Altered production of Aβ (amyloid-β peptide), derived from the proteolytic cleavage of APP (amyloid precursor protein), is believed to be central to the pathogenesis of AD (Alzheimer’s disease). Accumulating evidence reveals that APPc (APP C-terminal domain)-interacting proteins can influence APP processing. There is also evidence to suggest that APPc-interacting proteins work co-operatively and competitively to maintain normal APP functions and processing. Hence, identification of the full complement of APPc-interacting proteins is an important step for improving our understanding of APP processing. Using the yeast two-hybrid system, in the present study we identified GULP1 (engulfment adaptor protein 1) as a novel APPc-interacting protein. We found that the GULP1–APP interaction is mediated by the NPTY motif of APP and the GULP1 PTB (phosphotyrosine-binding) domain. Confocal microscopy revealed that a proportion of APP and GULP1 co-localized in neurons. In an APP–GAL4 reporter assay, we demonstrated that GULP1 altered the processing of APP. Moreover, overexpression of GULP1 enhanced the generation of APP CTFs (C-terminal fragments) and Aβ, whereas knockdown of GULP1 suppressed APP CTFs and Aβ production. The results of the present study reveal that GULP1 is a novel APPc-interacting protein that influences APP processing and Aβ production.

Key words: Alzheimer's disease, amyloid-β peptide (Aβ), amyloid precursor protein (APP), amyloid precursor protein intracellular domain (AICD), CED-6, engulfment adaptor protein 1 (GULP1).
processing and to maintain the proper levels of $\beta\beta$, APPc, and other APP cleavage fragments. To improve our understanding of the mechanism by which APP processing is regulated, it is therefore important to identify the full complement of proteins that bind to APPc. In the present study, we identify GULP1 (engulfment adaptor protein 1) as a novel APPc-interacting protein that alters APP processing and $\beta\beta$ production.

EXPERIMENTAL

All of the experiments were performed at least three times with similar results.

Yeast two-hybrid system

Yeast two-hybrid screens were performed essentially as described previously [14]. Briefly, sequences encoding human APPc (amino acids 649–695 of APP$_{695}$) were subcloned into the yeast ‘bait’ pY1 vector and then transformed into yeast AH109. Library screens were performed by mating the bait-containing yeast AH109 and yeast Y187 which contains a pre-transformed human brain cDNA library (Clontech). Following selection, vigorously growing clones were subjected to $\beta$-galactosidase assays. pACT2 plasmids were rescued by transformation into Escherichia coli DH5α cells and the inserts were sequenced.

Cell culture and transfection

CHO (Chinese-hamster ovary), HEK (human embryonic kidney)-293 and SH-SY5Y cells were cultured as described previously [15]. CHO cells stably expressing APP$_{695}$ (CHO-APP$_{695}$) were grown in Ham’s F12 medium (Hyclone, Thermo Scientific) supplemented with 10% fetal bovine serum (Hyclone, Thermo Scientific), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen), 2 mM glutamine (Invitrogen) and 250 μg/ml genetin (Roche). Primary rat cortical neurons were obtained from E18 (E is embryonic day) rat embryos (animal experimentation was carried out according to the CUHK animal experimentation Ethics Committee guidelines) and grown on culture plates coated with poly-D-lysine in Neurobasal medium and B27 supplement (Invitrogen) containing 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen) and 2 mM glutamine (Invitrogen). Neurons were cultured for 7 days prior to the analyses. For plasmid transfection, CHO, HEK-293 and SH-SY5Y cells were transfected with FuGENE® 6 (Roche), and rat cortical neurons were transfected with Lipofectamine™ 2000 (Invitrogen). siRNA (small interfering RNA) knockdown was performed using GULP1 and control non-targeting siRNAs (Dharmacon, Thermo Scientific). siRNAs were transfected into cells using Lipofectamine™ RNAiMAX (Invitrogen). The efficiency of knockdown was determined using immunoblot analysis.

Plasmids

The mammalian expression construct for APP$_{695}$ was as described previously [10]. HA (haemagglutinin)-tagged full-length GULP1 (pEBB-GULP1), untagged GULP1 (pEBG-GULP1) and GULP1$^{1-168}$ (pEBG-GULP1$^{1-168}$) mammalian expression constructs were as described previously [16]. The APP–GAL4 construct consisting of human APP$_{695}$ followed by the entire GAL4 transcription factor (pRc-CMV-APP$_{695}$) was as described previously [17]. The bacterial GST (glutathione transferase)–APPc expression construct was as described previously [10]. GST–APPc$_{NATA}$ and GULP1F$_{145V}$ mutants were generated using the QuickChange® XL site-directed mutagenesis kit (Stratagene). Bacterial His$_{6}$–GULP1 and His$_{6}$–GULP1$^{1-168}$ expression constructs were created by subcloning the corresponding human GULP1 cDNAs into pET-28a (Novagen). A mammalian expression construct of Myc-His-tagged BACE1 (β-site APP cleavage enzyme 1) was as described previously [18]. The GAL4 UAS-dependent firefly luciferase reporter pFR-Luc and transfection efficiency vector Renilla luciferase phRL-TK plasmids were obtained from Stratagene and Promega respectively.

Antibodies

The antibodies used were as follows: anti-HA (12CA5; Roche), anti-α-tubulin (DM1A; Sigma), anti-GST (Sigma), anti-APP (H-43; Santa Cruz Biotechnology), anti-APP (22C11; Millipore), anti-PDI (protein disulfide-isomerase) (RL77; Affinity BioReagents), anti-GM130 (cis-Golgi matrix protein of 130 kDa; BD Biosciences) and anti-Rab5 (Synaptic Systems). Rabbit anti-APP was as described previously [19]. The rat polyclonal antibody against GULP1 was created by immunization of animals with full-length human GULP1 expressed as a His$_{6}$-fusion protein (His$_{6}$–GULP1). In brief, 100 μg of His$_{6}$–GULP1 (1 μg/μl) was mixed with an equal volume of TiterMax adjuvant (Sigma) and then injected subcutaneously into Sprague–Dawley rats. Boost injections were given to the rats every 3 weeks. After four boost injections, blood samples were collected from the rats by terminal cardiac puncture. Rat sera were isolated by centrifugation (at 2000 g for 1 h at 4°C) and then affinity-purified against the antigen. The information for the antibodies used in the present study are listed in Supplementary Table S1 (at http://www.BiochemJ.org/bj/436/bj4360631add.htm).

Protein-binding assays

GST–APPc and GST–APPc$_{NATA}$ fusion proteins were expressed in E. coli BL21 and captured by glutathione–Sepharose 4B according to the manufacturer’s instructions (GE Healthcare). GST pull-down assays were performed essentially as described previously [19]. In brief, GST, GST–APPc and GST–APPc$_{NATA}$ ‘baits’ were used in pull-down assays from GULP1-transfected cells which were harvested in ice-cold lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and Complete™ protease inhibitor (Roche)]. The cell lysates were incubated with the baits at 4°C for 1 h. The captured proteins were then isolated by boiling in SDS/PAGE sample buffer, and were analysed by SDS/PAGE and immunoblotting as described previously [19]. For the direct protein-binding assay, His$_{6}$–GULP1$^{1-168}$ was expressed in E. coli BL21 cells, purified by Ni-NTA (Ni$^{2+}$-nitrilotriacetate) agarose (Qiagen) and incubated with purified GST, GST–APPc or GST–APPc$_{NATA}$ baits in ice-cold lysis buffer. The protein complexes were captured by glutathione–Sepharose 4B as described above and analysed by SDS/PAGE and Western blotting.

For immunoprecipitation, CHO cells transfected either with APP + HA-tagged GULP1 or HA-tagged GULP1 were harvested in ice-cold lysis buffer. APP was immunoprecipitated from cell lysates using a rabbit anti-APP antibody or non-immune rabbit IgGs for 16 h at 4°C. The antibody was captured by Protein G–Sepharose (Sigma) for 2 h at 4°C, and the immunoprecipitates were washed three times with ice-cold lysis buffer. Proteins in the immunoprecipitates were analysed by SDS/PAGE and Western blotting. The endogenous APP–GULP1 interaction was determined by immunoprecipitation of APP or non-immune rabbit IgGs from mouse brain lysate. APP and GULP1 in the immunoprecipitates were detected by a mouse anti-APP polyclonal antibody and a rat anti-GULP1 antibody respectively.
Indirect Immunofluorescence

Primary cortical neurons cultured on poly-D-lysine-coated glass coverslips were fixed and processed for immunofluorescence as described previously [19]. Endogenous GULP1 and APP were detected using a rat polyclonal anti-GULP1 antibody and rabbit anti-APP antibody respectively. PDI, GM130 and Rab5 antibodies respectively. Antibodies were visualized with highly cross-absorbed donkey anti-rabbit, anti-mouse and anti-rat Igs (i.e the secondary antibodies were solid-phase-absorbed highly cross-absorbed donkey anti-rabbit, anti-mouse and anti-rat Iggs respectively). Antibodies were visualized with Alexa Fluor® 488, 546 or 633 (Invitrogen). Images were captured by a confocal microscope (Zeiss). The images for the same antigen were captured using the same laser settings.

ELISA assays

Human Aβ1–40 and Aβ1–42 levels in the cell culture medium were analysed using the high-sensitivity human amyloid β40 and β42 ELISA kits (Millipore; http://www.millipore.com/catalogue/item/ezhs-set) essentially following the manufacturer’s instructions. In brief, CHO-APPΔ711 cells were transfected either with non-targeting control or GULP1 siRNAs. The cells were replenished with fresh medium 48 h post-transfection. After 7 h, the medium was collected, diluted as appropriate in sample diluent and added to the ELISA plate. After overnight incubation at 4°C with the primary antibody, the ELISA plate was washed five times with wash buffer and streptavidin-peroxidase-conjugate was added. Following a incubation for 1 h at room temperature (25°C) and washing, colorimetric substrate solution was added to the ELISA plate. The colorimetric signal development was then stopped by adding stop solution. Signals from ELISA were measured at 450 nm using a microplate reader (Bio-Rad Laboratories).

Tricine SDS/PAGE analysis for APP CTFs (C-terminal fragments)

APP CTFs were analysed by 16 % Tricine SDS/PAGE essentially as described previously [20]. The cells were harvested in 1× Tricine sample buffer containing 2 mM phenanthroline, 10 % 2-mercaptoethanol and Complete® protease inhibitor (Roche). Immunoblot analyses were performed using a rabbit anti-APP antibody that recognizes the last 21 amino acid residues of APP [19,20]. The relative amounts of APP CTFs on the immunoblots were analysed by densitometry using a densitometer and Quantity One software (Bio-Rad Laboratories).

Statistical analyses

Statistical analyses were performed using one-way ANOVA tests with LSD (least significant difference) post-hoc test. Significance is indicated between different treatments as *P < 0.0001 and **P < 0.001. Values are means ± S.D.

RESULTS

GULP1 interacts with APPc

From a yeast two-hybrid screen of a human brain cDNA library using APPc as ‘bait’, we isolated several partial cDNA clones of known APP-binding proteins (FE65, JIP-1 and X11β) and one clone for a potentially novel APP-binding protein, GULP1, which was chosen for further study. The interaction between APPc and GULP1 was confirmed by colony-lift filter β-galactosidase assays in yeast (Supplementary Figure S1 at http://www.BiochemJ.org/bj/436/bj4360631add.htm). GULP1 contains a PTB domain (residues 1–168) [16,21], and since a number of other APP-binding proteins also contain a PTB domain [3–5], it appeared likely that the interaction between APPc and GULP1 involved the YENPTY motif in APPc and the PTB domain of GULP1. To further confirm the interaction between APPc and GULP1 in other biochemical assays, GST-fusion protein pull-down assays using GST, GST–APPc and a GST–APPc mutant in which amino acids 684–687 (NPTY of APPα) were mutated to ATA (APPcATA) were performed. Mutation of these residues in APPc is known to abrogate the binding of other PTB-domain-containing proteins [4,22–25]. These GST fusion ‘baits’ were used to pull down GULP1 from GULP1-transfected CHO cells. Such pull-down assays revealed that the GST–APPc bait, but not GST or GST–APPcATA, bound to GULP1 (Figure 1A).

To complement the studies described above, we also performed GST pull-down assays with GST–GULP1 ‘baits’ to determine whether APP binds to these ligands. We prepared ‘baits’ comprising full-length GULP1 (GST–GULP1) and the GULP1 N-terminal PTB domain (residues 1–168) (GST–GULP11–168). Both GST–GULP1 and GULP11–168, but not GST alone, bound APP from APP-transfected CHO cells (Figure 1B). To further confirm the importance of the GULP1 PTB domain in mediating the interaction of GULP1 with APP, Phe145 within the GULP1 PTB domain was mutated to valine (GULP1F145V) and the ability of this mutant to bind to APPc in GST pull-down assays was tested. Similar mutations in other PTB-containing proteins have been shown to abrogate them binding to their ligands, and this includes binding of FE65 and X11 to APP [26,27]. GST–APPc bound to wild-type GULP1, but not to GULP1F145V, from transfected CHO cells (Figure 1C).

We next tested whether APP and GULP1 interact in immunoprecipitation assays. APP was transfected to CHO cells either alone or with HA-tagged GULP1. APP was immunoprecipitated using an anti-APP antibody. Immunoblotting revealed that GULP1 was co-immunoprecipitated with APP in APP+GULP1 co-transfected cells, but not in GULP1-only-transfected cells; control immunoprecipitation with non-immune rabbit Igs gave no signal (Figure 1D). We also tested whether GULP1 interacts with membrane-associated APP CTFs. Since overexpression of APP increases the level of APP CTFα [28], we therefore tested the GULP1–APP CTFα interaction in APP+GULP1 co-transfected CHO cells. HA-tagged GULP1 was immunoprecipitated from the transfected cell lysates using an anti-HA antibody. Immunoblotting showed that APP CTFα was co-immunoprecipitated with GULP1 in APP+GULP1 co-transfected cells, but not in APP single-transfected cells (Figure 1E). We further investigated whether endogenous APP bound to endogenous GULP1 in immunoprecipitation assays. APP was immunoprecipitated from mouse brain using an anti-APP 6A6 antibody, and GULP1 was detected using a rat anti-GULP1 antibody (see Figure 2). Control immunoprecipitations were also performed using non-immune rabbit Igs. We found that endogenous APP bound to GULP1 in this assay (Figure 1F).

The biochemical assays described above provide strong evidence that GULP1 interacts with APP. However, to formally exclude the possibility that APP and GULP1 interact via some intermediate molecule, we examined whether APPc and GULP1 interact directly by incubating purified His6–GULP11–168 with purified GST, GST–APPc and GST–APPcATA prepared in E. coli. The purity of the proteins were determined by HPLC analysis (only a single peak was observed in the elution profile of each preparation; results not shown) and SDS/PAGE. Again, in this in vitro interaction assay, GST–APPc, but not GST alone or GST–APPcATA, bound to His6–GULP1 (Figure 1G). Taken together,
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Figure 1 GULP1 interacts with APP

(A) E. coli expressing GST, GST–APPc and GST–APPcNATA were used as baits in pull-down assays from GULP1-transfected cells essentially as described previously [19]. Briefly, equimolar amounts of the baits were incubated with 200 μg of GULP1-transfected cell lysates. Following incubation for an hour at 4 °C, the GST ‘bait’ beads were pelleted by centrifugation at 500 g and washed three times with cell lysis buffer. The samples were then boiled in SDS sample buffer. GULP1 was detected using the HA sequence attached to the N-terminus of GULP1. (B) E. coli expressing GST, GST–GULP and GST–GULP11–168 were used as baits in pull-down assays from APP-transfected cells. (C) E. coli expressing GST and GST–APPc were used as baits in pull-down assays from GULP1- or GULP1F145V-transfected cells. GULP1 was detected using a rat anti-GULP1 antibody. (D) Immunoprecipitations were performed from cells either transfected with GULP1 + APP or GULP1, APP was immunoprecipitated by a rabbit anti-APP H-43 antibody. Immunoprecipitated APP was detected using a mouse anti-APP antibody (22C11) and co-immunoprecipitated GULP1 was detected using a mouse anti-HA antibody. ‘−’ and ‘+’ refer to the presence of non-immune rabbit Igs or anti-APP antibody in the immunoprecipitations. (E) Immunoprecipitations were performed from cells either transfected with APP or GULP1 + APP. HA-tagged GULP1 was immunoprecipitated using a mouse anti-HA antibody (12CA5). Immunoprecipitated GULP1 was detected using a rat anti-GULP1 antibody and co-immunoprecipitated APP CTFα was detected by a rabbit anti-APP antibody which recognizes the last 21 amino acid residues of APP [19]. ‘−’ and ‘+’ refer to the presence of non-immune mouse Igs or anti-HA antibody in the immunoprecipitations. Antibodies used in immunoprecipitations cross-react with the secondary antibody. The position of the IgG light chain is indicated. (F) Immunoprecipitations were performed from cells either transfected with APP or GULP1 + APP. HA-tagged GULP1 was immunoprecipitated using a mouse anti-HA antibody (12CA5). Immunoprecipitated GULP1 was detected using a rat anti-GULP1 antibody and co-immunoprecipitated APP CTFα was detected by a rabbit anti-APP antibody which recognizes the last 21 amino acid residues of APP [19]. ‘−’ and ‘+’ refer to the presence of non-immune mouse Igs or anti-HA antibody in the immunoprecipitations. Antibodies used in immunoprecipitations cross-react with the secondary antibody. The position of the IgG light chain is indicated. (F) Immunoprecipitations were performed from cells either transfected with APP or GULP1 + APP. HA-tagged GULP1 was immunoprecipitated using a mouse anti-HA antibody (12CA5). Immunoprecipitated GULP1 was detected using a rat anti-GULP1 antibody and co-immunoprecipitated APP CTFα was detected by a rabbit anti-APP antibody which recognizes the last 21 amino acid residues of APP [19]. ‘−’ and ‘+’ refer to the presence of non-immune mouse Igs or anti-HA antibody in the immunoprecipitations.

these results show that APP and GULP1 interact directly, and the interaction is mediated by the NPTY motif of APP and the GULP1 PTB domain.

A proportion of GULP1 and APP co-localize in neurons

In order to characterize the distribution of endogenous GULP1 within neurons, we generated a rat anti-GULP1 antibody. This antibody detected a single species of a molecular mass of 35 kDa on immunoblots of both non-transfected and GULP1-transfected CHO cells (the predicted mass of GULP1 is 35.5 kDa) and the signals were stronger in GULP1-transfected cells (Figure 2A). Moreover, absorption of the antibody with the antigen (His6–GULP1) abrogated these signals (Figure 2A, right-hand panel). The GULP1 antibody detected a 35 kDa species in a range of different tissues, including brain, and also in a number of cell-types, including primary rat cortical neurons (Figures 2B and 2C). The anti-GULP1 antibody was also characterized by immunostaining in rat cortical neurons (Supplementary Figure S2 at http://www.BiochemJ.org/bj/436/bj4360631add.htm). To determine the subcellular distributions of GULP1 and APP, immunostaining of 7 DIV (days in vitro) rat cortical neurons was performed. It was revealed that a proportion of both endogenous APP and GULP1 co-localized in perinuclear regions (Figures 2D and 2E). To identify the subcellular structures in neurons where APP and GULP1 co-localize, we co-stained for APP and GULP1.
GULP1 binds APP

A proportion of GULP1 co-localizes with APP in neurons

(A) Characterization of rat anti-GULP1 antibody. Untransfected (UT) and GULP1-transfected CHO cell lysates were probed with anti-GULP1 antibody (left-hand panel) or anti-GULP1 antibody pre-absorbed with the antigen, i.e. purified His6–GULP1 (right-hand panel). α-Tubulin immunoblots show equal loading of the gels. (B and C) Expression of GULP1 by immunoblotting in different mouse tissues (B) and cell lines (C). Approximately 15 μg of tissue and cell lysates was loaded into each lane. As a control, 1 μg of GULP1-transfected CHO cell lysate was used. The molecular mass in kDa is indicated on the left-hand side. (D–F) Confocal imaging showing the distributions of endogenous APP and GULP1 in DIV7 rat cortical neurons. (D and E) APP and GULP1 in neuronal cell bodies co-stained for ER (PDI) (D) or Golgi (GM130) (E). (F) A region of axon stained for APP, GULP1 and the endosomal marker Rab5. Arrows in (D), (E) and (F) indicate regions of triple co-localization of immunofluorescence signals. APP was detected using a rabbit anti-APP antibody, GULP1 was detected using a rat anti-GULP1 antibody, and PDI, GM130 and Rab5 were all detected with mouse antibodies. The scale bar in (D) and (E) is 10 μm, and in (F) is 5 μm.

with the ER (endoplasmic reticulum) marker PDI and the Golgi marker GM130. As reported previously, intense staining of APP was observed in both ER and Golgi, which was revealed by immunostaining for PDI and GM130 (Figures 2D and 2E) [29,30]. Although GULP1 was mainly cytoplasmic, we detected a proportion of GULP1 co-localized with APP, and some co-localization of the two proteins with GM130 and PDI (Figures 2D and 2E; indicated with arrows). Since GULP1 was reported to be found in early endosomes [31], we also triple-stained for APP, GULP1 and the early endosome marker Rab5 in axons. Again we detected co-localization of APP and GULP1 and, in addition, some localization of both of these proteins with Rab5 (Figure 2F; indicated with arrows). Taken together these findings suggest that a proportion of APP co-localizes with GULP1, and that this co-localization occurs in a variety of subcellular compartments.

GULP1 enhances cleavage of APP–GAL4

A number of studies have shown that APPc-interacting proteins can influence APP processing. We therefore examined whether GULP1 alters APP processing using, in the first instance, a previously reported APP–GAL4 reporter assay system [17,32]. In this system, the C-terminus of APP is fused to the yeast transcription factor GAL4 (including both the GAL4 DNA-binding and transactivation domains) and processing of APP by APP secretases releases APPc–GAL4 to enable its translocation to the nucleus where it can activate transcription of a GAL4-dependent firefly luciferase reporter gene. Overexpression of GULP1 stimulated APP–GAL4 dependent transcription by approximately 6–8-fold in this system in transfected CHO, HEK-293 and SH-SY5Y neuroblastoma cells (Figure 3A). To assist in determining the time for analysis post-siRNA knockdown, the half-life of GULP1 was analysed in pulse–chase experiments. These experiments demonstrated that the half-life of GULP1 is approximately 2.2 h (Supplementary Figure S3 at http://www.BiochemJ.org/bj/436/bj4360631add.htm). In agreement with the overexpression analysis, knockdown of GULP1 inhibited APP–GAL4-dependent transcription by approximately 50% in CHO, HEK-293 and SH-SY5Y cells (Figure 3B). siRNA-mediated knockdown of CHO, HEK-293
co-transfected cells suppressed the production of the APP CTFs. By contrast, knockdown of endogenous GULP1 in APP was increased significantly in the presence of GULP1 as compared with the control protein CAT pFR-Luc GAL4 reporter, phRL-TK and the constructs indicated. Transcription of the GAL4 reporter with GULP1 siRNA.

and SH-SY5Y cells reduced GULP1 expression by over 95%, as confirmed by immunoblotting (Figure 3C).

**GULP1 alters APP processing and increases Aβ secretion**

The stimulation of APP–GAL4-mediated transcription by GULP1 is in line with GULP1 altering processing of APP so as to permit release of APPc–GAL4 and its translocation to the nucleus. We therefore monitored APP processing in CHO cells in which GULP1 expression was modulated. To do so, we analysed cleavage patterns of APP-membrane-associated APP CTFs produced by BACE1 and α-secretase. BACE1 cleaves at two sites to produce β and β’ CTFs, and this leads to production of Aβ, whereas α-secretase cleavage precludes production of Aβ (see the reviews [1,2]). GULP1 expression was increased by transient transfection and decreased by the use of siRNAs. Transient overexpression of GULP1 in APP + BACE1 co-transfected CHO cells enhanced the generation of APP CTFs, including α, β and β’ CTFs (Figure 4A). In GULP1 co-transfected cells, the levels of CTFα and CTFβ were increased by 60% and 150% respectively. By contrast, knockdown of endogenous GULP1 in APP + BACE1 co-transfected cells suppressed the production of the APP CTFs (Figure 4B). CTFα was decreased by 30%, whereas CTFβ was lowered by 55%. Thus it appears that GULP1 increases processing of APP at both the α- and β-secretase sites. However, modulation of GULP1 expression had the most notable effect on APP processing at the BACE1 site (Figures 4A and 4B).

We next monitored the effect of GULP1 on secretion of Aβ by modulation of GULP1 expression in CHO cells stably expressing APPβ(31) (CHO-APPβ(31)), a widely used cell line for studying APP processing [33,34]. The amount of secreted Aβ1–40 and Aβ1–42 in the medium of GULP1-overexpressing and GULP1-knocked down CHO-APPβ(31) was measured using ELISAs. Overexpression of GULP1 enhanced the amounts of both secreted Aβ1–40 (1.6-fold) and Aβ1–42 (1.7-fold), as compared with the control protein CAT (chloramphenicol acetyltransferase) (Figure 4C). On the other hand, siRNA knockdown of GULP1 significantly lowered the levels of secreted Aβ1–40 (2.1-fold) and Aβ1–42 (1.7-fold) (Figure 4D). Efficient knockdown of GULP1 was confirmed by immunoblotting (Figure 4D). Thus GULP1 alters APP processing, and overexpression of GULP1 increases Aβ production, whereas loss of GULP1 reduces Aβ generation.

**DISCUSSION**

In the present study, we have shown that GULP1 is a new APP/APPc-interacting protein. The interaction between GULP1 and APP was confirmed by various biochemical assays, and they show overlapping subcellular distribution patterns in neurons. Human GULP1, the homologue of *Caenorhabditis elegans* CED-6, is an adaptor protein that contains an N-terminal PTB domain, a centrally located leucine zipper and a C-terminal proline/serine-rich region [16,35]. We have demonstrated in the present study that the interaction of the two proteins is mediated by the NPTY motif of APP and the GULP1 PTB domain. Although PTB domains were initially reported as phosphotyrosine-interacting motifs, it is now known that many PTB domains bind to their interactors in a phosphotyrosine-independent fashion, including a number of APPc-interacting proteins such as FE65 and X11s [26,36]. In our pull-down assays, bacterially expressed GST–APPc could efficiently interact with GULP1, which might suggest that tyrosine phosphorylation of APPc/APP within the NPTY motif is not crucial for the interaction between the two proteins.

In addition to APP, the PTB domain of GULP1 has been found to interact with molecules that are implicated in phagocytosis, including CED-1 and stabilins [16,37,38], and it is therefore believed that GULP1 is an important molecule for the process of phagocytosis. In fact, overexpression of CED-6 has been shown to rescue the engulfment defect of CED-6-null *C. elegans* [35]. Moreover, overexpression of GULP1 enhances the engulfment of apoptotic cells in the J774 macrophage cell line [39], whereas knockdown of GULP1 impairs stabilin-2-mediated phagocytosis [38]. Although the mechanism by which GULP1 enhances engulfment is not fully understood, it has been shown that GULP1 promotes reorganization of actin around apoptotic cells [40]. It is worth noting that APP has been shown to accelerate the rate of cell migration/movement, a process that involves actin dynamics [41]. Thus the identification of the interaction between GULP1 and APP opens up a novel avenue for studying the role of APP in actin organization. On the other hand, we and others have shown that GULP1 is widely expressed in different tissues and cell types, including brain and neurons [39,42,43] (please also see Figure 2). It is therefore very likely that the function of GULP1 is not only limited to engulfment and may also be involved in other cellular processes. In fact, we have demonstrated in the present study that the interaction between GULP1 and APP alters APP processing.
Figure 4  GULP1 influences APP processing and enhances Aβ secretion

(A) Immunoblot shows the levels of APP holoprotein and CTFs from CHO cells transiently transfected with APP, APP + BACE1 and APP + BACE1 + GULP1 (top panel). The expression of BACE1 (middle panel) and GULP1 (bottom panel) was confirmed. (B) Immunoblot analysis of APP CTFs from CHO cells transfected with APP alone or with APP + BACE1, plus either control siRNA or GULP1 siRNA (top panel). The expression of transfected BACE1 (middle panel) and endogenous GULP1 (bottom panel) were determined. APP CTFs were resolved on Tris-Tricine SDS/PAGE (16% gels). GULP1 and BACE1 were resolved on Tris-glycine SDS/PAGE (10% gels). The APP CTF band intensities were quantified by a Bio-Rad GS710 imaging densitometer and Quantity One software. The quantification data for (A) and (B) are shown in the histograms below the immunoblots. n = 6 for (A); n = 9 for (B); UT, untransfected. Values are means ± S.D. (C) CHO-APP751 cells were transfected either with control protein CAT or GULP1. The amount of Aβ secreted from the cells into the culture medium was determined by Aβ ELISA. n = 10; *P < 0.0001; values are means ± S.D. The expression of transfected GULP1 was analysed by resolving the cell lysates on Tris-glycine SDS/PAGE (10% gels) and followed by immunoblotting using an antibody against the HA tag on GULP1 (top panel). Four different samples, two CAT control and two GULP1 overexpressions are shown with α-tubulin as a loading control. (D) CHO-APP751 cells were transfected either with control siRNA or GULP1 siRNA. The amount of Aβ secreted from the cells into the culture medium was measured by Aβ ELISA. Both Aβ1-40 and Aβ1-42 decreased significantly in the GULP1-knockdown cells. n = 10; *P < 0.0001; values are means ± S.D. The expression level of endogenous GULP1 in control and GULP1 siRNA-transfected cells were analysed by resolving the cell lysates on Tris-glycine SDS/PAGE (10% gels) and then followed by immunoblot analysis using an anti-GULP1 antibody (top panel). Four different samples, two control and two GULP1 siRNA are shown with α-tubulin as a loading control. The experiments in (C) and (D) were repeated three times with similar results. The molecular mass in kDa is indicated on the left-hand side of the immunoblots.
The fact that knockdown of GULP1 led to suppression of APP CTFs and Aβ generation suggests that GULP1 is a modulator of APP processing. Although the mechanism by which GULP1 alters APP processing is not known, several APPc-interacting proteins have been shown to modulate APP trafficking and to alter APP processing [44–48]. Interestingly, GULP1 has been found to influence the endosomal trafficking of LRP [LDL (low-density lipoprotein) receptor-related protein], another GULP1-binding protein, ligands including αr-macroglobulin and prosaposin [31]. In fact, GULP1 has been suggested to play a role in endocytic trafficking as it binds to clathrin [42]. As endosomes are a major site for the generation of Aβ [2], it is therefore possible that the GULP1–APP interaction modulates trafficking of APP to endosomes. Moreover, GULP1 has been shown to regulate a small G-protein ADP-ribosylation factor 6 which is involved in endocytosis and recycling of membrane components between the plasma membrane and endosomes [49,50]. Therefore GULP1 may also alter APP processing indirectly through regulating cellular recycling endocytosis. Additionally, LRP has been proposed to play an essential role in APP processing and Aβ clearance [51]. The effect of LRP on APP processing might be mediated by the adaptor protein FE65 as it contains two PTB domains which could function as a ‘bridge’ between LRP and APP [52]. Although GULP1 only contains a single PTB domain, GULP1 has been shown to dimerize via the central leucine zipper [21]. Therefore GULP1 dimers may function as a linker between APP and LRP, which may in turn alter APP trafficking and processing.

Recently, GULP1 has been shown to bind APP in immuno-precipitation assays and to suppress Aβ production when overexpressed [43]. The results of the present studies extend these findings to show that the APP–GULP1 interaction can be detected using other biochemical assays (yeast two-hybrid and GST pull-down assays). Furthermore, we provide evidence that APP and GULP1 interact directly. Additionally, we demonstrate that GULP1 influences processing of APP using an APP–GAL4 reporter assay. However, in contrast with these earlier studies, we find that overexpression of GULP1 increases Aβ production, and support this finding by demonstrating that siRNA-mediated loss of GULP1 produces a complementary decrease in Aβ production. The reasons for the different findings between the results of the present study and the previous study [43] are unclear at this stage, but may involve the use of different cell types. Indeed, contradictory APP-processing effects have been reported for several other APP-interacting proteins, including FE65 [22,46]. It would therefore be interesting to investigate the effect of GULP1 overexpression and loss in vivo in transgenic mice in future studies. Nevertheless, both the present study and a previous study [43] demonstrate that GULP1 interacts with APP to influence its processing and, as such, provide insight into new routes for manipulating APP processing and Aβ production.

Taken together, the results of the present study identify GULP1 as an APP-binding protein. This interaction involves the YENPTY motif in the intracellular domain of APP and the PTB domain of GULP1. A proportion of APP and GULP1 co-localize in cells, and GULP1 modulates APP processing and Aβ production.

AUTHOR CONTRIBUTION
Candy Hao, Michael Perkinton and William Chan designed the study, performed the experiments and wrote the manuscript. Michael Perkinton, Ho Chan, Christopher Miller and Kwok-Fai Lau contributed to the planning of experiments, interpretation of results and the writing of the manuscript. All authors approved the final manuscript.

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REFERENCES
SUPPLEMENTARY ONLINE DATA

GULP1 is a novel APP-interacting protein that alters APP processing

Candy Yan HAO*, Michael S. PERKINTON†, William Wai-Lun CHAN*, Ho Yin Edwin CHAN*, Christopher C. J. MILLER† and Kwok-Fai LAU*‡†

*Biochemistry Programme, School of Life Sciences, the Chinese University of Hong Kong, Shatin, NT, Hong Kong SAR, †MRC Centre for Neurodegeneration Research, Department of Neuroscience, King’s College London, Institute of Psychiatry, Denmark Hill, London SE5 8AF, U.K., and ‡Molecular Biotechnology Programme, School of Life Sciences, the Chinese University of Hong Kong, Shatin, NT, Hong Kong SAR

EXPERIMENTAL

Colony-lift filter assay

A colony-lift filter assay for measuring β-galactosidase activity in transformed yeast was performed as described in the Yeast Protocols Handbook (Clontech). pY1-APPc+pACT2, pY1+pACT2-GULP1, pY1-APPc+pACT2-GULP1 or pCL1 (positive control vector that encodes the full-length GAL4) were transformed into the yeast strain AH109 (Clontech). The transformants were grown on SD agar (synthetic dropout agar) plates containing the appropriate dropout supplements at 30°C for 4 days. The yeast colonies on the plates were lifted up by nitrocellulose membranes (NEN Life Science Products). Following permeabilization of the yeast by freezing the membranes in liquid nitrogen for 10 s, the membranes were placed on 3 mm chromatography paper (Whatman) presoaked with Z buffer/X-gal solution [comprising 60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl and 1 mM MgSO4 (pH 7.0)] and 0.02 % 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The membranes were incubated for 4 h at 30°C for colour development.

siRNA knockdown in neurons

Knockdown of GULP1 in rat cortical neurons was performed using GULP1 Accell siRNA (Dharmacon, Thermo Scientific) as described previously [1]. In brief, GULP1 and non-targeting Accell siRNAs were added to rat cortical neuron culture to a final concentration of 1 μM on 4 DIV. The neurons were then analysed either by Western blotting or indirect immunofluorescence on 8 DIV.

Pulse–chase analysis

CHO cells were transfected with HA-tagged GULP1 by FuGENE® 6 (Roche). At 24 h post-transfection, the cells were incubated in methionine/cysteine-free medium (Invitrogen) for 30 min, and then replenished with methionine/cysteine-free medium containing 7.4 MBq/ml [35S]methionine (PerkinElmer) for 3 h. Following the ‘pulse’, the cells were ‘chased’ in normal culture medium and then collected in ice-cold lysis buffer at various times. HA-tagged GULP1 was immunoprecipitated from the cell lysates using the 12CA5 anti-HA antibody (Roche) for 16 h at 4°C. The antibody was captured by Protein G–Sepharose (Sigma) for 2 h at 4°C, and the immunoprecipitates were washed three times with ice-cold lysis buffer. The samples were analysed by SDS/PAGE and autoradiography. The presence of GULP1 in the immunoprecipitates was determined by immunoblotting.

Figure S1 APPc interacts with GULP1 in a yeast two-hybrid system

pY1-APPc + pACT2, pY1 + pACT2-GULP1, pY1-APPc + pACT2-GULP1 or pCL1 were transformed into the yeast stain AH109. A colony-lift filter β-galactosidase assay was performed for the transformants. Blue signals were observed in the yeast transformed with pY1-APPc + pACT2-GULP1, but not the ‘bait’-only or ‘prey’-only transformants. pCL1-transformed yeast was used as a positive control for the β-galactosidase assay.

1 To whom correspondence should be addressed (email kflau@cuhk.edu.hk).
Figure S2  Characterization of the rat anti-GULP1 antibody by indirect immunofluorescence in neurons

(A) Confocal imaging showing rat cortical neurons stained with anti-GULP1 antibody or anti-GULP1 antibody pre-absorbed with the antigen, purified His<sub>6</sub>–GULP1. Nuclei were stained by DAPI (4′,6-diamidino-2-phenylindole) (right-hand panel). (B) Western blot analysis shows knockdown of GULP1 in rat cortical neurons. Neurons were incubated with either control or GULP1 Accell siRNA at 4 DIV, and then harvested in SDS sample buffer on 8 DIV for immunoblotting. The molecular mass in kDa is indicated on the left-hand side. (C) Confocal imaging showing control and GULP1-knockdown neurons stained with the anti-GULP antibody. The right-hand panel shows staining of nuclei by DAPI. The images in (A) and (C) were captured using same laser intensity. The scale bar in (A) and (C) is 10 μM.

Figure S3  Determination of the half-life of GULP1 by pulse–chase analysis

(A) CHO cells transfected with HA-tagged GULP1 were pulse-labelled in methionine/cysteine-free medium containing 7.4 MBq/ml [35S]methionine for 4 h. The cells were then ‘chased’ at the time points indicated. HA–GULP1 in the cell lysates was immunoprecipitated by the anti-HA antibody 12CA5. GULP1 in the immunoprecipitates was adjusted to equal amounts, separated by Tris-glycine SDS/PAGE (10 % gel), and analysed by autoradiography (top panel). GULP1 in the adjusted immunoprecipitates was analysed by immunoblotting using an anti-GULP1 antibody (bottom panel). The molecular mass in kDa is indicated on the left-hand side. (B) The GULP1 signals on the autoradiograph were scanned and analysed using a Bio-Rad GS710 imaging densitometer and Quantity One software (Bio-Rad). The amount of [35S]-labelled GULP1 in (A) is expressed as a percentage of labelled protein at the different chase time points. [35S]-Labelled GULP1 could not be detected 12 h after chasing. The half-life of GULP1 is approximately 2.2 h (according to the data from t = 0 and t = 6). Two independent experiments were performed with similar results.
Table S1   Antibodies used in the present study

n/a, not available.

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