specific antibodies. In E: lanes 1–4, 30 µg of total protein from mTLT-1 HEK293 cell pellet, or conditioned culture media; lane 1, cell-associated mTLT-1/YFP; lane 2, soluble mTLT-1 isoform released from cells transfected with mTLT-1/YFP; lane 3, cell-associated mTLT-1; lane 4, soluble mTLT-1 isoform released from cells transfected with mTLT-1; lane 5, 5 µl of murine serum.
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FIGURE 3. A, schematic representation of the hTLT-1 immunoglobulin-like domain. Several loops connecting β-strands are labeled according to convention for the V-set immunoglobulin fold. Labels also indicate the CDR-equivalent loops and termini. Disulfide bonds connecting β-strands C to C, and β-strands D to F, are shown in orange. B, the C-terminus showing residues located at the tip of the loops connecting β-strands. C, superimposed models of TLT-1 (shown in blue) and hTREM-1 (red). D, superposition of the hTLT-1 monomer (shown in blue) and an antibody light chain variable domain (chain A in the PDB entry 1cr9).

FIGURE 4. Hypervariable regions including CDR-equivalent loops in hTLT1 (shown in blue), NKp44 (green), and mTREM-1 (cyan). A, residues just outside of CDR1 and the neighboring D–E loop (D and E refer to the β-strands’ annotation) have conserved interactions in hTLT-1 and NKp44 structures, but the interactions are not conserved in the mTREM-1 structure. B, structural differences at the second hypervariable region, including CDR2-equivalent residues. C, CDR3, located at the tip of the F–G loop, shows the greatest variation between structures of TREM family members.

code: 1NBQ) and hTLT-1 is 1.6 Å. For comparison, the r.m.s.d. values calculated for hTLT-1 and proteins with higher amino acid sequence homology, mTREM-1, hTREM-1, NKp44, and plgR, are 1.1, 1.1, 2.1, and 1.4 Å, respectively.

CDR-equivalent Loops—Complementarity determining regions (CDRs) have been identified in antibody V-domains as portions as antigen binding regions (30). In single-domain immunoglobulin-like molecules, the CDR-equivalent loops are referred to as CDR1, -2, and -3. The structure of hTLT-1 demonstrates variability in these loop structures, and these are the locations of the largest structural differences between hTLT-1 and related immunoglobulin-like crystal structures.

The conformations of CDR-equivalent loops in hTLT-1 were clearly defined in electron density, due in part to crystal contacts with neighboring molecules, which stabilized the positions of CDR2- and CDR3-equivalent loops. A section of the CDR1-equivalent loop forms a 3_10 helix (31) (Fig. 4A). In hTLT-1, the side chains of Tyr^{40} and Lys^{49} interact with the carboxylate oxygen atoms of D81, located on the D–E loop, probably stabilizing the orientations of strands βB and βC relative to strands βD and βE. Human TREM-1 and NKp44 are homologues at the positions equivalent to Tyr^{40}, Lys^{49}, and Asp^{81} in hTLT-1. In plgR the latter residue is asparagine, resulting in a similar pattern of interactions. In mTREM-1 the residues equivalent to Tyr^{40} and Asp^{81} (in hTLT-1) are phenylalanine (Phe^{45}) and histidine (His^{87}). Their relative orientations do not support a similar favorable interaction; however, the side chain of Lys^{35} is within hydrogen bond distance from a nitrogen atom in the side chain of His^{87} (Fig. 4).

The second hypervariable region in the TREM family includes CDR2-equivalent residues, linking strands βC’ and βC (Fig. 4B). In murine and human TREM-1, NKp44, and plgR, this hypervariable region extends from the end of βC’, through strand βC” to Arg^{76}, located in the C”–D loop. In TLT-1, conserved residues surrounding CDR2 include Ser^{65} (serine or threonine in related molecules) and Arg^{76}. The hydrogen bond between a side-chain equivalent to Ser^{65} and a backbone atom of strand βC is maintained in all known structures of TREM family members. Arg^{76}, in turn, forms a hydrogen bond and a salt bridge with the side chains of Gln^{95} and Asp^{81} from the nearby loop D–E. These interactions likely stabilize the overall structure of the β-sheets while allowing sequence diversity within the hypervariable loops.

In hTLT-1, the third CDR-equivalent loop is very short and consists of only 4 residues that do not show hydrogen bonds characteristic of β-strands (Fig. 4C). This loop shows the greatest conformational variability between TREM-1, NKp44, and TLT-1 (the only TREM family structures currently known). In fact, significant difference is observed between the two hTREM-1 crystal structures. The first structure (1Q8M) is a domain-swapped dimer in which the loop CDR3 (F–G loop), the amino-terminal half of βG, and the amino terminus were in extended conformations, forming a large portion of the dimeric interface. In murine and human TREM-1, the F–G loop is three residues longer than hTLT-1. In mTREM-1 and monomeric hTREM-1 struc-
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Although the deepest portions of this groove on the surface of TLT-1 are uncharged, the sides of the groove are lined with negatively charged glutamic acid residues that contribute by Asp^{107} near CDR3 on β-strand F, and Glu^{80} located in the C–C’ loop and the strand βC’ on the surface of mono-meric hTREM-1, this groove is essentially covered by side chains of the C–C’ and F–G loops, which are in closer proximity in TREM-1 compared with hTLT-1. Prominent on the surface of hTREM-1 (PDB code 1SMO), is a 5.5-Å approach of the carboxylate oxygen from Glu^{121} and the methyl group of Met^{65}. In hTLT-1, the F–G loop bends away from the C–C’ loop and contacts a neighboring molecule in the crystal. A more exposed groove on the surfaces of hTLT-1 and NKp44 relative to murine or human TREM-1 results from the differences in loop conformations.

Comparison with Active mTREM-1 Peptide—Residues 94–110 in hTLT-1 are structurally equivalent to mTREM-1 residues 103–119. A peptide composed of mTREM-1 residues 103–119 has been synthesized and shown to block pro-inflammatory cytokine production caused by intact, membrane-bound mTREM-1 in a mouse model of septic shock (12). In hTLT-1 and mTREM-1, this peptide encompasses roughly half of the E–F loop, the entire β-strand F, and part of the F–G loop (CDR3). Both ends of the active peptide from mTREM-1 form solvent-exposed loops, and much of the β-strand portion of the peptide contributes to the groove formed by twisting the GFCC’ β-sheet and protrusion of the C–C’ loop (Fig. 5). In hTLT-1, residues equivalent to the mTREM-1 peptide contribute negative charges to the electrostatic surface at the E–F loop, a property conserved in all known structures of TREM family members (NKp44, hTREM-1, mTREM-1, and hTLT-1). At the carboxyl end of the peptide, Arg^{110} of hTLT-1 contributes a positive charge to the molecular surface at CDR3, whereas murine and human TREM-1 have proline in the equivalent location.

This 17-amino acid peptide is highly conserved between murine and human TREM-1, with only 3 amino acid substitutions in this region. The sequence of hTLT-1 shows additional variation. Only 6 of 17 amino acids are conserved, all near the amino terminus of the peptide, i.e. the farthest from CDR3 (Fig. 6). The backbone atoms superimpose well between mTREM-1 and hTLT-1 over residues in the N-terminal half of the peptide corresponding to the E–F loop and most of strand F, but toward the C-terminal end of the peptide, the sequences and backbone traces diverge. The divergence between the two structures reflects the sequence diversity and conformational differences of CDR3. Thus, the 17-amino acid peptide comprises nearly equal portions of a conserved structural element, and one that is unique to each different protein.

DISCUSSION

Platelet activation is one of the first steps in a cascade of thrombolytic events, including clot formation and retraction (32). Here we show that the extracellular domain of murine and human TLT-1 is released from the surface of activated platelets. From murine platelets and serum we observed a single band, or tight doublet with an apparent molecular mass of 25 kDa, whereas the full-length murine TLT-1 could be observed at 45 kDa. Soluble hTLT-1 isoforms appeared at apparent masses of 12 and 14 kDa, whereas the heaviest hTLT-1 isoform observed ran at an apparent mass of 35 kDa. In both human and murine TLT-1, the mass difference between full-length and truncated forms is roughly 20 kDa (judged by SDS-PAGE). The molecular masses of mTLT-1 and hTLT-1, calculated from their amino acid sequences (omitting residues 1–20 as signal peptides) are 31.5 and 30.7 kDa, respectively. The difference between observed and calculated masses, therefore, likely results from different post-translational modifications in mice relative to humans.
The soluble species of mTLT-1 released from platelets and detected in serum likely contain modifications, as an unmodified 25-kDa form of mTLT-1 would retain its membrane-spanning region and most of the cytoplasmic domain. Intact hTLT-1, however, is observed at an apparent mass much closer to its predicted molecular mass than mTLT-1, and the soluble forms of hTLT-1 observed are significantly smaller. Apparent fragments of the hTLT-1 extracellular domain of masses 12 and 14 kDa correspond roughly to 100 and 110 amino acids of the extracellular domain, or approximately the complete immunoglobulin-like domain (105 residues total, 20–125). Although the smaller forms of TL-T-1 may result from alternative transcription, release of recombinant murine or human TLT-1 extracellular domain from HEK293 cells strongly suggests that the stalk region of TLT-1 is susceptible to hydrolysis.

Immunoblots were probed with two different antibodies specific for the extracellular domain of mTLT-1, and a panel of control antibodies suggests that the observed forms are actually mTLT-1 (Fig. 1D), and not the result of nonspecific binding interactions observed with a single antibody. Our observation that TLT-1 can be detected in soluble forms released from platelets suggests the potential for biological activity of these apparent fragments. In the context of platelet function, surface-associated TLT-1 could enhance platelet aggregation or clot formation, and shedding of TLT-1 could participate in clot retraction by helping release platelets from the clot. Soluble TLT-1 species may bind the natural TLT-1 ligand and prevent productive engagement of surface-associated TLT-1. The crystal structure of this recombinant fragment of hTLT-1 (residues 20–125) includes the most likely ligand binding portion of the extracellular domain and is similar to the soluble isoforms identified from natural sources.

The crystal structure of the immunoglobulin-like domain of hTLT-1 demonstrates a close relationship with other TREM receptors (5–8) but also with pIgR (22) and the mouse myeloid cell receptor Clm-1 (1ZOX). Structural similarities between hTLT-1 and antibody variable domains, T-cell receptors, and cell adhesion molecules suggest that TLT-1 can form a complex with one or more protein or peptide ligands. Comparing β-sheet residues of hTLT-1 with murine and human TREM-1, and NKP44, indicates a closer relationship with TREM-1 than NKP44, because the r.m.s.d. is nearly twice as high between hTLT-1 and NKP44, compared with hTLT-1 and either murine or human TREM-1. This was unexpected, because the structure confirmed that hTLT-1 and NKP44 share a second disulfide bond not common to hTLT-1 and TREM-1.

Antibodies use CDR loops to tightly bind protein or peptide antigens (30). CDR loops show the greatest variability in length and conformation with β-strands conserving the immunoglobulin V-type fold (Figs. 3 and 4), partly due to interactions between β-strands and β-sheets, which hold adjacent parts of the structure in a conserved relative orientation. Even within the conserved portions of the immunoglobulin-like domain, electrostatic surfaces demonstrate that hTLT-1 is unique, with sequence variability resulting in differences in surface shape and electrostatic properties (Fig. 5).

Recently, a recombinant mTREM-1 immunoglobulin-like domain was shown to block mTREM-1-mediated pro-inflammatory cytokine production (12). In addition to the intact immunoglobulin-like domain, a 17-amino acid fragment of this domain reduced pro-inflammatory cytokine production and death in a murine model of septic shock (11). The activity of this peptide in apparently blocking mTREM-1 activity is interesting, because the N-terminal portion of this peptide is highly conserved in sequence and structure within the TREM family, and the carboxyl portion of the peptide is unique between members of the TREM family. The location of this peptide-forming part of CDR3, its prominent exposure on the molecular surface, and its anti-mTREM-1 activity suggest that the peptide includes an important binding site for the natural ligand of TREM-1. The peptide may block productive binding between the ligand and surface-associated TREM-1 (11). We hypothesize that an equivalent fragment formed from hTLT-1 residues functions analogously to block hTLT-1 binding to its natural partner, thereby inhibiting hTLT-1 signaling. Once an hTLT-1 activity is established, we can test whether the intact immunoglobulin-like domain or smaller fragments of that domain can block the activity.

The overall structural conservation within immunoglobulin-like domains and the unique surface and electrostatic properties demonstrate how nature uses the immunoglobulin-like domain as a scaffold for building biological molecules with extreme ligand binding specificity. The presence of a naturally occurring soluble form of murine and human TLT-1 released from platelets suggests a TLT-1 function not directly related to its unique cytoplasmic domain. The hTLT-1 structure suggests experiments to uncover the mechanism of TLT-1 action, once such an activity is established. Together, these details extend the molecular understanding of TLT-1 and provide a foundation to explain continuing biochemical analysis of TLT-1 function.

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