The prompt clearance of cells undergoing apoptosis is critical during embryonic development and normal tissue turnover, as well as during inflammation and autoimmune responses. We recently demonstrated that stabilin-2 is a phosphatidylserine receptor that mediates the clearance of apoptotic cells, thereby releasing the anti-inflammatory cytokine, transforming growth factor-β. However, the downstream signaling components of stabilin-2-mediated phagocytosis are not known. Here, we provide evidence that the adaptor protein, GULP, physically and functionally interacts with the stabilin-2 cytosolic tail. Using fluorescent resonance energy transfer analysis and biochemical approaches, we show that GULP directly binds to the cytosolic tail of stabilin-2. Knockdown of endogenous GULP expression significantly decreased stabilin-2-mediated phagocytosis. Conversely, overexpression of GULP caused an increase in aged cell engulfment. The phosphotyrosine binding (PTB) domain of GULP was sufficient for the interaction with stabilin-2; therefore, transduction of TAT fusion PTB domain acts as a dominant negative, resulting in impaired engulfment of aged red blood cells in stabilin-2 expressing cells. In addition, the PTB domain of GULP was able to specifically interact with the NPXY motif of the stabilin-2 cytosolic tail. Taken together, these results indicate that GULP is a likely downstream molecule in the stabilin-2-mediated signaling pathway and plays an important role in stabilin-2-mediated phagocytosis.

The quick clearance of apoptotic cells occurs throughout the life span of all multicellular organisms and is critical for the prevention of serious consequences as a result of inflammatory and autoimmune diseases (1). Genetic studies conducted in Caenorhabditis elegans have identified seven genes that act in two partially redundant pathways to control the clearance of apoptotic cells. In one pathway, CED-2/CrkII, CED-5/CED-10, which in turn promotes cytoskeletal reorganization during corpse engulfment (2, 3). In the other pathway, CED-1/MEGF10, CED-7/ABCA1, and CED-6/GULP act together (4–7). Both pathways converge at CED-10 to mediate actin rearrangement and subsequent engulfment of cell corpses (8).

GULP, which is the mammalian orthologue of CED-6, functions as a cytoplasmic adaptor protein in the engulfment of apoptotic corpses (7, 9). GULP is composed of a phosphotyrosine binding (PTB) domain at its N terminus, a central leucine zipper, and a C-terminal serine/proline-rich region. Previously, it was observed that the engulfment defect in ced-6-deficient worms was rescued by the expression of human CED-6/GULP as a transgene, which showed the conservation of the function of CED-6 through evolution (10), suggesting that the signaling pathways mediated by CED-6/GULP are likely conserved from worms to humans. Additionally, GULP has been shown to directly interact with CED-1 and its human homologues (LRP1 and MEGF10) and is known to be involved in the engulfment of apoptotic cells (5, 11). Furthermore, draper, the Drosophila orthologue of CED-1, and Dced-6 mediate the removal of apoptotic cells both in vivo and in the Drosophila central nervous system (12).

Stabilin-2/FEEL-2/HARE is a large multifunctional glycoprotein that is reportedly a hyaluronan receptor for endocytosis (13–15) and a scavenger receptor that binds bacteria and endocytoses modified low density lipoprotein and glycation end products (16, 17). We recently demonstrated for the first time that stabilin-2 also functions as a membrane receptor involved in the engulfment of apoptotic cells (18). In addition, we previously showed that stabilin-2 can stereospecifically recognize PS, and that activation of stabilin-2 by anti-stabilin-2 antibody led to a release of an anti-inflammatory cytokine, transforming growth factor-β, in macrophages, which suggests that stabilin-2 is a bona fide PS receptor for cell corpse clearance in macrophages. This finding prompted us to investigate the intracellular signaling pathway that is mediated by stabilin-2. Stabilin-2 contains a large extracellular portion that encodes for seven FAS1 domains, four epidermal growth factor-like domain repeats, a Link domain, a transmembrane region, and a short cytosolic region. The cytoplasmic domain of stabilin-2 has 2

The abbreviations used are: PTB, phosphotyrosine binding; PS, phosphatidylserine; GST, glutathione S-transferase; FRET, fluorescence resonance energy transfer; eGFP, enhanced green fluorescence protein; siRNA, small interfering RNA; ANOVA, analysis of variance; RBC, red blood cell; DMEM, Dulbecco’s modified Eagle’s medium; Ni-NTA, nickel-nitrilotriacetic acid; RT, reverse transcription.
Roles of GLUP during Stabilin-2-mediated Phagocytosis

NPXY and XXXL motifs (potential binding sites for both PTB and Src homology 2 domains, respectively) that are known to be critical for CED-1-mediated phagocytosis (6), and GLUP/ced-6 has the PTB domain, which is known to interact with the NPXY motif. Therefore, we evaluated GLUP to determine whether it interacts with the NPXY motif of stabilin-2 and transduces the signal for the stabilin-2-mediated engulfment of apoptotic cells.

In this study, we demonstrate that the PTB domain of the GLUP protein directly binds to the NPXY motif of the cytoplasmic tail of stabilin-2, and we provide the first evidence for a requisite function of GLUP in stabilin-2-mediated phagocytosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—L cells that were stably transfected with stabilin-2-Myc (L/Stab-2 cells) or an empty vector (L/Mock cells) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) that was supplemented with 10% heat-inactivated fetal bovine serum and appropriate antibiotics, as described previously (18). CHO-K1 cells were maintained in F-12 supplemented with 10% fetal bovine serum and the appropriate antibiotics. COS7 and PT67 cells were maintained in DMEM (high glucose) supplemented with 10% fetal bovine serum and the appropriate antibiotics.

Antibodies—Monoclonal antibody directed against c-Myc (clone 9E10) was purchased from Santa Cruz Biotechnology. Monoclonal antibody directed against FLAG (clone M2) was obtained from Sigma. To produce human and mouse GLUP antibodies, His-tagged recombinant human and mouse GLUP proteins were expressed in Escherichia coli and purified using a Ni-NTA column, in accordance with the manufacturer’s instructions (Qiagen). Antibodies were raised in rabbits by subcutaneous injection of the recombinant protein (200 µg). The antiserum was further purified via affinity chromatography in accordance with the manufacturer’s protocols (Amersham Biosciences).

Plasmids—The cDNAs encoding the full-length GLUP (amino acids 1–304), PTB domain (amino acids 11–159), and C-terminal region (leucine zipper and proline-rich domain, amino acids 159–304) were amplified by PCR and subcloned into pGEX4T-1 (for expression as GST fusion proteins) and p3XFLAGCMV (for expression as FLAG-tagged proteins). Plasmid encoding mCherry was a generous gift from Takeharu Nagai (Hokkaido University). The cDNA encoding the full-length stabilin-2 was cloned into pcDNA3.1/myc-His (18). Plasmids encoding stabilin-2 mutants (NPX, APX, and APX) were generated by site-directed mutagenesis. The cDNA corresponding to the cytoplasmic tail of stabilin-2 (amino acids 2461–2551) was amplified by PCR and subcloned into pET28a (Novagen). For generation of bacterial constructs expressing the stabilin-2 cytoplasmic tail with mutations in the NPXY motif (NPX, APX, and APX), the regions corresponding to the cytoplasmic tail of each individual stabilin-2 mutant from the mammalian expression vectors mentioned above were amplified by PCR and were then subcloned into pET28a.

Protein Expression and Purification—GST fusion proteins were expressed in BL-21 cells and purified using glutathione-Sepharose 4B (Amersham Biosciences) in accordance with manufacturer’s instructions. The His-tagged stabilin-2 cytoplasmic tail and the mutants were expressed in BL-21 cells and then purified using a Ni-NTA column (Qiagen) as described previously (19).

In Vitro Binding Experiments—Bacterially produced His-tagged proteins corresponding to the stabilin-2 cytoplasmic tail (wild type or NPX mutants) were incubated with GST fusion GLUP protein immobilized on glutathione-Sepharose beads (Amersham Biosciences) in a binding buffer (100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, and 100 µg of bovine serum albumin). The reaction was allowed to proceed for 1–2 h at 4 °C with rocking. The beads were then collected by centrifugation and washed five times with binding buffer without bovine serum albumin. Next, the beads were resuspended in 20 µl of SDS-PAGE sample buffer and boiled for 5 min. The bound His-tagged stabilin-2 protein was then resolved by SDS-PAGE and subsequently immunoblotted with anti-His antibody. GST fusion proteins used in the experiments were visualized by Coomassie Blue staining.

FRET Analysis—For FRET imaging experiments in living cells, COS7 cells were cultured on a collagen-coated 35-mm glass-base dish (Asahi Techno Glass, Tokyo, Japan) and then transfected with pcDNA-stabilin-2-eGFP and pcDNA-mCherry-GULP using Lipofectamine, in accordance with the manufacturer’s instructions. Confocal FRET images were obtained using a Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Cascade 512B (EMCCD) camera (Roper Scientific, Trenton, NJ), a CSU-10 spinning Nipkow disk confocal unit (Yokogawa Electric, Tokyo, Japan), an emission filter wheel (MAC5000, Ludi Electronic Products, Hawthorne, NY), and a krypton/argon laser. All systems were controlled using the MetaMorph software program (Universal Imaging, Downingtown, PA). Fluorescent images were acquired sequentially through eGFP, mCherry, and FRET filter channels. Filter sets used were purchased from Chroma Technology (Rockingham, VT). Images were acquired by using the 2 × 2 binning mode and 200-ms exposure time. FRET analysis was carried out as described previously (20). Corrected FRET (FRET‘) was calculated by using the following equation: FRET‘ = FRET − (0.16 × eGFP) − (0.2 × mCherry). FRET‘ images were displayed in the pseudocolor mode.

Retroviral Infection—Human GULP cDNA was amplified by PCR, cloned at the Xhol-EcoRI site of the pMSCVpuro retroviral vector, and then designated as pMSCV-GULP. To generate recombinant retrovirus for the purpose of infecting L/Mock and L/Stab-2 cells, PT67 packaging cells (Clontech) were transfected with pMSCV-GULP or an empty vector using Metafectine (Biontex). 48 h after transfection, the cells were replated and grown in maintenance medium supplemented with 2 µg/ml puromycin for 2 weeks. Retrovirus-containing medium was then filtered through a 0.45-µm syringe filter, supplemented with 6 µg/ml hexadimethrine bromide (Sigma), and used to infect pre-confluent L/Mock or L/Stab-2 cells. Stable cell lines were selected further using 7 µg/ml puromycin.
Preparation of Small Interfering RNA (siRNA)—siRNAs representing GULP cDNA nucleotides 532–1364 and green fluorescence protein (GFP) cDNA nucleotides 128–1045 were generated in vitro by using a Dicer siRNA Generation kit in accordance with the manufacturer’s instructions (Gene Therapy Systems). Briefly, PCR products with T7 promoter sequences were transcribed in vitro, and the resulting double-stranded RNA was digested with recombinant Dicer enzyme to convert it to siRNA. L/Stab-2 cells were then transfected with GULP siRNA or GFP siRNA using GeneSilencer reagent (Gene Therapy Systems). After 48 h, the cells were subjected to the phagocytosis assays. GFP siRNA was used as a control for GULP siRNA, and disruption of GULP expression was estimated by comparing the ratio of GULP and β-actin band intensities between control and GULP siRNA transfections via RT-PCR and Western blotting.

RNA Isolation and Reverse Transcription-PCR—Total RNA was extracted from cells using TRizol reagent in accordance with the manufacturer’s instructions (Invitrogen), and the quantity and quality of the isolated RNA were then determined by measuring the absorbance at 260 and 280 nm. The reverse transcription reaction was performed in a mixture with a final volume of 30 μl that contained 2 μg of total RNA, 200 ng of oligo(dT)15 primer, 1× reverse transcription buffer, 0.5 mM deoxyribonucleotide triphosphate mixture, RNasin recombinant ribonuclease inhibitor (Promega), and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). After incubation for 50 min at 42 °C, the reverse transcription reaction was terminated by heating it for 15 min at 70 °C. The newly synthesized cDNA was then amplified by PCR in a reaction mixture consisting of 2 μl of cDNA template, 1.5 mM MgCl2, 1 unit of Taq polymerase, and 0.3 μM of mouse GULP primers (sense 5′-GGA ACA GAA GTT GTG AGA GAT G-3′; antisense 5′-CTG ATC CAC AGT TAA ATG GGT C-3′). Mouse β-actin primers (sense 5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′; antisense 5′-GGG TGC CAC AGG ATT CCA TAC CCA TAC-3′) were used as an internal control. The amplification was performed under the following conditions: 94 °C for 2 min (initial denaturation); 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s (30 cycles); and 72 °C for 7 min (final extension). PCR products were electrophoresed on 2% agarose gels and then visualized by ethidium bromide staining.

Immunoprecipitations and GST Pulldown Assays—CHO-K1 cells were transiently transfected with Myc-tagged stabilin-2 (wild type or mutants) and FLAG-tagged GULP. The cells were then lysed on tissue culture plates using immunoprecipitation (IP) buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM each aprotinin, leupeptin, and pepstatin). The lysates were then cleared by centrifugation at 10,000 × g for 10 min at 4 °C, which was followed by incubation with anti-Myc or anti-FLAG antibody for 1 h at 4 °C and precipitation with protein A-conjugated agarose (Invitrogen) at 4 °C overnight. The immunoprecipitates were then washed three times with wash buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM each aprotinin, leupeptin, and pepstatin). The coprecipitated stabilin-2 or GULP was then detected by immunoblotting using anti-stabilin-2 or anti-FLAG antibody, respectively.

For the GST pulldown assay, the lysates from L/Stab-2 cells were incubated with GST fusion proteins immobilized on glutathione-Sepharose beads for 4 h at 4 °C. Next, the beads were then washed three times with the wash buffer, and the precipitated Myc-tagged stabilin-2 protein was then detected using anti-Myc antibody. GST fusion proteins used in the experiments were visualized by Coomassie Blue staining.

Transduction of TAT Fusion Proteins—The PTB domain of GULP was cloned in-frame into a bacterial expression vector, pTAT-GFP, to produce TAT-PTB-GFP proteins. The vector pTAT-GFP has an N-terminal 11-amino acid TAT protein transduction domain followed by a polylinker, the coding region corresponding to GFP protein, and the His6 tag. GFP-TAT protein was used as a control. The purification protocol was adapted from a published procedure using a Ni-NTA column (21). Briefly, bacterial pellets were resuspended in buffer (100 mM NaCl, 20 mM Hepes (pH 8.0), and 8 M urea) and then sonicated. Addition of 8 M urea to the buffer allows for the isolation of insoluble proteins in bacterial inclusion bodies and efficient transduction into cells. The lysates were cleared by centrifugation at 10,000 × g for 10 min at 4 °C and then loaded onto a Ni-NTA column (Qiagen). Bound proteins were eluted by the stepwise addition of 100, 250, 500, and 1000 mM imidazole in the above buffer. Urea was removed by rapid dialysis using a desalting PD-10 column (Amersham Biosciences), in accordance with the manufacturer’s instructions.

For the transduction of TAT fusion protein, cells were seeded in 6-well plates and grown for 24 h. The cells were then kept in serum-free DMEM for 2 h. TAT fusion proteins were added to a final concentration of 500 nM in serum-free media and then incubated for 5 h at 37 °C. After extensive washing, to identify the uptake of TAT fusion proteins, fluorescence derived from bound proteins was quenched using trypan blue. The uptake of TAT fusion proteins was then confirmed via flow cytometry on a FACSCalibur cytometer (BD Biosciences).

Phagocytosis of Aged RBCs—Aged red blood cells were prepared by incubating the cells in phosphate-buffered saline (20% hematocrit) at 37 °C as described previously (22). Exposure of PS on the cell surface was detected via annexin V-fluorescein isothiocyanate using an annexin V apoptosis detection kit (Santa Cruz Biotechnology). Next, aged RBCs were added to the L/Stab-2 cells and then incubated for 1 h at 37 °C. After washing away the unbound RBCs, the uningested RBCs were lysed via the addition of deionized H2O for 10 s, which was followed by immediate replacement with DMEM as described previously (23). The cells were then fixed with methanol and stained using a Diff Quick staining kit (IMEB Inc.). The phagocytosis of the aged RBCs was then quantified via light microscopy following hypotonic lysis. The percentages of phagocytosis were determined as the percentages of phagocytes that were positive for engulfment as described previously (18). At least 100 cells were scored per well, and all experiments were repeated at least three times. In certain experiments, TAT fusion proteins were preincubated with L/Stab-2 cells for 5 h at 37 °C. After extensive
washing, aged RBCs were added, and the percentages of phagocytosis were determined as above.

Yeast Two-hybrid Interaction Assays—The stabilin-2 cDNA corresponding to the cytoplasmic region of stabilin-2 (wild type, amino acids 2461–2551), as well as the APXA mutant form, was subcloned into the pGBK7 to produce Gal4 transcription binding domain fusion proteins. The GULP cDNA corresponding to the PTB domain of GULP (amino acids 1–145) was subcloned into pGBD7 to produce Gal4 transcription activation domain fusion protein. Specific pairs of constructs expressing activation domain and binding domain fusion proteins were transformed into the yeast strain AH109 (Clontech). Transformants were selected on synthetic dropout (SD) medium that lacked leucine and tryptophan (SD/−Leu/−Trp). Individual colonies were streaked on high stringency medium (SD/−Ade/−His/−Leu/−Trp/X-α-galactosidase) in the presence of 10 mM 3-amino-1,2,4-triazole to test for activation of the reporter genes ADE2, HIS3, and MEL1.

Statistical Analysis—The statistical significance was assessed by the ANOVA test. A p value of <0.05 was considered to be statistically significant.

RESULTS

GULP Directly Binds to Stabilin-2—To examine the interaction of GULP with stabilin-2, we transiently transfected CHO-K1 cells with Myc-tagged stabilin-2 and FLAG-tagged GULP constructs, and then tested whether stabilin-2 and GULP could form a complex. As shown in Fig. 1A (left panels), a Myc tag-specific antibody, but not an isotype-matched control antibody, resulted in the precipitation of Myc-stabilin-2 with FLAG-GULP. In addition, when reverse experiments were conducted, Myc-stabilin-2 was co-immunoprecipitated with FLAG-GULP using a FLAG tag-specific antibody, but this did not occur when an isotype-matched control IgG was used (Fig. 1A, middle panels). To further test whether stabilin-2 and GULP bind directly, we expressed a His-tagged stabilin-2 protein corresponding to a cytoplasmic region and a GST fusion GULP protein in bacteria, and we then performed an in vitro binding assay using these recombinant proteins. The results showed that His-tagged stabilin-2 cytoplasmic tail associated with the GST fusion GULP but not with control GST protein (Fig. 1B), which suggests that there is a direct interaction between these two proteins.

To directly show the interaction between stabilin-2 and GULP in cells, we performed a fluorescent resonance energy transfer (FRET) analysis using eGFP tagged to the C terminus of the stabilin-2 (stabilin-2-eGFP) as a donor and mCherry tagged to the N terminus of GULP (mCherry-GULP) as an acceptor. This pair of probes was co-expressed in COS7 cells and subjected to confocal microscopic analysis. The digital images were acquired through eGFP, mCherry, and FRET channels from a single cell. Because FRET from eGFP to mCherry only occurs if the two proteins are in very close proximity (<50 Å), stabilin-2–GULP interactions should be assessed by measuring a corrected FRET (FRET<sup>C</sup>) value that is calculated for the entire image on a pixel-by-pixel basis using a three-filter “micro-FRET” method and is presented as a quantitative pseudocolor image (20). Increased FRET<sup>C</sup> was detected at the plasma membranes in COS7 cells expressing stabilin-2-eGFP and mCherry-GULP (Fig. 1C); however, such an increase in FRET<sup>C</sup> at the membrane sites was not observed when the control eGFP protein was used as donor. Taken together, these results indicate that stabilin-2 directly interacts with GULP in cells.

GULP Is Required for Stabilin-2-mediated Phagocytosis—To test the functional requirement of GULP in stabilin-2-mediated phagocytosis, we designed siRNAs against the mouse GULP using the Dicer system, and then conducted experiments to assess the effects of the siRNA on the expression of endogenous

![FIGURE 1. GULP interacts directly with stabilin-2. A, CHO-K1 cells were transiently transfected with stabilin-2-Myc and FLAG-GULP plasmids. Next, the lysates were immunoprecipitated (IP) with an anti-Myc antibody or isotype-matched control IgG, and the immunoprecipitates were then analyzed by immunoblot analysis using anti-stabilin-2 and anti-FLAG antibodies (left panels). In reverse experiments, the lysates were immunoprecipitated with an anti-FLAG antibody or isotype control IgG, and the immunoprecipitates were then analyzed by immunoblot analysis using anti-stabilin-2 and anti-FLAG antibodies (middle panels). Comparable expression of stabilin-2 and GULP proteins was confirmed by immunoblotting (right panels). A representative result of three independent experiments is shown. WCL, whole cell lysate. B, GST and GST fusion GULP were produced in bacteria and then assayed for their ability to bind to recombinant His-tagged protein corresponding to the stabilin-2 cytoplasmic tail. Bound proteins were visualized by immunoblotting using anti-His antibody. Coomassie Blue staining demonstrated that the same amount of GST fusion proteins was used. A representative result of three independent experiments is shown. C, interaction between stabilin-2 and GULP was assessed by FRET analysis. Three fluorescent images of COS7 cells expressing stabilin-2-GFP and mCherry-GULP were obtained, and FRET<sup>C</sup> was calculated and displayed using quantitative pseudocolor (see “Experimental Procedures”).]
Roles of GULP during Stabilin-2-mediated Phagocytosis

FIGURE 2. Knockdown of GULP impaired the engulfment of aged RBC in L/Stab-2 cells. A and B, expression of endogenous GULP mRNA (A) and protein (B) in GULP-siRNA-treated cells. L/Stab-2 cells were transfected with control (phosphate-buffered saline), GFP-siRNA, or GULP-siRNA. At 48 h post-transfection, the cells were harvested, and the expression levels of GULP mRNA and protein were analyzed via RT-PCR and immunoblotting, respectively. GFP siRNA was used as a control for GULP siRNA. A representative result of three independent experiments is shown. C, representative images of aged RBC engulfment in parental, GFP-siRNA treated, and GULP-siRNA treated L/Stab-2 cells. A representative result of three independent experiments is shown. D, effects of GULP knockdown in stabilin-2-mediated phagocytosis. A phagocytosis assay was performed in parental, GFP-siRNA-treated, or GULP-siRNA-treated L/Stab-2 cells, and the percentages of cells that carried aged RBCs were then determined. The results are expressed as the means ± S.D. of at least three experiments. ANOVA: *, p < 0.01.

GULP mRNA and protein using RT-PCR and immunoblot analysis, respectively. Treatment with GULP siRNA significantly inhibited the expression of GULP mRNA (Fig. 2A) and reduced the expression of GULP protein by ~60% (Fig. 2B). However, no reduction was observed in the expression of stabilin-2 or actin in the same cell lysates. To investigate the functional role of stabilin-2-mediated phagocytosis, we examined the effect of GULP siRNA on the phagocytic activity of stabilin-2-expressing cells (L/Stab-2 cells). We selected aged RBCs as target cells because they do not bind to phagocytes without apoptotic signals and allow the distinction between binding and engulfment via hypotonic lysis (24). Therefore, all aged RBCs after hypotonic lysis (Fig. 2C, arrowheads). Pretreatment with GULP siRNA significantly inhibited the uptake of aged RBCs in stabilin-2-expressing cells (L/Stab-2 cells) (Fig. 2, C and D); however, these effects were not observed when L/Stab-2 cells were treated with control GFP siRNA. This result suggests that GULP plays a key role in stabilin-2-mediated phagocytosis. To further investigate a functional link between stabilin-2 and GULP, we used an alternative approach to assess the effects of overexpressing GULP on the phagocytic activity of L/Stab-2 cells. We generated recombinant retrovirus expressing human GULP for infection into L/Mock or L/Stab-2 cells. Overexpression of GULP increased the uptake of aged RBCs by ~30% in L/Stab-2 cells when compared with control infection (Fig. 3, A and B). Conversely, L/Mock cells did not engulf aged RBCs in the presence or absence of GULP. These results corroborate our findings that the suppression of endogenous GULP expression results in a significant reduction of aged RBC engulfment and indicates that GULP functions as an adaptor molecule that facilitates the stabilin-2-mediated phagocytosis and signal transduction.

The PTB Domain of GULP Mediates the Interaction with Stabilin-2—Next, to evaluate the region of GULP required for binding to stabilin-2, two different GULP deletion mutants expressing the PTB domain and the C-terminal region of GULP (leucine zipper and proline-rich domain, ΔPTB) were produced as GST fusion proteins (Fig. 4A). Full-length GULP and its PTB domain were able to pull down stabilin-2 protein, whereas the C-terminal fragment (ΔPTB) of GULP failed to do so (Fig. 4B). Furthermore, we generated FLAG-tagged constructs corresponding to the PTB domain and the C-terminal region of GULP (Fig. 4A), and we then tested their ability to bind to stabilin-2. Myc-tagged stabilin-2 was co-immunoprecipitated by full-length GULP and its PTB domain but not by GULP mutant that lacked PTB domain (Fig. 4C), indicating that, in the absence of the PTB domain, there is no binding to stabilin-2, which is in agreement with the results of GST-pulldown assays. Taken together, our results suggest that the major interaction site of stabilin-2 in GULP lies in the PTB domain of the N-terminal region of GULP. To further analyze the functional role of the PTB domain of the GULP protein, we examined the effect of the exogenous PTB domain via an HIV-Tat-mediated delivery method as described previously (21). The GULP cDNA corresponding to its PTB domain was cloned into bacterial expression vector containing GFP, pTAT-GFP, to produce TAT fusion proteins (Fig. 5A). To analyze the ability of TAT fusion proteins to transduce into cells, TAT-GFP, TAT-PTB-GFP, or control GFP lacking TAT was added to the culture media of L/Stab-2 cells, and their uptake was then analyzed by flow cytometry. TAT-GFP and TAT-PTB-GFP proteins were effec-

FIGURE 3. Overexpression of GULP enhances the engulfment of aged RBCs in L/Stab-2 cells. A, L/Mock or L/Stab-2 cells were infected with retrovirus expressing GULP cDNA or an empty vector. The expression of GULP was evaluated via immunoblotting (IB) with anti-FLAG antibody. A representative result of three independent experiments is shown. B, phagocytosis assays were performed in L/Mock, and L/Stab-2 cells were infected with retrovirus expressing GULP cDNA or empty vector, and the percentages of cells that carried aged RBCs were determined. The results are expressed as the means ± S.D. of three independent experiments. ANOVA: *, p < 0.01.
Roles of GLUP during Stabilin-2-mediated Phagocytosis

Figure 4. GULP interacts with the cytoplasmic tail of stabilin-2 via its PTB domain. A, schematic diagrams of GULP and its deletion mutants with N-terminal GST or Flag tag. B, GST, GST-GULP, GST-PTB, or GST-ΔPTB proteins immobilized on Sepharose beads were incubated with L/Stab-2 cell lysates. Associated stabilin-2 protein was then detected by immunoblot (IB) analysis using anti-Myc antibody. Coomassie Blue staining demonstrated that the same amount of GST fusion proteins was used. Input represents 5% of the total cellular lysate. C, CHO-K1 cells were transiently transfected with stabilin-2-Myc and Flag-GULP (wild type or mutants). The lysates were then immunoprecipitated (IP) with an anti-Myc antibody, and the immunoprecipitates were analyzed by immunoblot analysis using anti-stabilin-2 and anti-FLAG antibodies (left panels). Comparable expression of stabilin-2 and GULP proteins was confirmed by immunoblotting (right panels). WCL, whole cell lysate.

Figure 5. Transduction of TAT fusion PTB domain inhibited the engulfment of aged RBCs in L/Stab-2 cells. A, schematic diagrams of TAT fusion proteins. B, L/Stab-2 cells were incubated with GFP, TAT-GFP, or TAT-PTB-GFP protein for 5 h at 37°C. Nonspecific cell surface fluorescence was quenched by trypan blue staining, and the transmembrane uptake of TAT fusion proteins was then determined by flow cytometry. C, 500 nM TAT-GFP or TAT-PTB-GFP proteins were transduced into L/Stab-2 cells. Phagocytosis assays were then performed, and the percentages of cells ingesting the aged cells were determined. The results are expressed as means ± S.D. of at least three experiments. ANOVA: *, p < 0.01.

Proteins containing the PTB domain are known to bind directly to the sequence NPX(Y/F). In addition, a recent study proposed that integrin β tails can bind PTB domains from 17 different proteins using their conserved NPX(Y/F) motifs (25). Therefore, to test whether the PTB domain of GULP may interact with the NPX motif of stabilin-2 protein, we constructed an NPX double mutant (APXA), which contains Ala substitutions at both the Asn2516 and Tyr2519 positions in stabilin-2. Wild type or APXA mutant stabilin-2 was co-expressed with GULP in CHO-K1 cells, and their interaction was then assayed via co-immunoprecipitation experiments. As shown in Fig. 6, A and B, wild type stabilin-2 was able to bind to GULP, whereas APXA mutant failed to bind to GULP, confirming that GULP binds to the NPX(Y) motif within the stabilin-2 cytoplasmic tail. Furthermore, to identify the direct interaction between GULP and the NPX motif, we performed an in vitro binding assay using recombinant proteins corresponding to the cytoplasmic tail with mutations in the NPX motif (Fig. 6C). Replacement of the asparagine with alanine resulted in only a slight decrease in GULP binding. In contrast, alteration of the tyrosine to alanine caused a dramatic decrease in GULP binding. The interaction between the NPX motif in stabilin-2 and GULP was further solidified by yeast two-hybrid assays. The cytoplasmic tail of stabilin-2 was cloned into the pGBKTT7 bait vector, whereas the PTB domain of GULP was cloned into the pGADT-2 prey vector. As shown in Fig. 6E, the PTB domain of GULP was able to interact with the cytoplasmic tail of stabilin-2. We also tested the mutant cytoplasmic tail, which contains Ala substitutions at both Asn2516 and Tyr2519 positions of the NPX motif, and found that it does not interact with the PTB domain of GULP (Fig. 6E). Taken together, these data indicate that the NPX motif is necessary to mediate the binding to GULP.
The rapid clearance of apoptotic cells is crucial for development during embryogenesis, the maintenance of tissue integrity, prevention of autoimmunity, and the resolution of inflammation (1). We recently demonstrated that stabilin-2 is a PS receptor that is involved in rapid cell corpse clearance (18). However, the signaling pathway mediated by stabilin-2 has been not investigated and therefore we conducted this study, the results of which provided evidence that PS receptor stabilin-2 is linked to hCED-6/GULP, which is known to include a phosphotyrosine-independent interaction. The results of this study also showed that the interaction between stabilin-2 and GULP involves the NPXY motif and the PTB domain of each, respectively, both of which have been shown to be important in phagocytosis (5, 6).

In C. elegans, two partially redundant pathways mediate cell corpse removal, with the ced-1, ced-6, and ced-7 genes functioning in one pathway and the ced-2, ced-5, ced-10, and ced-12 genes acting in the other (26–29). CED-1 is a putative receptor that is required only by the engulfing cells and likely participates in the recognition of apoptotic cells. CED-1 contains two amino acid sequence motifs in its cytoplasmic tail, NPXY and YXXL, which 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 (6).

In this study, we provide evidence that GULP is a signaling molecule involved in stabilin-2-mediated phagocytosis. For example, knockdown of endogenous GULP expression resulted in a significant reduction in stabilin-2-mediated phagocytosis, whereas overexpression of GULP caused an increase of the engulfment of aged RBC in L/Stab-2 cells. In addition, results from experiments using HIV-TAT fusion protein suggest that transduction of dominant negative GULP causes a significant decrease in aged cell clearance, indicating that GULP is a signaling molecule involved in stabilin-2-mediated phagocytosis.

The PTB domain was first identified in Shc, a signaling adaptor protein that mediates phosphotyrosine-dependent interactions between growth factor receptor tyrosine kinases and their downstream effectors (31). However, because many PTB domains have been identified and their binding sites characterized, it seems likely that other PTB domains can also bind to NPXY motifs without a requirement for phosphorylation of the tyrosine (32). Su et al. (5) suggest that the key residues that form the phosphotyrosine binding pocket are most likely missing in GULP. Consistent with this finding, the cytoplasmic tail of stabilin-2 produced in bacteria bound to the PTB domain of GULP, suggesting that the interaction between stabilin-2 and GULP might be a phosphotyrosine-independent interaction. Stabilin-2 also contains the YXXL motif, which has been shown to be functionally important in corpse clearance in vivo (6). We found that the YXXL motif also plays a key role in the engulfment of apoptotic cells by stabilin-2, which suggests that another adaptor molecule is most likely recruited to this site, perhaps through an Src homology 2 domain. In this study, we found that the PTB domain of GULP is necessary for interaction with stabilin-2 to occur. Previous studies have shown that the leucine zipper domain of GULP facilitates its homodimerization and could therefore affect ligand binding via the PTB domain (33). We assumed that stabilin-2 can exist as a homodimer or multimer on the cell surface, which suggests that its PTB domain was able to specifically interact with one specific NPXY motif in the CD91 cytoplasmic tail (5, 30). Consistent with these findings, in this study, stabilin-2 was also found to directly bind to the PTB domain of GULP via the NPXY motif in its cytoplasmic tail, suggesting that stabilin-2 operates via similar intracellular signal transduction.

The PTB domain of GULP interacts directly with the NPXY motif in the stabilin-2 cytoplasmic tail. A and B, CHO-K1 cells were transiently transfected with FLAG-GULP and stabilin-2-Myc (wild type or mutant). Next, the lysates were immunoprecipitated (IP) with an anti-Myc antibody or isotype-matched control IgG, and the immunoprecipitates were then analyzed by immunoblot analysis using anti-stabilin-2 and anti-FLAG antibodies (A, left panels). Comparable expression of stabilin-2 and GULP proteins was confirmed by immunoblotting (A and B, right panels). WCL, whole cell lysate. C, schematic diagram of GST fusion PTB protein and His-tagged stabilin-2 cytoplasmic tails with mutation in the NPXY motif. D, His-tagged stabilin-2 cytoplasmic tail and the mutants were produced in bacteria and were then assayed for their ability to bind to GST-PTB protein. Bound proteins were visualized by immunoblot analysis using anti-His antibody.

DISCUSSION

Roles of GULP during Stabilin-2-mediated Phagocytosis

The rapid clearance of apoptotic cells by CED-1, CD91/LRP, and MEGF10 and that its PTB domain was able to specifically interact with one specific NPXY motif in the CD91 cytoplasmic tail (5, 30). Consistent with these findings, in this study, stabilin-2 was also found to directly bind to the PTB domain of GULP via the NPXY motif in its cytoplasmic tail, suggesting that stabilin-2 operates via similar intracellular signal transduction. In this study, we provide evidence that GULP is a downstream adaptor molecule involved in stabilin-2-mediated phagocytosis. For example, knockdown of endogenous GULP expression resulted in a significant reduction in stabilin-2-mediated phagocytosis, whereas overexpression of GULP caused an increase of the engulfment of aged RBC in L/Stab-2 cells. In addition, results from experiments using HIV-TAT fusion protein suggest that transduction of dominant negative GULP causes a significant decrease in aged cell clearance, indicating that GULP is a signaling molecule involved in stabilin-2-mediated phagocytosis.
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that homodimerization of GULP via the leucine zipper could help in the dimerization or clustering of stabilin-2 receptors. In addition, the results of this study showed that the PTB domain of GULP acts as a dominant negative mutant in stabilin-2-mediated phagocytosis, suggesting that the proline-rich motif in the C-terminal of GULP may interact with the Src homology 3 domains of other signaling proteins in the downstream signaling pathway.

Recently, two engulfment signaling pathways were found to converge at ced-10 to mediate actin rearrangement and corpse removal (8). Based on these findings, we suspect that the interaction of stabilin-2 and GULP requires an activated form of stabilin-2 induced by ligand engagement, which in turn allows Rac1 activation and rearrangement of the actin cytoskeleton. Our preliminary data showed that stabilin-2-mediated phagocytosis is also dependent on the Rho family GTPase Rac1.3 The activity of Rho GTPases is controlled by guanine nucleotide exchange factors that activate the rate-limiting reaction, namely the exchange of GDP for GTP. A recent study reported that GULP did not bind to Rac1 in the preferentially GTP-bound or GDP-bound forms (34). Therefore, it appears more likely that an existing or yet to be identified Rac1-GEF activity is associated with GULP (34). In addition, it has been reported that homodimerization of GULP via the leucine zipper could regulate Rac1 activity through negative regulation of the Rac1-GAP activity. However, the signaling molecules that act downstream of GULP in the stabilin-2-mediated pathway and could regulate Rac1-activity remain to be determined.

In summary, the data presented here show that an adaptor protein, GULP, directly interacts with the NPXY motif of stabilin-2 via its PTB domain and plays a key role in stabilin-2-mediated phagocytosis. Thus, our results provide a framework for further characterization of the stabilin-2-mediated signaling pathway.

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