Interaction of CED-6/GULP, an Adapter Protein Involved in Engulfment of Apoptotic Cells with CED-1 and CD91/Low Density Lipoprotein Receptor-related Protein (LRP)*

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Hua Poo Su‡, Kumiko Nakada-Tsukui‡, Annie-Carole Tosello-Trampont‡, Yonghe Li§, Guojun Bu§, Peter M. Henson§, and Kodimangalam S. Ravichandran¶‡

From the ‡Beine Carter Center for Immunology Research and the Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908, the ¶Washington University School of Medicine, St. Louis, Missouri 63110, and the ¶National Jewish Medical and Research Center, Denver, Colorado 80206

The prompt clearance of cells undergoing apoptosis is critical during embryonic development, normal tissue turnover, as well as inflammation and autoimmunity. The molecular details of the engulfment of apoptotic cells are not fully understood. *ced*-6 and its human homologue *gulp*, encode an adapter protein, whose function in engulfment is highly evolutionarily conserved; however, the upstream and downstream components of CED-6 mediated signaling are not known. Recently, *ced-1* has been shown to encode a transmembrane protein on phagocytic cells, with two functional sequence motifs in its cytoplasmic tail that are important for engulfment. In this study, using a combination of biochemical approaches and yeast two-hybrid analysis, we present evidence for a physical interaction between GULP/CED-6 and one of the two motifs (NPXY motif) in the cytoplasmic tail of CED-1. The phosphotyrosine binding domain of GULP was necessary and sufficient for this interaction. Since the precise mammalian homologue of CED-1 is not known, we undertook a database search for human proteins that contain the motifs shown to be important for CED-1 function and identified CD91/LRP (low density lipoprotein receptor-related protein) as one candidate. Interestingly, recent studies have also identified CD91/LRP as a receptor involved in the phagocytosis of apoptotic cells in mammals. The GULP phosphotyrosine binding domain was able to specifically interact with one specific NPXY motif in the CD91 cytoplasmic tail. During these studies we have also identified the mouse GULP sequence. These studies suggest a physical link between CED-1 or CD91/LRP and the adapter protein CED-6/GULP during engulfment of apoptotic cells and further elucidate the pathway suggested by the genetic studies.

In multicellular organisms, cells die by apoptosis throughout life (1). This is required for processes ranging from embryonic development to routine tissue maintenance. When cells undergo programmed cell death within an organism, the final act is the efficient removal of the apoptotic cells (2). Without such removal, the release of toxic contents of these dying cells could lead to inflammation and subsequent pathologies within the organism (3). In fact, humans with deficiencies in genes involved in the phagocytosis of apoptotic cells and mice with targeted disruptions of specific genes develop symptoms characteristic of autoimmune disease (4–6). Although cells that die by either necrosis or apoptosis are eventually engulfed by the same phagocytic effector cells, uptake of apoptotic cells elicits anti-inflammatory cytokine production, in contrast to cells that die by necrosis (7–11). This is most likely due to differences in the signaling mechanisms activated during the recognition and/or processing of dying cells by the phagocyte. Thus, our understanding the molecular mechanism involved in clearance of apoptotic cells is of fundamental importance and could likely have a direct clinical relevance.

A number of cell surface receptors have been implicated in the phagocytosis of apoptotic cells in mammals, and these include scavenger receptors, integrins, the phosphatidylycerine receptor, and complement receptors (2). There is an apparent redundancy in the receptors required for engulfment, since blocking of individual receptors with antibodies appears to diminish but not completely abrogate engulfment of apoptotic targets. Differences in utilization of these receptors depending on the phagocytic cell as well as their state of activation has also been documented (2, 12, 13). Thus, the relative significance of the individual receptors has remained difficult to assess. Moreover, relatively little is known about the cytoplasmic proteins recruited downstream of these receptors that regulate engulfment of apoptotic cells in mammals. However, recent discoveries through elegant genetic studies in the nematode *Caenorhabditis elegans* system have provided insight into some of the critical players involved in engulfment (14–16). To date, seven genes required for the engulfment of apoptotic cells have been identified in the worm. The recent cloning of all of the seven genes in the worm and the existence of mammalian homologues for six of the seven genes, have provided an exciting opportunity to better delineate the signaling pathways during engulfment of apoptotic cells.

The seven genes identified in the worm have been classified into two partially redundant functional pathways, based on the severity of the phenotypes in double-mutants within these two groups. One group of genes consists of the genes *ced*-2, *ced*-5, *ced*-10, and *ced*-12, and their respective mammalian homologues have been identified as *crkII*, *dock180*, *rac*, and *elmo* (17–22). Analysis of this group of genes in *C. elegans*, as well as their homologues in Drosophila and mammals indicate that all of these four cytoplasmic signaling proteins play a role in organizing and controlling cytoskeletal rearrangement during engulfment and cell migration (17–20, 22). The receptor(s) that
function upstream of this group of genes remains to be identified.

The other group includes the genes ced-6/gulp, ced-1, and ced-7/abc1 (23–28). ced-6, as well as its Drosophila and human homologues, encode an evolutionarily conserved adapter protein that is required specifically in the engulfing cells and not in the dying cells. CED-6 and its homologues contain an N-terminal phosphotyrosine binding (PTB) domain, a central leucine zipper, and a proline-rich C-terminal region (25–27). The conservation of CED-6 function through evolution was best exemplified by the rescue of the engulfment defect in ced-6-deficient worms through expression of human ced-6/gulp as a transgene (26). This suggested that the interacting partners as well as some of the signaling pathways downstream of CED-6 are likely conserved from worm to humans. Overexpression of human CED-6 in the macrophage line J774 also promotes engulfment (27). Presently, there are no known binding partners for CED-6 or its homologues. Interestingly, overexpression of ced-6 in the ced-1- and ced-7-deficient backgrounds partially rescues the engulfment defect (25), suggesting that CED-6 might function as a signaling adapter downstream of CED-1 and CED-7, both of which are membrane proteins. However, whether CED-1 or CED-7 interacts with CED-6 and how such an interaction might be mediated has not yet been determined.

The molecular nature of ced-1 was revealed very recently. ced-1 encodes a putative receptor required only on the engulfing cells and likely participates in the recognition of apoptotic cells (24). CED-1 contains a large extracellular portion encoding 16 atypical EGF-like repeats, a transmembrane region, and a short cytoplasmic region. Two amino acid sequence motifs in the CED-1 cytoplasmic tail, NPX and YXXL motifs, have been shown to be important for function based on the rescue of ced-1-deficient worms using wild type or mutated forms of CED-1. This also suggested that CED-1 could transduce signals during engulfment, since NPX motifs can serve as binding targets for PTB domains, while the YXXL motifs can serve as targets for Src-homology 2 (SH2) domains. Currently, the precise mammalian homologue of CED-1 is unknown. The mammalian proteins, MEGF-6 and SREC, identified as most similar to CED-1 (24), fail to show any homology in the cytoplasmic region with CED-1 and do not contain the NPXY or YXXL motifs.

The homologue of CED-7 (the third member of this group that contains CED-1 and CED-6) is the 12-transmembrane

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The abbreviations used are: PTB, phosphotyrosine binding; LRP, low density lipoprotein receptor-related protein; EGF, epidermal growth factor; SH2, Src homology 2; GULP, enGULfment adaPter protein; GST, glutathione S-transferase; HA, hemagglutinin; GFP, green fluorescent protein; LZ, leucine zipper domain; CED, cell death abnormal.

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**FIG. 1.** Sequence alignment of murine GULP with human GULP and worm CED-6. The sequence of mouse GULP, as compiled from sequences in the database, was aligned with human GULP and CED-6. The dark bar below the sequences represents the PTB domain, while the leucine zipper is shown in gray bar. The human and murine GULP proteins are 93% identical with only a two-residue difference in the PTB domain. Preceding the aligned PTB domain, CED-6 contains an additional 34 residues not shared by GULP. Similarly the C terminus of CED-6 is also longer with about 150 additional residues not found in either GULP.
ATP-binding cassette transporter protein ABC1, which appears to be required on the engulfing as well as the dying cells (23, 28, 29). However, how CED-7/ABC1 proteins signal and the manner in which they functionally interact with other members of this functional genetic pathway are not known.

In this study, using several approaches, we observe the binding of the PTB domain of worm and mammalian CED-6 proteins to the NPYX motif in the cytoplasmic tail of CED-1. The PTB domain of CED-6 appears to be necessary and sufficient to mediate the interaction with CED-1. We also observe the binding of GULP to CD91/LRP, which has recently been found to play a role in the phagocytosis of apoptotic cells (30). These studies further elucidate the pathway suggested by the genetic studies in C. elegans and also confirm and extend them in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The DNA coding sequence for the peptide constructs were cloned by making pairs of oligonucleotides, which contain a 5′-NotI and a 3′-NotI site and cloned into a modified pEBG eukaryotic expression vector (31). GULP and CED-1 cytoplasmic tail expression constructs were obtained by PCR amplification of the relevant cDNAs and cloned into the modified pEBG eukaryotic expression vectors (for expression as a glutathione S-transferase (GST) fusion protein) and pEBB-HA (for expression of hemagglutinin (HA)-tagged proteins) using 5′-NotI and 3′-NotI restriction sites. To generate the CD16-CED-1-GFP fusion construct, first the cytoplasmic region of CED-1 was amplified and cloned into the pEBB-CD16 vector as described previously (32). The enhanced green fluorescent protein (EGFP) coding sequence was subcloned from pEGFP-N1 (CLONTECH, Palo Alto, CA) as a NotI-NotI fragment in-frame at the 3′ end of the CED-1 coding sequence. The cDNA constructs of CD91/LRP minireceptors have been described previously (37).

**Cell Culture and Transfection**—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% bovine calf serum, 5% fetal calf serum, and penicillin/streptomycin/glutamin (Invitrogen). COS-7 cells were transfected using Superfect or Polyfect (Qiagen, Valencia, CA) according to the manufacturer’s recommendation. All transient transfections were performed using 2–10 μg of the appropriate expression plasmid and analyzed 24–48 h posttransfection.

**Immunoprecipitations and Immunoblotting**—Cells were lysed on the tissue culture plates with lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 10 mM each aprotinin, leupeptin, pepstatin, AEBSF, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1% Nonidet P-40). GST-tagged proteins were precipitated with glutathione-Sepharose (Amersham Biosciences, Inc.). Precipitates were washed four times with buffer containing 20 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM each aprotinin, leupeptin, pepstatin, AEBSF, 5 mM sodium fluoride, 1 mM sodium orthovanadate, and 0.1% Nonidet P-40. Antibodies for Western blotting GFP, HA, and GST-tagged proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated to horseradish peroxidase were purchased from Amersham Biosciences, Inc. All immunoblots were developed using enhanced chemiluminescence (Pierce, Rockford, IL). All experiments were performed at least three times.

**Two-hybrid Assay**—The CytoTrap two-hybrid system was purchased from Stratagene (La Jolla, CA). The cod-6 constructs were cloned in frame of the pHSOS fusion protein (pSos), and the cod-1 constructs were cloned in-frame with the myristoylation membrane localization signal (pMyr). The plasmids were verified by DNA sequencing. Transformation of the Saccharomyces cerevisiae strain, cdc25H, was performed according to the manufacturer’s directions. The interaction between the proteins encoded by the introduced plasmids was scored by growth of the yeast transformants at the non-permissive temperature on galactose plates. The specificity of the interactions was verified by growth on glucose plates as well as cotransformation with plasmids encoding mutant proteins or control proteins.

**Database Searches**—The file containing predicted human proteins was downloaded from www.ensembl.org (33). The searches were performed on the April freeze dataset using a script written by us, which scans the database for a given motif and removes all sequences that do not contain the motif. The sequences that were not removed were scanned for all the other motifs. The remaining sequences were subjected to BLAST searches to determine the identity of the genes.

The mouse GULP sequence was derived from clones represented by GenBank™ accession numbers, AK014093 and AK017798, and the publicly available draft mouse genomic sequence. Comparison with human GULP suggests that AK014093 encodes all but the first two residues of mouse GULP; however, these two were present in AK017798. Additionally, two residues that did not align between human GULP and AK014093 are found to align between human GULP and AK017798, but were confirmed by the mouse genomic draft sequences. The C-terminal 20-amino acids of AK017798 appear to be derived from genomic intron sequences and did not align with the human GULP or the AK014093 clone.

**RESULTS**

**Identification of Mouse GULP and Comparison of CED-6/GULP Proteins**—While the genetic studies clearly show a role for CED-6 in engulfment of apoptotic cells, the binding partners of CED-6 are not known. Specifically, we were interested in interacting partners for the CED-6 PTB domain. These previous studies had identified the C. elegans, Drosophila melanogaster, and Homo sapiens ced-6 genes, but the identity of the murine ced-6 gene was not known (26, 27). During the course of the studies described below, we identified the murine ced-6 homologue from database searches. By assembling two murine ced-6 expressed sequence tags and comparison with the draft mouse genomic sequence we have compiled the cDNA sequence encoding murine CED-6 (Fig. 1). The mouse ced-6 gene encodes a 304-amino acid protein with the same overall architecture as the human and worm CED-6 proteins: an N-terminal PTB domain, immediately followed by a leucine zipper domain and a C-terminal proline-rich region. The mouse and human CED-6 proteins are highly homologous (93% amino acid identity) and have only one residue difference within the PTB domain. Comparison of C. elegans CED-6 with the mammalian CED-6 proteins reveals 34 extra residues at the N-terminal end and an additional 150 amino acids at the C-terminal end (Fig. 1). Previously, the human CED-6 protein was simply referred to as hCED-6 (26, 27). To avoid confusion between worm and mammalian proteins, we have renamed the mammalian CED-6 proteins as GULP (enGULfment adPter protein).

**GULP Binds to the NPYX Motif of CED-1—PTB domains have previously been shown to be capable of interacting with sequences that contain a core NPYX motif, although the specificity of particular PTB domains depends on neighboring sequences (38, 39).** The recent cloning of the cod-1 gene reveals that it encodes a transmembrane receptor containing cytoplasmic NPYX and YXXL motifs (potential binding sites for both PTB and SH2 domains, respectively) that were shown to be critical for CED-1-dependent phagocytosis (24). Since CED-1

**TABLE I**

| SOS fused bait protein/target protein | GULP PTB only (1–168) | GULP PTB+1-LZ (1–202) | GULP C-terminal domain (191–304) | CED-6 PTB domain only (36–200) |
|-------------------------------------|-----------------------|-----------------------|---------------------------------|-----------------------------|
| Lamin C (negative control)           | –a                    | –                     | –                               | –                           |
| SOS binding protein (positive control) | + + +                | + + +                 | + + + + +                       | + + +                       |
| CED-1 CT (931–111)                  | + + +                 | + + +                 | + + + + +                       | + + +                       |
| CED-1 CT (931–111) N962A            | + + +                 | +                     | + + + + +                       | + + +                       |

a, no detectable interaction.
b, CT, cytoplasmic tail.
and CED-6 belong to the functional genetic pathway (14, 24) and CED-6 contains a PTB domain (25), we tested whether the CED-1 NPLY sequence (surrounding tyrosine 965) may serve as a ligand for the CED-6/GULP PTB domains. We generated eukaryotic expression vectors encoding GST fused to a 13-amino acid CED-1 sequence containing the NPXY motif. The ability of HA-tagged GULP to bind to this CED-1-derived peptide was tested. After transient transfection into COS-7 cells, the GST-tagged peptide was coexpressed with the HA-tagged GULP plasmid in COS-7 cells. Following glutathione precipitation, the coprecipitated HA-GULP was detected by anti-HA immunoblotting. Anti-GST immunoblotting revealed comparable levels of precipitated GST-tagged peptides. Expression of similar levels of HA-GULP in all conditions was confirmed by immunoblotting of the total lysates (right panel). C, schematic diagram of GST-tagged CED-1 cytoplasmic tail constructs and HA-tagged GULP. D, following coexpression of the CED-1 cytoplasmic constructs with GULP, the proteins were precipitated with glutathione-Sepharose, and the presence of coprecipitated GULP was detected by anti-HA tag immunoblotting. Mutation of the NPXY motif abrogates binding to GULP (second lane). Equivalent expression of HA-GULP and precipitation of CED-1 constructs are shown. E, schematic diagram of the GST-tagged CED-1 constructs and the HA-tagged GULP constructs. Mutation of leucine to proline at amino acids 176 and 183 has been shown to disrupt the leucine zipper and prohibit dimerization of GULP. F, following precipitation of the GST tag, coprecipitation of GULP by the cytoplasmic tail of CED-1 is detected by anti-HA immunoblotting.

To determine whether GULP can interact with the NPXY motif in the intact CED-1 cytoplasmic tail and to determine the relative contribution of NPXY versus YXXL motifs, a construct encoding the entire cytoplasmic region of CED-1 fused to a GST tag was generated. In addition, mutations of the asparagine of the NPXY motif (N962A mutant) and the tyrosine in the YXXL motif (Y1019A mutant) were also performed (Fig. 2C). When coexpressed with GULP in COS-7 cells, both the wild type and the Y1019A mutant bound to GULP, but the N962A mutant of CED-1 showed a severe reduction in binding (Fig. 2D), suggesting that the interaction most likely occurs through GULP binding to the NPXY motif on CED-1.

We have previously identified a leucine zipper domain immediately following the PTB domain of GULP, which mediates homodimerization (31). We tested whether dimerization of GULP is required for interaction with CED-1 using a leucine zipper mutant of GULP (mLZ), shown in Fig. 2E, that we have shown fails to dimerize (31). This mutant LZ construct bound CED-1 comparable with wild type GULP (Fig. 2F), suggesting that under these conditions, the leucine zipper-dependent

**FIG. 2.** Interaction of human GULP with CED-1-derived NPXY peptide and the cytoplasmic tail of CED-1. A, the sequence of the NPXY peptide derived from the CED-1 cytoplasmic tail and the two mutants. The NPXY motif is shown in gray. B, the GST-tagged peptide constructs were coexpressed with the HA-tagged GULP plasmid in COS-7 cells. Following glutathione precipitation, the coprecipitated HA-GULP was detected by anti-HA immunoblotting. Anti-GST immunoblotting revealed comparable levels of precipitated GST-tagged peptides. Expression of similar levels of HA-GULP in all conditions was confirmed by immunoblotting of the total lysates (right panel). C, schematic diagram of GST-tagged CED-1 cytoplasmic tail constructs and HA-tagged GULP. D, following coexpression of the CED-1 cytoplasmic constructs with GULP, the proteins were precipitated with glutathione-Sepharose, and the presence of coprecipitated GULP was detected by anti-HA tag immunoblotting. Mutation of the NPXY motif abrogates binding to GULP (second lane). Equivalent expression of HA-GULP and precipitation of CED-1 constructs are shown. E, schematic diagram of the GST-tagged CED-1 constructs and the HA-tagged GULP constructs. Mutation of leucine to proline at amino acids 176 and 183 has been shown to disrupt the leucine zipper and prohibit dimerization of GULP. F, following precipitation of the GST tag, coprecipitation of GULP by the cytoplasmic tail of CED-1 is detected by anti-HA immunoblotting.
The PTB Domain of GULP/CED-6 Binds to CED-1—Next, we wished to test whether GULP can interact with membrane-bound CED-1. Our multiple efforts to express full-length CED-1 on the cell surface were unsuccessful, and the reason for this unclear. To assess the interaction of GULP with a membrane-bound CED-1, we fused the cytoplasmic tail of CED-1 to the extracellular domain of CD16 (34). We added a GFP tag to the C terminus of the CD16-CED-1 sequence to verify that the fusion protein was expressed on the cell surface (data not shown) as well as providing a tag for immunoblotting. When coexpressed, GULP was able to coprecipitate the CD16-CED-1-GFP fusion protein (Fig. 3A). We also tested whether the isolated PTB domain of GULP can interact with CED-1. Two different GULP PTB constructs (one that terminates just prior to the leucine zipper and another that contains the core PTB sequence based on alignment with other PTB domains) were both capable of precipitating the CD16-CED-1-GFP fusion protein. Taken together, these data suggest that GULP, via its PTB domain, can interact with a membrane-bound CED-1.

We then tested whether the C. elegans CED-6 PTB domain can also interact with the NPXY sequence motif in the CED-1 cytoplasmic tail. Although the PTB domains of GULP and CED-6 are quite similar, the worm protein has an additional 35 residues N-terminal to the homology with GULP PTB. Thus, we generated two versions of CED-6 PTB coding for amino acids 1–200 and 36–200 (Fig. 3C). Both of these constructs were able to interact with the wild type CED-1 cytoplasmic tail as shown in Fig. 3D. The interaction of CED-6 PTB was severely diminished with the N962A mutant of CED-1. These data suggested that the PTB domain of GULP and CED-6 have similar ligand binding characteristics, which is consistent with the observation that the human GULP was able to rescue the cell corpse engulfment defect in the ced-6 mutant worm (26).

The PTB Domains of GULP and CED-6 Interact with the Cytoplasmic Domain of CED-1 in Yeast Two-hybrid Assays—We also tested the CED-6/GULP interaction with CED-1 using the CytoTrap yeast two-hybrid assay. The PTB domains of GULP and CED-6 were cloned into the pSos bait plasmid, while the cytoplasmic domain of CED-1 (wild type or the N962A mutant) were cloned into the pMyr target vector. This two-hybrid system uses a yeast strain with a temperature-sensitive mutation in cdc25. The interaction between the bait and target proteins are detected through recruitment of the human SOS-bait fusion to the myristoylated target protein, which allows the growth of the yeast at non-permissive temperatures. As shown in Table I, both GULP and CED-6 PTB domains were able to interact with the CED-1 tail in this two-hybrid analysis, and the binding was again dependent on the presence of an intact NPXY motif. We also tested other GULP constructs that contain the PTB+LZ or the C terminus alone. While the PTB+LZ was capable of interacting with CED-1, the C terminus that lacks the PTB failed to interact with CED-1. These data suggested that the GULP/CED-6 PTB is necessary and sufficient to mediate binding to CED-1.

A Database Search for Putative “Functional Orthologues” of CED-1—When ced-1 was originally cloned, the mammalian proteins MEGF-6 and SREC were found to be most similar to CED-1 (24). However, the cytoplasmic tails of these two proteins do not contain the NPXY or XXXL motifs that were found to be critical for CED-1 function, suggesting that MEGF-6 and SREC may not represent the mammalian homologues of CED-1. The large extracellular domain of CED-1 and the numerous atypical EGF-like repeats may have biased the database searches. Our searches using the cytoplasmic region of

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**Fig. 3.** The PTB domain of GULP/CED-6 mediates binding to the CED-1 cytoplasmic tail. A, GULP constructs were fused to GST as shown. To localize the cytoplasmic region of CED-1 to the plasma membrane, it was fused to extracellular domain of CD16. GFP was fused to the CED-1 tail to aid detection. CD16-CED-1-GFP was expressed at the membrane as detected by microscopy (data not shown). B, following precipitation of GST-tagged GULP constructs, blotting with anti-GFP antibodies revealed the coprecipitated CD16-CED-1-GFP. C, schematic diagram of the CED-1 cytoplasmic tail constructs fused to GST and the PTB domain of CED-6 fused to the HA tag. D, following coexpression in COS-7 cells, the CED-1 tail was coprecipitated with glutathione-Sepharose. Coprecipitated CED-6 PTB domain was detected by anti-HA immunoblotting. Equivalent levels of expression of the CED-6 PTB constructs were shown by anti-HA immunoblotting of the total cell lysates (right panel). Anti-GST immunoblotting revealed that comparable levels of CED-1 were precipitated in the different lanes (bottom panel).
Interaction of CED-6/GULP with CED-1 and CD91/LRP

Recently, the LRP/CD91 has been shown to play a role in engulfment of apoptotic cells (30). CD91/LRP serves as a receptor for calreticulin, which binds to apoptotic cells via a group of molecules termed collectins. In addition to the presence of an NPXY motif (a criteria used in the above database search), the CD91/LRP cytoplasmic tail also contains the YXXL motif that was found to be functionally important for CED-1 mediated engulfment. To test the ability of CD91/LRP to bind to GULP, short peptides encoding either of the two NPXY motifs within the CD91/LRP cytoplasmic tail were constructed as GST fusion proteins (Fig. 4A). As shown in Fig. 4B, the second NPXY motif of CD91/LRP appears to interact with GULP. This binding is comparable with that of the peptide derived from CED-1. There was negligible binding to the first NPXY motif. Moreover, a peptide containing the NPXY motif from the integrin β3, previously implicated in engulfment (2), was also unable to bind to GULP, suggesting additional specificity outside of the core NPXY motif.

We used the previously characterized set of CD91/LRP mini-receptors, which encode a HA-tagged transmembrane and cytoplasmic domain of CD91/LRP (as a surrogate for membrane bound CD91/LRP) to further test the interaction between CD91/LRP and GULP (37). As shown in Fig. 4D, GULP was able bind to the wild type CD91/LRP version. We also compared GULP binding to the N26A and N60A mutants, which carry substitutions at the N(−3) positions of the first and second NPXY motifs, using a script written by us. Coexpression of wild type CD91/LRP with GST tag alone (negative control) is shown in the first lane. Anti-HA immunoblotting of total cell lysates showed comparable expression of the CD91/LRP constructs.

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Fig. 4. Binding of GULP to CD91/LRP. A, the sequences of the peptides used to test binding to GULP. The peptides derived from CD91/LRP and integrin β3 as well as the positive control CED-1 peptide are shown. The common phenylalanine in the −5 position relative to the tyrosine is highlighted. B, the GST-tagged peptide constructs were coexpressed with HA-GULP plasmids in COS-7 cells. The peptides were precipitated with glutathione-Sepharose, and the coprecipitated GULP was revealed by anti-HA immunoblotting. Comparable expression of the CD91/LRP constructs was revealed by blotting of total cell lysates (right panel). C, the GULP and CD91/LRP constructs are shown schematically. The CD91/LRP minireceptor contains an N-terminal HA tag, which is fused to the extracellular portion of CD91/LRP. Within the cytoplasmic region of CD91/LRP, the mutations in the first and second NPXY motifs are also shown. D, GST-tagged GULP was coexpressed with the indicated CD91/LRP constructs. Following anti-GST precipitation, the coprecipitated CD91/LRP constructs were detected by anti-HA immunoblotting. Coexpression of wild type CD91/LRP with GST tag alone (negative control) is shown in the first lane. Anti-HA immunoblotting of total cell lysates showed comparable expression of the CD91/LRP constructs.
NPXY motifs, respectively (Fig. 4C). GULP was able to bind to the N26A construct, but failed to bind to the N60A, confirming that GULP binds to the second NPXY motif within the CD91/LRP cytoplasmic tail.

DISCUSSION

Several lines of evidence presented in this report suggest that GULP/CED-6 interacts with CED-1 and CD91/LRP, two receptors that have been shown to participate in the phagocytosis of apoptotic cells. The full-length GULP, as well as the isolated PTB domain of GULP, were able to bind specific peptides derived from the cytoplasmic tail, the entire cytoplasmic tail of CED-1, or a membrane-bound form of the CED-1 tail. GULP and CED-6 bind to an NPXY motif that has previously been shown to be important in CED-1-dependent phagocytosis in the worm (24). The interaction between CED-1 and the PTB domain of GULP/CED-6 was further confirmed by yeast two-hybrid analysis. Analogously, specific peptides derived from the CD91/LRP tail as well a tagged form of CD91/LRP efficiently interacted with GULP. The failure of the first NPXY motif in CD91/LRP or the NPXY motif from the β3 integrin to bind to GULP provided additional confirmation for the specificity of the GULP/CD91 interaction.

Our data suggest that the PTB domain of GULP/CED-6 is necessary and sufficient for this interaction. The 14-residue peptide motifs derived from CED-1 and CD91/LRP suggest that GULP PTB can bind to these proteins independently of the context of the rest of the molecule. Comparisons between the peptides that can interact with GULP/CED-6 suggest a preference for a phenylalanine at the −5 position relative to the tyrosine. As has been seen previously with other PTB domains (38, 39), it likely that the GULP PTB domain also makes additional contacts beyond the core NPXY motif to attain specificity. Consistent with this, GULP fails to interact with an NPXY motif derived from the β3 integrin or the first NPXY motif from CD91/LRP. However, there appears to be redundancy in the binding of PTB domains to some ligands (40–42).

CD91/LRP has been shown to bind to the PTB domains of Shc and Dab1, although it has not been determined which of the NPXY motifs act as targets to the different PTB domains (40, 42, 43).

While the original phosphotyrosine-binding domain identified in the Shc and IRS-1 proteins required phosphorylation of the tyrosine residue, subsequently, many other PTB domains have been shown to be capable of binding to NPXY motifs without a requirement for phosphorylation of the tyrosine (39). The interaction of the GULP PTB domain with ligands does not appear to require phosphorylation of the tyrosine residue (data not shown), and we could also readily detect this interaction in yeast. Moreover, a comparison of the primary sequence and predicted secondary structure of GULP with the known Shc three-dimensional structure (44) suggests that the key residues that form the phosphotyrosine binding pocket are missing in GULP/CD91/6. Despite the lack of requirement for phosphorylation, the interaction between GULP/CD91/6 and either CED-1 or CD91/LRP could still be induced by ligand binding to the receptors.

We have also shown that there is no significant binding of GULP/CD91/6 to the YXXL motif within the cytoplasmic domain of CED-1, which has also been shown to be functionally important in corpse clearance in vivo (24). This suggests that another molecule is most likely recruited to this site, perhaps through an SH2-dependent interaction. It is interesting to note that the mutation of the leucine zipper, which is immediately adjacent to the PTB domain of GULP, did not affect binding to CED-1. We had previously shown that this leucine zipper facilitates homodimerization of GULP and had predicted that perhaps the LZ could affect ligand binding via the GULP PTB (31). Under the conditions tested, this appears not to be the case. However, it is still possible that GULP/CD91/6 could help in the clustering of receptors. Based on our data, it is also formally possible that while one of the two GULP monomers could bind to CED-1 or CD91/LRP, the other “free” PTB domain could recruit another protein into the complex and thereby affect signaling.

Although SREC, a scavenger receptor, was suggested as a possible homologue of mammalian CED-1 (24), SREC and CED-1 have no homology in their cytoplasmic tails. Since no obvious orthologues were identified through routine searches using the CED-1 cytoplasmic tail in the genomic, non-redundant, or expressed sequence tag databases of mouse or human (or Drosophila), we attempted to identify the human homologue of CED-1 using its “functional” motifs. In this process, we have identified CD91/LRP as a ligand for GULP. It is tempting to suggest that CD91/LRP replaces the role of CED-1 in mammals and that differences in this pathway may reflect evolutionary changes in the outcomes of the phagocytic process. However, it is not known if the other molecules identified as possible CED-1 homologues during our searches play a role in engulfment or interact with GULP.

While the genetic studies in the worm have placed ced-1, ced-6, and ced-7 in the same functional genetic pathway (14, 24) and ced-6 has been suggested to function downstream of ced-1 (25), the connection between these proteins has been unclear. Our data suggest a model whereby GULP/CED-6 would interact with CED-1 or its mammalian equivalents (such as CD91/LRP), either basally or inducibly during recognition of apoptotic cells and regulate downstream signaling events. Interestingly, CED-1 mutants that lacked a cytoplasmic domain, when expressed in worms lacking CED-1, were fully capable of colocalizing at the interface between the phagocytic cell and the apoptotic target; however, unlike the wild type protein, the apoptotic cell is not engulfed or seen in vesicles (24). Given that several PTB domain-containing proteins have been shown to alter intracellular trafficking of their ligands (42, 43, 45, 46), an intriguing possibility is that GULP/CED-6-dependent signaling alters the trafficking of the receptor itself or the targeting of the “engulfed cargo” for further processing. In mammals, the role of cytokine production following phagocytosis is becoming more prominent due to the importance of the studies linking failure to clear apoptotic cells with the onset of autoimmune phenotypes (6, 7, 47). Whether GULP binding to CD91/LRP or CED-1 homologues regulates the anti-inflammatory signaling and what role other proteins that interact with the C terminus of GULP might play during engulfment remain to be determined.

Our identification of CED-1 or CD91/LRP interaction with GULP/CD91/6 begins to provide a framework for further characterization of this signaling pathway.

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