Structural study of TTR-52 reveals the mechanism by which a bridging molecule mediates apoptotic cell engulfment

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During apoptosis, apoptotic cells are removed by professional phagocytes or neighboring engulfing cells either directly through phagocytic receptors or indirectly through bridging molecules that cross-link dying cells to phagocytes. However, how bridging molecules recognize “eat me” signals and phagocytic receptors to mediate engulfment remains unclear. Here, we report the structural and functional studies of Caenorhabditis elegans TTR-52, a recently identified bridging molecule that cross-links surface-exposed phosphatidylserine (PtdSer) on apoptotic cells to the CED-1 receptor on phagocytes. Crystal structure studies show that TTR-52 has an open β-barrel-like structure with some similarities to the PKCα-C2 domain. TTR-52 is proposed to bind PtdSer via an “ion-mediating” PtdSer-binding mode. Intensive functional studies show that CED-1 binds TTR-52 through its N-terminal EMI domain and that the hydrophobic region of the TTR-52 C terminus is involved in this interaction. In addition, unlike other PtdSer-binding domains, TTR-52 forms dimers, and its dimerization is important for its function in vivo. Our results reveal the first full-length structure of a bridging molecule and the mechanism underlying bridging molecule-mediated apoptotic cell recognition.

[Keywords: apoptotic cell engulfment; bridging molecule; crystal structure; TTR-52]

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cytoplasmic region of CED-1 contains two motifs characterized by NPXY and YXXL, which are important for signal transduction [Su et al. 2002].

PtdSer is an evolutionarily conserved “eat me” signal exposed on the surface of apoptotic cells. The recognition of PtdSer by phagocytic receptor TIM4, the C2 domain of the bridging molecule MFG-E8 [MGF-E8-C2 domain], or intracellular PtdSer-binding proteins that are not involved in apoptotic cell recognition, such as Annexin V, the C2 domains of synaptotagmins, or PKCα, has been extensively studied by structural biology approaches (Sutton et al. 1995; Swairjo et al. 1995; Santiago et al. 2007; Shao et al. 2008; Guerrero-Valero et al. 2009). The C2 domains of synaptotagmins and PKCα, has been extensively studied by structural biology approaches (Sutton et al. 1995; Swairjo et al. 1995; Santiago et al. 2007; Shao et al. 2008; Guerrero-Valero et al. 2009). The C2 domains of synaptotagmins and PKCα fold into a β-sandwich structure containing eight β strands. One characterized feature of this domain is the successive β-bulge folds of some of these strands [Sutton et al. 1995]. Annexin V is a helix bundle protein with channel-forming membrane characteristics. The PtdSer-binding domains of TIM4 have immunoglobulin-like features, while the C2 domain at the MGF-E8 N terminus has a β-sandwich structure that has some significant differences from that of PKCα-C2. With the exception of MFG-E8, all of these proteins have been reported to have a common “ion-mediating” mechanism such as calcium-bridging for PtdSer binding, although they fold into different structures. Although numerous extracellular bridging molecules were identified, how they cross-link apoptotic cells to phagocytes remains unclear, largely due to the lack of a complete structure of full-length bridging molecules.

TTR-52 is a secreted protein identified recently in C. elegans that recognizes surface-exposed PtdSer and binds to the extracellular domain of CED-1 [Wang et al. 2010]. It therefore serves as a bridging molecule that mediates recognition of apoptotic cells by cross-linking the “eat me” signal PtdSer with the CED-1 receptor. Based on sequence analysis, TTR-52 has been predicted to have a common “ion-mediating” mechanism such as calcium-bridging for PtdSer binding, although they fold into different structures. Although numerous extracellular bridging molecules were identified, how they cross-link apoptotic cells to phagocytes remains unclear, largely due to the lack of a complete structure of full-length bridging molecules.

Results

Full-length TTR-52 is composed of 135 amino acids, the first 20 residues being a signal peptide. To begin with, our work was hampered because aggregation of the wild-type protein purified from Escherichia coli prevented its crystallization [Fig. 1A]. We evaluated the baculovirus expression system and the 293T mammalian cell expression system for production of large amounts of TTR-52, but both systems failed due to low expression of TTR-52. To overcome this problem, a series of mutant proteins were analyzed using alanine-block scanning mutagenesis. Although dozens of mutant proteins were tested, only one in which residues 50–55 [50EDSLPL55] were replaced by six successive alanines [termed TTR-52 [M5]] produced some proteins that were found to be stable dimers by gel filtration chromatography [Fig. 1A]. Well-diffracted crystals were obtained using this protein.

The 2.01 Å crystal structure of TTR-52 [M5] was determined using the single isomorphous replacement with anomalous scattering [SIRAS] method [Fig. 1; Supplemental Table S1]. A summary of the crystallographic data and refinement statistics for TTR-52 [M5] is shown in Supplemental Table S1. The structure was refined to a final R value of 17.7% and a free R value of 23.8%. A clear and continuous electron density map indicates that the structure was properly defined [Supplemental Fig. S1].

Overall structure of the TTR-52 monomer

The TTR-52 [M5] monomer consists of seven β strands (S1–S7) that are connected by five loops [L1–L5] and one β turn (T1) [Fig. 1B]. These β strands fold into an open β-barrel-like structure, with the sixth and seventh strands forming edges around the opening. Loops linking these β strands are located at the two sides of the barrel. Loop 2 [L2, between S2 and S3], loop 3 [L3, between S4 and S5], and loop 5 [L5, between S6 and S7] are located in the N-terminal region of the barrel, and loop 1 [L1, between S1 and S2], loop 4 [L4, between S5 and S6], and turn 1 (T1, between S3 and S4) are located in the C-terminal region. The six consecutive alanine residues introduced by mutagenesis form a short α helix (α1) in L2. We propose that helix α1 is an artifact resulting from mutation of those residues to alanine and that the corresponding region in wild-type TTR-52 would be a loop, according to the secondary structure prediction by PsiPred [Bryson et al. 2005]. We further assume that this six-alanine peptide does not change the overall folding of TTR-52, since it is located on the flexible loop that is far away from the β strands. In order to perform structural and functional analysis of the wild-type protein, the structure of wild-type TTR-52, in which the six alanines were mutated back to their original residues, was built using Modeller [Eswar et al. 2006] based on the structure of TTR-52 [M5]. No additional changes were introduced to other parts of the structure, except that helix α1 was modeled as a short loop. Overall, the structure exhibits extensive negatively charged and hydrophobic surfaces [Fig. 2B].

Structural comparison of TTR-52 [M5] with transthyretin

Based on sequence homology, it was predicted that TTR-52 may have a transthyretin protein fold [Wang et al. 2010]. Structural alignment using DALI [Dietmann et al. 2001] showed that these two protein structures have an overall root mean square deviation (R.M.S.D.) of ~3.0 Å. We found three major differences between these two
structures. First, there is a hydrophobic region on the C terminus of TTR-52 that might serve as a functional surface (Fig. 1B) that is not present in transthyretin. Second, transthyretin has a β-sandwich structure in which strands 4, 1, 7, and 8 and strands 3, 2, 5, and 6 form two layers of the twisted β sheets (Hornberg et al. 2000). However, topological analysis showed that TTR-52 has only seven β strands, corresponding to the N-terminal seven strands in transthyretin (Fig. 1C). In TTR-52, strand 3 connects the second and fourth strands by backbone hydrogen bonds [Supplemental Fig. S1]. Compared with the third and fourth strands in transthyretin, the corresponding strands in TTR-52 are located closer to the center of the structure and to each other, making the TTR-52 structure like an open β barrel (Fig. 1D). Third, intramolecular interactions are also different. Transthyretin monomers assemble into stable dimers via hydrogen bonds between the sixth and eighth strands (Hornberg et al. 2000). Two dimers then interact further to form a tetramer through hydrophobic interactions (Connely et al. 2010). In contrast, gel filtration analysis showed that recombinant wild-type TTR-52 purified from 293T cells exists as dimers [Supplemental Fig. S2]. In our structure, while TTR-52 [M5] is also shown to exist as a dimer, its monomer–monomer interaction mode is different from that of transthyretin. The monomers in the dimer of TTR-52 interact back to back, mainly through the side chains on the third and fourth strands (Fig. 6A, below).

Mechanism of TTR-52 binding to PtdSer
When DALI searching was used to identify proteins with a structure similar to TTR-52, none of them were PtdSer-binding proteins. When compared with the reported structures of PtdSer-binding proteins [Sutton et al. 1995; Swairjo et al. 1995; Gaboriaud et al. 2003; Shao et al. 2008], we found that TTR-52 has a structure similar to the PKCa-C2 domain, which is known for its intracellular PtdSer-binding ability, although sequence identity of the PKCa-C2 domain and TTR-52 is <20% [Supplemental Fig. S3]. We compared the structure of TTR-52 with that of the PKCa-C2 domain [Guerrero-Valero et al. 2009]. The PKCa-C2 domain has eight strands and folds into a β-sandwich structure. The seven-stranded TTR-52 has a topology similar to the C-terminal seven strands [strands 1–7] of the PKCa-C2 domain [for convenience, we refer to the first N-terminal strand of PKCa-C2 as S0] [Fig. 1C]. The successive β-bulge folds that characterize
 residues and the main chain of the other two residues. Two calcium ions coordinated by five aspartate residues in C. elegans embryos. Wild-type TTR-52::mCherry forms bright rings around dying cells (arrows), but point mutations D51A, D36A/E38A, or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abolish recognition of apoptotic cells by TTR-52 and that D36A/E38A or R100A/D101A did not completely abol
another possibility that the N-terminal region (L2, 49–59 amino acids; L3, 79–88 amino acids) is required for PtdSer binding. We made point mutations (D51A, D80A, and N85A) in this region and found that all three point mutations almost completely abolished the clustering of apoptotic cells by TTR-52::mCherry and failed to rescue the cell corpse phenotype of ttr-52 (tm2078) mutants (Figs. 2C, 3). They also showed decreased membrane labeling in yeast cells (Supplemental Fig. S5) and greatly reduced PtdSer binding in a membrane lipid strip assay (Fig. 4). These results suggest that the loops in the N-terminal region, rather than those in the C-terminal region, may play a crucial role in PtdSer binding.

PtdSer is bound in a cation ion-dependent manner in TIM4, Annexin V, and the PKCo-C2 domain. Particularly, PtdSer is bound by the C2 domain between two loops at one end of the β-sandwich structure that mimic the two arms of a clamp (Verdaguer et al. 1999). Although no sequence similarity can be found between the L2 and L3 loops of TTR-52 and those corresponding regions in the C2 domain [Supplemental Fig. S3], the acidic residues in L2 and L3 may mimic the “ion-mediating” PtdSer-binding properties, similar to TIM4, Annexin V, and the PKCo-C2 domain [Supplemental Fig. S6]. Therefore, removal of the predicted cation ions bound to TTR-52 may disrupt the binding of this protein to PtdSer. To test this, Flag beads were washed with TBS-E buffer [50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA] to chelate divalent cations after affinity purification of TTR-52-mCherry-Flag from the culture medium of 293T cells transfected with pCMV-TTR-52-mCherry-Flag. The eluted proteins were assayed for PtdSer binding using a membrane lipid strip. Instead of binding PtdSer, cation-free TTR-52 showed strong binding activity toward phosphatidic acid. The binding of TTR-52 to PtdSer was not restored even when the TTR-52 protein solution was equilibrated in buffers containing 0.2 mM Ca\(^{2+}\), 0.2 mM Zn\(^{2+}\), or 0.2 mM Ca\(^{2+}\)/0.1 mM Zn\(^{2+}\) (Fig. 4), suggesting that removal of cation ions by EDTA may alter the local conformation of the cation ion-binding loops, which is required for specific binding of TTR-52 to PtdSer and is not recovered efficiently by adding back cation ions.

In addition to the negatively charged residues, we also studied the functions of the continuous stretches of
hydrophobic residues on the tips of L2 and L3: 53LPL55 and 81FGPI84. Point mutations L53A, P54A, and L55A in L2 had a much lower ability to recognize dying cells in vivo compared with the wild type, although they were able to partially (P54A) or fully (L53A and L55A) rescue the ttr-52 (tm2078) mutant phenotype [Fig. 3]. However, point mutation P83D in L3 had a severe phenotype; i.e., it could not form a ring around dying cells [Fig. 2C] and could not rescue the ttr-52 (tm2078) mutant phenotype [Fig. 3]. It is important to note that there are no hydrophobic residues corresponding to those mentioned above on the tips of L2 and L3 in the PKCa-C2 domain. TIM4 has also been reported previously to use hydrophobic residues on the tips of loops to interact with the cell membrane [Santiago et al. 2007]. We propose that the hydrophobic residues on the tips of the PtdSer-binding loops in TTR-52 may have a similar function; these hydrophobic residues may be located in close contact with the membranes of dying cells. These results indicate that PtdSer binding and membrane insertion are both required for the recognition of dying cells.

Interactions between TTR-52 and CED-1

As we showed previously, TTR-52 binds to the extracellular domain of CED-1 [Wang et al. 2010]. Here, we generated different truncations of CED-1 and analyzed their interactions with TTR-52 by an in vitro pull-down assay [Fig. 5A–C]. The EMI domain at the N terminus of CED-1 was found to be sufficient for TTR-52 binding [Fig. 5]. We further tested the importance of the EMI domain for CED-1 function in vivo and found that a ced-1 truncation without the EMI domain [Pced-1,CED-1ΔEMI-GFP] could not rescue the mutant phenotype of ced-1 (e1735), a null allele of ced-1 [Fig. 5D; Zhou et al. 2001]. We then overexpressed the EMI domain under the control of heat-shock promoters [PheatSS40-EMI-GFP, secretion form of EMI-GFP] or the ced-1 promoter [Pced-1SS40-EMI-TM-GFP, membrane-
anchored EMI by CED-1 transmembrane domain) in a wild-type unc-76 (e911) worm and found that they caused a dominant-negative effect, i.e., transgenic worms showed a cell corpse engulfment defect similar to that of the ttr-52 mutant [Fig. 5D].

We next investigated the potential CED-1-binding sites on TTR-52. TTR-52 folds into an open β-barrel conformation where strands 6 and 7 form the edges around the opening. Together with the C-terminal tail, the C terminus forms a highly hydrophobic region [Fig. 1B]. Some of the hydrophobic residues from this region in our structure are exposed to the solvent. This architecture implies that strands 6 and 7 plus the C-terminal end may be involved in CED-1 binding. We made a double point mutation, I113R/L132K, and a small deletion, 131YLEDY135 (TTR-52 ΔC), in the C terminus of TTR-52, both of which recognized cell corpses normally but failed to rescue the persistent cell corpse phenotype of ttr-52 (tm2078) mutants [Figs. 2C, 3]. TTR-52 interacts with the CED-1 receptor to induce its recognition of apoptotic cells [Wang et al. 2010]. The percentage of cell corpses clustered by CED-1::GFP in wild-type embryos was 60%, while that in ttr-52 (tm2078) mutants was reduced to 41% [Table 1]. We found that expression of TTR-52-I113R/L132K or TTR-52 ΔC failed to rescue the decreased clustering of CED-1 around apoptotic corpses in ttr-52 (tm2078) embryos and that <40% of cell corpses were labeled by CED-1::GFP [Table 1]. These results indicate that the C-terminal residues of TTR-52 are important for mediating recognition and binding of apoptotic cells by CED-1.

**TTR-52 dimer formation is important for its function**

TTR-52 [M5] was found to exist as a dimer in our structure, consistent with the behavior of wild-type proteins expressed in 293T cells. Both wild-type TTR-52 proteins purified from the medium of 293T cells transfected with pCMV-TTR-52-Flag and TTR-52 [M5] purified from E. coli were present as dimers in gel filtration chromatography experiments [Fig. 1; Suplemental Fig. S2]. In our structure, adjacent TTR-52 [M5] molecules interact back to back to form a butterfly-like dimer structure [Fig. 6A]. The interaction surface of the two monomers is ~700 Å² in total, as determined by PISA [Krissinel and Henrick 2007]. The interaction of adjacent TTR-52 monomers is mainly mediated by residues D58, R63, W65, Q73, and T75 on the third and fourth strands [Fig. 6A].

**Table 1. Quantification of CED-1::GFP rings formed around dying cells in TTR-52 transgenic worms**

<table>
<thead>
<tr>
<th>Strains</th>
<th>CED-1::GFP clustering</th>
</tr>
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<tbody>
<tr>
<td>smls34, P_hsp TTR-52-mCherry</td>
<td>L1: 59.3% (<em>n</em> = 162)</td>
</tr>
<tr>
<td>smls34, P_hsp TTR-52-ΔC-mCherry</td>
<td>L2: 39.6% (<em>n</em> = 192)</td>
</tr>
<tr>
<td>smls34, P_hsp TTR-52-R63D/W65Q/Q73A-mCherry</td>
<td>L3: 39.0% (<em>n</em> = 236)</td>
</tr>
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Transgenic worms from two independent lines carrying P_hsp TTR-52-mCherry, P_hsp TTR-52-ΔC-mCherry, or P_hsp TTR-52-R63D/W65Q/Q73A-mCherry were crossed with smls34 [P_adh-1::CED-1-GFP], and the percentage of cell corpses surrounded by CED-1::GFP was determined by analyzing serial optical sections of the 1.5-fold stage embryos. More than 100 corpses per embryo were scored for a total of 15 embryos in each experiment.

**Discussion**

In a previous study [Wang et al. 2010], we reported that TTR-52 functions as a bridging molecule that binds PtdSer exposed on the surface of dying cells and the extracellular domain of CED-1 receptor on engulfing cells, thus cross-linking dying cells to engulfing cells and promoting cell corpse engulfment. In this study, we demonstrate how TTR-52 acts in this process by structural and cell biological studies.

While many methods are available for obtaining sufficient amounts of soluble proteins for crystallographic studies [Pantazatos et al. 2004; Keenan et al. 2005], they are not effective in all cases, and new methods are still
required. Here, we used alanine-block scanning, a mutagenesis technique that has been broadly applied in protein functional analysis, for recombinant protein optimization and crystallization trials. One mutant of TTR-52—TTR-52 (M5), in which six consecutive residues (EDSLPL) were mutated to six alanines—gave well-diffracted crystals, and its structure was determined. We also attempted to purify protein from mutants in which fewer residues in these regions were mutated, but without success (data not shown). We hypothesize that the success of this mutation could be due to its location on a loop in which there are several hydrophobic residues. These hydrophobic residues would lead to the aggregation of this protein when expressed in E. coli. Thus, when this loop was substituted by a stable helix, the folding process for this mutant protein may have been much easier. Thus, we demonstrate here that alanine-block mutagenesis can be successfully applied as a new method in protein crystallization.

The TTR-52 (M5) monomer has an open β-barrel architecture with seven strands connected by five loops. Our data demonstrate that loops L2 and L3 in the N-terminal region are responsible for PtdSer binding because mutations in this region (D51A, D80A, and N85A) greatly reduce its binding affinity to PtdSer in vitro and apoptotic cells in vivo (Figs. 2C, 4; Supplemental Fig. S5) and abolish its function in apoptotic cell engulfment in vivo (Fig. 3). Since many residues in this cleft are negatively charged acidic amino acids, we suspected that cations may mediate PtdSer binding as in other PtdSer-binding proteins, such as TIM4, Annexin V, and the PKCa-C2 domain. However, in spite of its high resolution, we did not find a divalent ion in the TTR-52 (M5) crystal structure, either in the electron density map or by anomalous signal screening, likely due to mutations in predicted cation-binding sites. We therefore verified this hypothesis by chelating divalent cations with EDTA using affinity-purified TTR-52-mCherry-Flag and found that the cation-free protein lost its ability to bind PtdSer, which was not restored when the protein was equilibrated with divalent cations (Fig. 4), implicating its importance in stabilizing protein structure. Although we showed the importance of divalent cations to TTR-52, we were not able to determine which cations are involved. We predicted that calcium is important because of the structural similarity between TTR-52 and the PKCa-C2 domain, in which PtdSer binding has been shown to be calcium-dependent. In contrast to the PKCa-C2 domain, the hydrophobic residues on the tips of loop 2 and loop 3 of TTR-52 are also involved in PtdSer binding; mutations at L53, P54, and L55 in loop 2 and at P83 in loop 3 affected cell corpse recognition, and the latter even abolished TTR-52’s function in cell corpse removal (Fig. 3). These hydrophobic residues are predicted to bind directly to the membranes of dying cells, as is the case for TIM4, which is expressed on the surface of antigen-presenting cells and mediates the phagocytosis of apoptotic cells. We propose that these hydrophobic residues may assist the binding of this protein to the surface of dying cells.

The L1 and L4 loops of TTR-52 also contain some negatively charged residues, but our results show that these residues are not essential to the binding of PtdSer by TTR-52 (Supplemental Figs. S4, S5). Combined mutation of these residues has a limited effect on apoptotic cell engulfment in vivo (Fig. 3). No cation ion was observed in this area in the structure. The exact function of this region is thus unclear. Notably, in the structure of synaptotagmin, the prototypical C2 domain-containing protein, there are also some clustered acidic residues in the loop region opposite the PtdSer-binding region (Sutton et al. 1995). This similarity suggests that the
additional acidic regions in the two proteins may have important functions.

The presence of a large hydrophobic region in the C terminus of TTR-52—containing strand 6, strand 7, and the C-terminal tail—suggests that it may be the binding site for the extracellular domain of CED-1. Both double mutation of I113R/L132K and deletion of the C-terminal five residues failed to rescue the ttr-52 mutant phenotype [Fig. 3], and the percentage of CED-1::GFP rings around dying cells was greatly decreased when these mutations were expressed [Table 1]. This implies that the C-terminal hydrophobic region of TTR-52 may interact with the CED-1 extracellular domain (CED-1 EX), although we could not prove this by using either ex vivo pull-down assays with recombinant proteins purified from E. coli or coimmunoprecipitation in transfected 293T cells. On the other hand, using a truncated protein pull-down assay, we found that the EMI domain in the extracellular region of CED-1 is responsible for its interaction with TTR-52. The EMI domain, which has been reported to be involved in protein–protein interactions [Doliana et al. 2000], is necessary and sufficient for the interaction of CED-1 with TTR-52 in vitro. We further confirmed this result in vivo; overexpression of the EMI domain of CED-1 alone in wild-type worms caused dominant-negative effects, giving a number of corpses in transgenic worms similar to that in the ttr-52 mutant [Fig. 5D].

Unlike other PtdSer-binding proteins, TTR-52 exists as a dimer in both the crystal structure and solution, as confirmed by gel filtration, sedimentation velocity, and cross-linking experiments [Figs. 1A, 6B, Supplemental Fig. S7]. When the crucial residues R63, W65, and Q73 between the dimer interface were mutated, TTR-52 [M5] lost its dimer state and behaved as a monomer. When this mutated form of TTR-52 was introduced into worms, it resulted in decreased levels of CED-1::GFP ring formation around dying cells [Table 1] and could not rescue the ttr-52 mutant phenotype, although PtdSer labeling on the surface of dying cells was not affected [Figs. 2C, 3]. It is thus clear that the interaction between TTR-52 molecules is important for its function, especially in inducing cell corpse recognition by CED-1. In addition, loss of TTR-52 reduces but does not totally block recognition of apoptotic cells by CED-1 [Wang et al. 2010], suggesting that multiple factors may be involved in mediating cell corpse engulfment through CED-1. It is therefore interesting to determine whether other PtdSer-binding proteins are involved and similar mechanisms are employed.

Based on the structural and functional studies reported here, we propose a model for the role of TTR-52 in mediating cell engulfment. TTR-52 forms a dimer in cells and the dimer provides two possible PtdSer-binding sites. The two PtdSer-binding sites of one TTR-52 dimer mimic the two upper wings of the butterfly-like structure of TTR-52 [Fig. 6A] and extend out from the dimer in the same direction, suggesting that the two PtdSer-binding sites can bind to apoptotic cells simultaneously, likely increasing the binding affinity of this protein to the apoptotic cell membrane. The two large hydrophobic regions in the C terminus of the dimer, mimicking the lower wings of the butterfly, provide binding sites for the EMI domain of CED-1. Thus, the apoptotic cells and phagocytes cells are cross-linked via the bridging molecule.

In summary, we report the first full-length crystal structure of a secreted bridging molecule that can be recruited by PtdSer exposed on the surface of dying cells to trigger CED-1-dependent cell corpse engulfment. These results reveal a potential mechanism of the TTR-52 bridging molecule in cell corpse engulfment in C. elegans. Since the bridging molecule-mediated engulfment phenomenon is conserved in higher organisms, this work provides useful information for studies of engulfment in mammalian cells.

Materials and methods

Plasmid construction

Standard methods of PCR amplification, cloning, and sequencing were used. A detailed description is provided in the Supplemental Material. All constructs generated using PCR-amplified fragments were verified by DNA sequencing.

Protein expression and purification

Plasmid pHET21b-TTR-52 ΔN-His was transformed into E. coli strain BL21 (DE3) [Invitrogen]. Cells were cultured in LB medium at 37°C with 100 mg/L ampicillin. When the OD 600 reached 0.6–0.8, the culture was induced by addition of isopropyl-thio-D-glactosidase (IPTG) [Sigma] to a final concentration of 0.5 mM for 20 h at 16°C. Cells were harvested by centrifugation at 5000 rpm for 10 min. Pellets were resuspended in Tris buffer [20 mM Tris at pH 8.0, 150 mM NaCl] and lysed by sonication. The lysate was separated by centrifugation at 16,000 rpm for 30 min, and the recovered supernatant was applied to a Ni-NTA affinity column [Qiagen], followed by intensive washing with washing buffer [20 mM Tris at pH 8.0, 150 mM NaCl, 50 mM imidazole]. Recombinant TTR-52 was eluted from the Ni-NTA affinity column using elution buffer [20 mM Tris at pH 8.0, 150 mM NaCl, 500 mM imidazole] and further purified by gel filtration with a Superdex200 column [GE Healthcare] using Tris buffer as described above on an FPLC protein purification system.

Crystallization and structure determination

The protein was concentrated for crystallization in spin concentrators [Millipore] to a concentration of ~3–4 mg/mL. SDS-PAGE was used to determine the purity of the proteins. Crystallization trials were set up using crystal screen kits (Hampton Research) using the hanging-drop method by mixing 1 μL of protein solution with an equal volume of a reservoir solution. Crystals of a usable size were obtained in a buffer condition with 0.2 M ammonium sulfate, 0.1 M sodium acetate [pH 4.5], and 35% PEGME2000. PtCl4 salt was soaked into the crystals to obtain electron density map. About 90% of the

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residues were built automatically by ARP/wARP (Morris et al. 2003), and the additional missing residues were added manually in COOT (Emsley et al. 2010). The final model was refined with PHENIX (Adams et al. 2010). In the Ramachandran plot generated using PROCHECK (Laskowski et al. 1993), 95.58% and 4.42% of the amino acids in the final atomic model were in the most favorable and additional allowed regions, respectively. No amino acids were in the disallowed region. The atomic coordinates and diffraction data have been deposited in the Protein Data Bank (3UAF). All structural figures were prepared with COOT and PyMOL (http://www.pymol.org).

**Strains and culture conditions**

*C. elegans* strains were maintained using standard methods (Brenner 1974). The N2 Bristol strain was used as the wild-type strain. All of the alleles used in this study have been described previously [Ellis et al. 1991; Riddle et al. 1997] except the *ttr-52* (tm2078), which is an engulfment defect mutant in this study (Supplemental Fig. S8).

**Quantification of cell corpses**

The number of cell corpses in early stage embryos was scored using Nomarski optics as described before (Stanfield and Horvitz 2000).

**Fluorescence microscopy and quantification of the CED-1::GFP clustering around apoptotic cells**

To score the percentage of cell corpses labeled by CED-1::GFP, Nomarski and fluorescent images of embryos in a 20-μm series (1 μm per section) were captured with a Zeiss Axioimager M1 equipped with epifluorescence and an AxioCam monochrome digital camera. Serial optical sections of embryos were analyzed, first to identify cell corpses by their raised disc-like morphology in 1.5-fold-stage embryos, and then to determine the clustering of CED-1::GFP.

**Heat-shock treatment**

Animals at various stages were incubated for 1 h at 33°C, followed by recovery at 20°C. Cell corpses and TTR-52 expression were examined 3.5 h after the heat-shock treatment.

**PtdSer-binding assays**

Wild-type *Saccharomyces cerevisiae* strain BY4743 (a gift from Y. Zhang, National Institute of Biological Sciences, Beijing, China) was streaked on YPAD plates and cultured at 30°C. *Y. Zhang, National Institute of Biological Sciences, Beijing, China* was a gift from Y. Zhang, National Institute of Biological Sciences, Beijing, China; *C. elegans* strains were obtained from the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health (NIH). The N2 Bristol strain was used as the wild-type strain. All of the alleles used in this study have been described previously [Ellis et al. 1991; Riddle et al. 1997] except the *ttr-52* (tm2078), which is an engulfment defect mutant in this study (Supplemental Fig. S8).

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**GST fusion protein pull-down assays**

Purified GST, GST-CED-1 EX, GST-CED-1 F1, GST-CED-1 EMI, and GST-CED-1 F1 EMI (1 μg of each) immobilized, respectively, on glutathione-agarose beads (Sigma) were incubated with 2.5 μg TTR-52 AN-His6 in a 200-μL reaction volume containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM dithiothreitol (DTT) at 4°C. The beads were washed five times with the same buffer, and bound proteins were resolved by 15% SDS-PAGE and detected by Western blotting with an antibody against the hexahistidine tag (Santa Cruz Biotechnology).

**Cross-linking assays**

Recombinant proteins TTR-52 (M5) and TTR-52 (M5)-R63D/W65Q/Q73A were purified from *E. coli* as described above. Ethylene glycol bis-succinimidyl succinate (EGS) (1 mM or 5 mM) (Sigma) was added to the recombinant proteins (2 μg) in 15 μL of 1× PBS buffer. Cross-linking reactions were carried out for 30 min at room temperature and stopped by adding EDTA. Samples were separated by 15% SDS-PAGE and stained by Coomassie blue.

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**References**


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**Supplemental Material**
http://genesdev.cshlp.org/content/suppl/2012/06/19/26.12.1339.DC1

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