Introduction

Cell-cell communication plays an important role in the development of multicellular organisms, especially with regard to their organization into tissues, as well as in control of cell growth and death and coordination of cell functions in such organisms. Animal cells communicate in two major ways: (1) they secrete chemicals that signal to cells located at various distances, and (2) they display signaling molecules at the cell surface that bind to receptors on adjacent cells. The latter mechanism is more suitable for precise and directional cell-cell communication than is the former. Indeed, the latter mechanism is widely adopted in the nervous and immune systems, where it contributes to axon guidance, antigen recognition and cell death by apoptosis. In general, the binding of a ligand to its receptor is followed by removal of the ligand-receptor complex from the cell surface and termination of the cell response. Complexes of soluble ligands and their receptors undergo endocytosis and subsequent degradation in lysosomes. By contrast, it has remained unclear how signaling is terminated after interaction of membrane-bound ligands with their receptors.

The CD47–SHPS-1 system is a cell-cell communication system that consists of the transmembrane proteins CD47 and signal-regulatory protein alpha (SIRPA; also known as Src-homology-2-domain-containing protein tyrosine phosphatase substrate 1, hereafter referred to as SHPS-1) (Jiang et al., 1999; Seiffert et al., 1999). CD47 and SHPS-1 interact with each other through their extracellular regions and are thought to initiate intracellular signaling in a bidirectional manner. CD47 is a member of the immunoglobulin (Ig) superfamily, possessing a V-type Ig-like extracellular domain, five putative membrane-spanning segments, and a short cytoplasmic tail (Brown and Frazier, 2001). CD47, also named IAP (integrin-associated protein), was originally identified in association with the integrin αvβ3. Some CD47-elicited cellular responses are thus probably mediated by integrins (Brown and Frazier, 2001). By contrast, SHPS-1, also known as SIRPα, BIT or P84, is a transmembrane protein whose extracellular region consists of three Ig-like domains (Fujioka et al., 1996; van Beek et al., 2005). Its cytoplasmic region contains four putative tyrosine phosphorylation sites that serve as binding sites for the Src homology 2 domains of the tyrosine phosphatases SHP-1 and SHP-2 (also known as PTPN6 and PTPN11, respectively). SHPS-1 undergoes tyrosine phosphorylation and binds SHP tyrosine phosphatases in response to growth factors, cytokines or integrin-mediated cell adhesion (Takada et al., 1998; Tsuda et al., 1998; van Beek et al., 2005). SHP-1 and SHP-2 are thus thought to participate downstream of SHPS-1 in specific biological functions mediated by the CD47–SHPS-1 system.

The CD47–SHPS-1 system participates in regulation of a variety of biological functions, particularly in the immune and nervous systems. The binding of CD47 on red blood cells to SHPS-1 on splenic macrophages is thought to prevent phagocytosis of the former cells by the latter, with this effect being mediated through SHP-1 complexed with SHPS-1 (Oldenborg et al., 2000; Okazawa et al., 2005; Ishikawa-Sekigami et al., 2006). Interaction of SHPS-1 with CD47 expressed on the surface of melanoma cells inhibits cell migration (Motegi et al., 2003), suggesting that the CD47–SHPS-1 system mediates contact inhibition of cell migration. By contrast, SHPS-1 on dendritic cells, through its interaction with CD47 on T cells, is implicated in positive regulation of T cell survival (Brown and Frazier, 2001). CD47, also named IAP (integrin-associated protein), was originally identified in association with the integrin αvβ3. Some CD47-elicited cellular responses are thus probably mediated by integrins (Brown and Frazier, 2001). By contrast, SHPS-1, also known as SIRPα, BIT or P84, is a transmembrane protein whose extracellular region consists of three Ig-like domains (Fujioka et al., 1996; van Beek et al., 2005). Its cytoplasmic region contains four putative tyrosine phosphorylation sites that serve as binding sites for the Src homology 2 domains of the tyrosine phosphatases SHP-1 and SHP-2 (also known as PTPN6 and PTPN11, respectively). SHPS-1 undergoes tyrosine phosphorylation and binds SHP tyrosine phosphatases in response to growth factors, cytokines or integrin-mediated cell adhesion (Takada et al., 1998; Tsuda et al., 1998; van Beek et al., 2005). SHP-1 and SHP-2 are thus thought to participate downstream of SHPS-1 in specific biological functions mediated by the CD47–SHPS-1 system.

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activation in a manner functionally similar to that apparent for costimulatory molecules on dendritic cells and T cells (Seiffert et al., 2001; Tomizawa et al., 2007). Both CD47 and SHPS-1 are highly expressed throughout the brain, and the regions in which they are especially abundant overlap to a substantial extent (Ohnishi et al., 2005). Engagement of CD47 on the surface of neurons by SHPS-1 promotes neurite or filopodium formation in a manner dependent on activation of Src and the small GTP-binding proteins Rac and Cdc42 (Miyashita et al., 2004; Murata et al., 2006), suggesting that the CD47–SHPS-1 system promotes neuronal development.

The mechanism by which the complex of CD47 and SHPS-1 is removed from the cell surface in order to terminate the induced cell responses has remained unknown. Although ectodomain shedding of SHPS-1 is one mechanism by which such responses might be terminated, it was found not to occur after the binding of CD47 to SHPS-1 (Ohnishi et al., 2004). We now show that coculture of CD47-expressing cells and SHPS-1-expressing cells results in endocytosis of the ligand-receptor complex into either cell type. We have also investigated the molecular mechanism and physiological roles of such endocytosis.

**Results**

Bidirectional trans-endocytosis of CD47 and SHPS-1

To examine the fate of the CD47–SHPS-1 complex, we studied two CHO cell lines that stably express mouse CD47 (CHO-CD47 cells) or mouse SHPS-1 (CHO–SHPS-1 cells). These CHO cell lines were originally designed to express a high level of exogenous protein as a result of transformation with an active form of H-Ras (CHO-Ras cells) (Sato et al., 2003). The two types of cells were cocultured for 1 hour and then subjected to immunofluorescence analysis with a monoclonal antibody (mAb) to CD47 (Fig. 1A-F). The cells were also stained with Rhodamine-conjugated phalloidin to reveal the overall morphology of CHO–SHPS-1 cells, which were not recognized by the mAb to CD47, as well as that of CHO-CD47 cells. We frequently observed that both types of cell formed contacts through multiple filopodium-like protrusions, with those extended by CHO-CD47 cells being labeled with the mAb to CD47 (Fig. 1A-C). The two cell types also formed linear contact sites (Fig. 1D-F). By contrast, the filopodium-like protrusions were rarely observed in cocultures of CHO-CD47 cells and parental CHO-Ras cells (Fig. 1G-I). We recently showed that engagement of CD47 by SHPS-1 promotes filopodium formation in a neuroblastoma cell line and in neurons (Miyashita et al., 2004; Murata et al., 2006). These previous and our present observations thus suggested that CHO-CD47 cells extend filopodium-like protrusions toward CHO–SHPS-1 cells as a result of interaction of CD47 with SHPS-1. Permeabilization of cells with Triton X-100 before staining revealed the presence of multiple CD47-positive vesicle-like structures in CHO–SHPS-1 cells that had formed contacts with CHO-CD47 cells (Fig. 1A,C,D,F). Quantitative analysis by an assay (assay A) in which cells containing more than three CD47-positive vesicle-like structures were defined as having CD47-positive vesicles revealed that 29.3±2.3% of CHO–SHPS-1 cells that formed contacts with CHO-CD47 cells were positive for such vesicles under nonpermeabilizing conditions. By contrast, 82.8±2.8% of such CHO–SHPS-1 cells contained CD47-positive vesicles under permeabilizing conditions (Fig. 1M). Quantitative analysis by another assay (assay B) in which the cells were sequentially stained with secondary polyclonal antibodies (pAbs) with two different labels and the numbers of CD47-positive vesicle-like structures either at the surface or inside of cells were evaluated separately

![Image](image_url)
CHO-CD47 cells was markedly greater than that at the cell surface (Fig. 1J-L,N).

CD47-positive vesicles were rarely observed in parental CHO-Ras cells cocultured with CHO-CD47 cells (Fig. 1G,J). Furthermore, pretreatment of CHO-CD47 cells with a mAb to CD47 (miap301), which blocks the binding of CD47 to SHPS-1, markedly reduced the number of CHO–SHPS-1 cells that contained CD47-positive vesicles under either permeabilized or nonpermeabilized conditions (Fig. 1M). These results thus suggested that contact of CHO-CD47 cells with CHO–SHPS-1 cells results in translocation of CD47 from the former cells and its subsequent internalization into the latter cells. In addition, such trans-endocytosis of CD47 appears to be mediated by the trans-interaction of CD47 (on CHO-CD47 cells) with SHPS-1 (on CHO–SHPS-1 cells).

We also found that cell-cell contact between CD47-expressing cells and SHPS-1-expressing cells induced translocation of SHPS-1 and its subsequent internalization into CD47-expressing cells (supplementary material Fig. S1A-H). Trans-endocytosis of CD47 into SHPS-1-expressing cells occurred more efficiently than did that of SHPS-1 into CD47-expressing cells (Fig. 1M; supplementary material Fig. S1G). However, the direction of trans-endocytosis could depend on relative expression levels of SHPS-1 and CD47 in two neighboring cells. We thus compared expression levels of CD47 and SHPS-1 by immunoblot analysis (supplementary material Fig. S2). The results suggest that expression of SHPS-1 is approximately twofold higher than that of CD47 (supplementary material Fig. S2C). Thus, predominant trans-endocytosis of CD47 from CHO-CD47 cells into CHO–SHPS-1 cells might be attributable to the higher expression level of SHPS-1 (in CHO–SHPS-1 cells) compared to that of CD47 in CHO-CD47 cells.

In subsequent experiments, we focused on characterization of the trans-endocytosis of CD47 into SHPS-1-expressing cells. To examine if the whole molecule of CD47 is endocytosed, CHO-Ras cells were transiently transfected with an expression vector for CD47 tagged at its C-terminus with the Myc epitope, and cocultured with CHO–SHPS-1 cells. We found that trans-endocytosed vesicles were stained with two different antibodies, miap301, which recognized the extracellular region of CD47, and 9E10, which recognized the Myc-tagged C-terminal region of CD47 (supplementary material Fig. S1I-K). In addition, SHPS-1 was colocalized with trans-endocytosed CD47 (supplementary material Fig. S1L-N). These results suggested that the entire CD47 molecule undergoes trans-endocytosis by neighboring SHPS-1-expressing cells and that SHPS-1, after its binding of CD47, is endocytosed together with CD47.

To further characterize the structure of the CD47-positive vesicles, immunogold labeling of ultrathin section with mAbs to CD47 was performed (Fig. 2A). We often found that multivesicular bodies (MVBs) in typical late endosome/lysosomes were CD47-labeled in CHO–SHPS-1 cells but not in CHO-Ras cells, when in contact with CHO-CD47 cells (Fig. 2A,B). In addition, such labeling of gold particles were confined to the inner vesicles of the structure rather than to the limiting membrane. The MVB is a typical structure of endosomes in the lysosomal pathway (van der Goot and Gruenberg, 2006). In mammalian cells, sorting to the MVB pathway begins at the early endosome, which either matures to, or fuses with, the late endosome and results in its appearance as a multivesicular structure (van der Goot and Gruenberg, 2006). We also found that staining of cathepsin D, a late endosome/lysosome marker, was frequently localized at the CD47-positive vesicles in CHO–SHPS-1 cells, in contact with CHO-CD47 cells (Fig. 2C,D). These results suggest that trans-endocytosed CD47 is sorted into the general endocytic pathway from early endosome to lysosome (van der Goot and Gruenberg, 2006).

Promotion of trans-endocytosis of CD47 by clathrin, epsin and dynamin

The binding of soluble ligands to their receptors at the cell surface results in receptor clustering and subsequent endocytosis of the ligand-receptor complex mediated by the formation of clathrin-coated pits (Mousavi et al., 2004). A small proportion of ligand-receptor complexes is also endocytosed through a clathrin-independent mechanism (Mousavi et al., 2004). We therefore examined the effect of depletion of endogenous clathrin heavy chain (CHC) by RNA interference on trans-endocytosis of CD47. Transfection of HEK293T cells with an expression vector (siRNAchc-1 or siRNAchc-2) that encodes both mouse SHPS-1 and epsin mRNA (siRNAchc-1 for nucleotides 1065-1083; siRNAchc-2 for nucleotides 4820-4838) resulted both in expression of exogenous SHPS-1 and in a marked decrease in the amount of endogenous CHC, compared with the amount of CHC apparent in surrounding non-transfected HEK293T cells (Fig. 3A-C; data for siRNAchc-2 are not shown). The transfected HEK293T cells were then cocultured with CHO-CD47 cells. In this and the following experiments, we used assay B for quantification of CD47-positive vesicles either at the surface or inside of cells. Compared with cells transfected with a control vector, transfection of HEK293T cells with either siRNAchc-1 or siRNAchc-2 reduced the number of CD47-positive vesicles apparent inside CHO–SHPS-1 cells adjacent to CHO-CD47 cells but did not affect the number of such vesicles at the cell surface (Fig. 3D). In addition to the clathrin-mediated pathway, a caveolin-mediated pathway is also implicated.

![Fig. 2](image)
to participate in endocytosis of the ligand-receptor complex (Nabi and Le, 2003). However, nystatin, a lipid-raft/caveolae disrupting reagent (Smart and Anderson, 2002), did not prevent trans-endocytosis of CD47 (data not shown). Therefore, the caveolin-mediated pathway may not participate in regulation of trans-endocytosis of CD47.

Epsin is also thought to contribute to clathrin-mediated endocytosis by binding to phospholipids, clathrin, and the AP2 complex (Wendland, 2002). We therefore tested the effect on CD47 trans-endocytosis of a dominant negative (DN) mutant of epsin, epsin(1-205), which comprises only amino acids 1-205 and thus lacks the binding site for both clathrin and the AP2 complex. Forced expression of epsin(1-205) together with SHPS-1 in CHO-Ras cells resulted in a marked reduction in the number of CD47-positive vesicles inside such cells adjacent to CHO-CD47 cells without affecting the number of these vesicles at the cell surface (Fig. 3E). Moreover, forced expression of the mutant epsin(204-458), which lacks the N-terminal portion of the protein responsible for binding to phospholipids, also reduced the number of CD47-positive vesicles inside SHPS-1-expressing CHO-Ras cells positioned adjacent to CHO-CD47 cells (Fig. 3E). These results thus suggested that trans-endocytosis of CD47 is mediated in a manner dependent on clathrin and epsin.

Dynamin is a small GTP-binding protein that is thought to participate in pinching off of coated pits from the cell membrane (Mousavi et al., 2004). Forced expression of dynamin 1(K44A), a DN mutant of dynamin 1 (Damke et al., 1994), in SHPS-1-expressing CHO-Ras cells also resulted in a marked reduction in the number of CD47-positive vesicles inside these cells located adjacent to CHO-CD47 cells (Fig. 3E). This finding thus also implicated dynamin in the trans-endocytosis of CD47.

A juxtamembrane region of SHPS-1 is required for efficient trans-endocytosis of CD47

Endocytosis of receptors, especially through the constitutive pathway, is mediated by motifs present within the cytoplasmic regions of the target proteins (Sorkin, 2004). These motifs include two tyrosine-based sequences, YxxØ and NPxY, as well as leucine-based sequences, and they provide the binding sites for the AP2 complex, which mediates the clathrin-dependent endocytosis of receptors (Sorkin, 2004). The cytoplasmic region of SHPS-1 contains four YxxL/I/V motifs; the tyrosine residues in the two N-terminal motifs do not undergo phosphorylation, whereas those in the two C-terminal motifs are phosphorylated and serve as the binding sites for SHP-1 and SHP-2 (Takada et al., 1998) (Fig. 4A).

We therefore next examined whether the cytoplasmic region of SHPS-1 participates in the trans-endocytosis of CD47 by SHPS-1-expressing cells. To this end, we studied CHO cell lines that stably express various deletion mutants of the cytoplasmic region of SHPS-1 as well as a CHO cell line (CHO–SHPS-1–4F) that expresses a mutant form of SHPS-1 in which all four tyrosine residues in the cytoplasmic region are replaced by phenylalanine (Liu et al., 2002). Thus, the surface expression of the wild-type or mutant SHPS-1 was also evaluated by immunofluorescence analysis with a mAb to SHPS-1, which reacted with the extracellular region of SHPS-1, under nonpermeabilized condition. The extent of surface SHPS-1 expression corresponded well to those of total expression of this protein in each cell line (supplementary material Fig. S3). CHO-CD47 cells were cocultured for 1 hour with either CHO–SHPS-1

Fig. 3. Participation of clathrin, epsin, and dynamin in trans-endocytosis of CD47. (A-C) HEK293T cells were transfected with the vector siRNAchc-1, which encoded both mouse SHPS-1 and a siRNA specific for human CHC mRNA. Twenty-four hours after transfection, the cells were isolated, plated on new culture dishes, and incubated for 48 hour before the addition of CHO-CD47 cells. After coculture for 1 hour, the cells were fixed and subjected to two-color immunostaining with mAbs to SHPS-1 (green; A) and to CHC (red; B). The merged image is shown in C. Asterisks indicate cells in which CHC was depleted and exogenous SHPS-1 was expressed. Bar, 20 µm. (D) HEK293T cells transfected with siRNAchc-1 or siRNAchc-2 were treated as in A-C and then fixed and stained as in Fig. 1J-L. The cells were also stained with pAbs to SHPS-1 and AMCA-conjugated goat pAbs to rabbit IgG in order to confirm the expression of SHPS-1. A control, HEK293T cells were transfected with the corresponding SHPS-1 expression vector without the insert for CHO-CD47 cells. The numbers of surface, internalized, and total CD47-positive vesicles in transfected HEK293T cells located adjacent to CHO-CD47 cells were determined by assay B. Data in D and E are means ± s.e.m. from three separate experiments. *P<0.05, **P<0.01 versus corresponding control value.
cells or CHO-Ras cells expressing SHPS-1 mutants, after which the trans-endocytosis of CD47 from CHO-CD47 cells into neighboring SHPS-1-expressing cells was evaluated by assay B. Given that the expression level of SHPS-1 was found to influence the number of trans-endocytosed vesicles containing CD47 in SHPS-1-expressing cells (supplementary material Fig. S4), the extent of CD47 trans-endocytosis was determined as a percentage of the total number of CD47-positive vesicles (N_total) that were present at the cell surface (N_sur) or intracellularly (N_int). Data are means ± s.e.m. from three separate experiments. **P < 0.01 versus the corresponding value for cells expressing wild-type SHPS-1.

Regulation of CD47 trans-endocytosis by Rac, Cdc42 and Rab5

Small GTP-binding proteins of the Rho family are implicated in regulation of clathrin-dependent and -independent endocytosis (Symons and Rusk, 2003). We therefore next examined whether Rho family proteins participate in trans-endocytosis of CD47 by SHPS-1-expressing cells. Forced expression of a DN mutant of Rac1 [Rac(T17N)] together with SHPS-1 in CHO-Ras cells resulted in a marked reduction in the number of CD47-positive vesicles observed inside such cells adjacent to CHO-CD47 cells (Fig. 5A). The CRIB domain of NWASP (NWASP-CRIB) specifically binds the GTP-bound (active) form of Cdc42 and thereby inhibits its activity (Miyashita et al., 2004; Murata et al., 2006). Forced expression of NWASP-CRIB also reduced the number of CD47-positive vesicles detected inside SHPS-1-expressing CHO-Ras cells adjacent to CHO-CD47 cells (Fig. 5A).

Fig. 4. Role of a juxtamembrane region of SHPS-1 in trans-endocytosis of CD47. (A) Schematic representation of mouse SHPS-1 mutants. Black, gray and white boxes indicate the extracellular (Ex), transmembrane (TM) and cytoplasmic (Cyto) regions of SHPS-1, respectively. WT, wild type. Y and F indicate the positions of tyrosine and phenylalanine, respectively. (B) Lysates (15 μg of protein) of CHO-Ras cell lines expressing wild-type or mutant forms of SHPS-1 were subjected to immunoblot analysis with the p84 mAb to SHPS-1. (C) CHO-Ras cells expressing wild-type or mutant forms of SHPS-1 were cocultured with CHO-CD47 cells, fixed, and stained as in Fig. 1J-L. The numbers of surface, internalized, and total CD47-positive vesicles in SHPS-1-expressing cells located adjacent to CHO-CD47 cells were determined by assay B. The extent of CD47 trans-endocytosis in each cell line was determined as the percentage of the total number of CD47-positive vesicles (N_total) that were present at the cell surface (N_sur) or intracellularly (N_int). Data are means ± s.e.m. from three separate experiments. **P < 0.01 versus the corresponding control value. (B-D) CHO–SHPS-1 cells were transfected with an expression vector for Rab5(Q79L; C) or for Rab5(S34N; D). Twenty-four hours after transfection, the cells were cocultured with CHO-CD47 cells for 1 hour, fixed, and subjected to immunostaining with mAbs to CD47 (red; B-D) and to the HA epitope tag of the Rab5 mutants (green; (C, D). In a control experiment (B), CHO–SHPS-1 cells were transfected with a vector for GFP and then analyzed as for the cells expressing the Rab5 mutants, with the exception that GFP was detected on the basis of its intrinsic fluorescence (green). Bar, 20 μm.

Fig. 5. Regulation of trans-endocytosis of CD47 by Rac, Cdc42 and Rab5. (A) CHO-Ras cells were cotransfected with an expression vector for SHPS-1 and with a vector for either Rac(T17N) or NWASP-CRIB (or with the corresponding empty vector). Twenty-four hours after transfection, the cells were cocultured with CHO-CD47 cells for 1 hour. CHO–SHPS-1 cells were also treated with 0.5 μM cytochalasin D (CytoD) or with dimethyl sulfoxide (DMSO) vehicle for 30 minutes before coculture with CHO-CD47 cells. All cells were then fixed and stained as in Fig. 1J-L. The numbers of surface, internalized, and total CD47-positive vesicles in SHPS-1-expressing cells adjacent to CD47-expressing cells were determined by assay B. Cells were also stained with a mAb to the Myc epitope in order to confirm the expression of Myc-tagged Rac(T17N) or NWASP-CRIB. Data are means ± s.e.m. from three separate experiments. *P < 0.05, **P < 0.01 versus the corresponding control value. (B-D) CHO–SHPS-1 cells were transfected with an expression vector for Rab5(Q79L; C) or for Rab5(S34N; D). Twenty-four hours after transfection, the cells were cocultured with CHO-CD47 cells for 1 hour, fixed, and subjected to immunostaining with mAbs to CD47 (red; B-D) and to the HA epitope tag of the Rab5 mutants (green; (D). In a control experiment (B), CHO–SHPS-1 cells were transfected with a vector for GFP and then analyzed as for the cells expressing the Rab5 mutants, with the exception that GFP was detected on the basis of its intrinsic fluorescence (green). Bar, 20 μm.

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change the cell-surface expression of SHPS-1 (supplementary material Fig. S5). Cytochalasin D, an inhibitor of actin polymerization, also reduced the number of CD47-positive vesicles inside CHO–SHPS-1 cells located adjacent to CHO-CD47 cells (Fig. 5A). These data thus suggested that Rac and Cdc42, but not Rho, promote trans-endocytosis of CD47. In addition, reorganization of the actin cytoskeleton appears to be required for this process. We also examined the effects of nocodazole (an inhibitor of microtubule polymerization), wortmannin (an inhibitor of phosphoinositide 3-kinase) or PP2 (an inhibitor of Src family tyrosine kinase). However, none of these inhibitors prevented trans-endocytosis of CD47 (data not shown).

Rab5, a member of the Rab family of small GTP-binding proteins, is also thought to regulate early endocytic transport after constitutive or ligand-activated internalization of various types of receptor (Stenmark et al., 1994). Forced expression of the dominant active mutant Rab5(Q79L) in CHO–SHPS-1 cells resulted in enlargement of CD47-positive vesicles in such cells positioned adjacent to CHO-CD47 cells (Fig. 5B,C). By contrast, forced expression of the DN mutant Rab5(S34N) resulted in a marked reduction in the size of CD47-positive vesicles in such CHO–SHPS-1 cells (Fig. 5B,D). These results thus suggest that Rab5 may promote the fusion of CD47-containing vesicles.

Time-lapse imaging of CD47 trans-endocytosis
We next examined CD47 trans-endocytosis by time-lapse imaging. CHO-Ras cells were transfected with a vector either for CD47 tagged with yellow fluorescent protein (CD47-YFP) or for SHPS-1 tagged with cyan fluorescent protein (SHPS-1–CFP). The cells were subsequently harvested, cocultured for 30 minutes, and subjected thereafter to time-lapse imaging. In the series of images shown in Fig. 6A-J (see also Movie 1 in supplementary material), a cell expressing CD47-YFP first extends a lamellipodium-like protrusion toward a cell expressing SHPS-1–CFP (Fig. 6A,B). The former cell subsequently extends a filopodium-like protrusion that makes contact with the surface of the latter cell (Fig. 6C,D). The extension of this filopodium-like protrusion from the CD47-expressing cell toward the SHPS-1-expressing cell is reminiscent of the results shown in Fig. 1A-I. Several vesicle-like structures positive for both YFP and CFP fluorescence are then generated at the site of cell–cell contact and subsequently move into the intracellular space of the SHPS-1-expressing cell (Fig. 6E-H). After vesicle endocytosis at the contact site, the filopodium-like protrusion of the CD47-expressing cell retracts and cell-cell contact is almost disrupted (Fig. 6I,J), suggesting that endocytosis of the CD47–SHPS-1 complex into an SHPS-1-expressing cell terminates cell-cell contact mediated by the interaction of CD47 with SHPS-1. By contrast, forced expression of the DN mutant dynamin 1(K44A) in SHPS-1-expressing cells prevented trans-endocytosis of CD47 as well as disruption of sites of cell-cell contact, which remained apparent for up to 60 minutes after their formation (Fig. 6K-N, see also Movie 2 in supplementary material).

Transient formation of cell aggregates by CHO-CD47 and CHO–SHPS-1 cells
We next examined whether trans-endocytosis of CD47 regulates cell-cell adhesion mediated by the CD47–SHPS-1 complex. Incubation of CHO-CD47 cells with CHO–SHPS-1 cells resulted in formation of large cell aggregates that were apparent after 5-15 minutes (Fig. 7A,B). The extent of this aggregation subsequently decreased and was abolished after incubation for 120-240 minutes (Fig. 7A,B). Moreover, immunoblot analysis showed that the amounts of CD47 and SHPS-1 were reduced 120 minutes after the onset of coculture (supplementary material Fig. S6). These results are consistent with a notion that the trans-endocytosed CD47 is sorted into the lysosomal pathway as mentioned in Fig. 2. We therefore next examined the effect of prevention of CD47 trans-endocytosis on cell aggregation. CHO-CD47 and CHO–SHPS-1–ΔC404 cells formed aggregates that persisted for a much longer period of time than did those formed by CHO-CD47 and CHO–SHPS-1 cells.

Fig. 6. Time-lapse imaging of CD47 trans-endocytosis. (A-J) CHO-Ras cells, which were transfected with an expression vector for either SHPS-1–CFP or CD47–YFP, were cocultured, and CFP and YFP images were acquired every minute for 75 minutes. Images obtained at the indicated times are shown. Filled white (A,B) and open (C,D) arrowheads indicate lamellipodium-like and filopodium-like protrusions, respectively, formed by a CD47-YFP-expressing cell. White arrows (E-H) indicate vesicle-like structures labeled with both CFP and YFP in a cell expressing SHPS-1–CFP. Endocytosis of vesicles at the contact site was followed by retraction of the filopodium-like protrusion by the CD47-expressing cell and the near disruption of cell-cell contact (yellow arrowheads; I,J). Also see Movie 1 in supplementary material. (K-N) CHO-Ras cells were treated as in A-J with the exception that an expression vector for dynamin 1(K44A) was introduced together with that for SHPS-1–CFP. CFP and YFP images were acquired as in A-J. Images obtained at 0, 10, 20 and 60 minutes are shown. Also see Movie 2 in supplementary material. Bars, 20 μm.
Trans-endocytosis of CD47 and SHPS-1

Furthermore, cytochalasin D stabilized the aggregates formed by the two latter cell types (Fig. 7C). These results thus suggested that trans-endocytosis of CD47 together with SHPS-1 contributes to the removal of the CD47–SHPS-1 complex from the cell surface and results in the subsequent degradation of both proteins, thereby regulating the formation of cell aggregates.

Trans-endocytosis of CD47 on primary cultured hippocampal neurons by neighboring glial cells

We next examined whether endogenous CD47 expressed on the surface of primary cultured hippocampal neurons might be a target for trans-endocytosis by neighboring SHPS-1-expressing cells. Mouse hippocampal neurons were cocultured for 1 hour with HEK–SHPS-1 cells that had been further transfected with an expression vector for GFP to allow visualization of cell shape. The cells were then fixed, stained in the presence of 0.1% Triton X-100 with a mAb to CD47 (red; A), and examined for GFP fluorescence (green; B). A merged image is shown in C. Arrows indicate CD47-positive vesicles in a HEK–SHPS-1 cell. Bar, 20 μm. (D-I) Mouse hippocampal neurons (and glial cells) were cocultured for 3 hour with HEK293T cells that had been transfected with an expression vector for CD47-YFP. Expression of CD47-YFP was confirmed by detection of YFP fluorescence (green; D, G). Merged images are shown in F and I. Arrows indicate CD47-positive vesicles in neighboring GFAP-positive cells. Bar, 20 μm.

Fig. 7. Transient aggregation of CHO-CD47 and CHO–SHPS-1 cells. (A) CHO–SHPS-1 cells were mixed with CHO-CD47 cells, incubated for the indicated times, fixed, and examined by phase-contrast microscopy. Bar, 20 μm. (B,C) CHO–SHPS-1, CHO–SHPS-1–ΔC404, or CHO-Ras cells were mixed with CHO-CD47 cells and incubated for the indicated times (B). Alternatively, CHO–SHPS-1 cells were pretreated with 0.5 μM cytochalasin D or DMSO for 30 minutes, mixed with CHO-CD47 cells or control CHO-Ras cells, and incubated for the indicated times (C). Cells were then fixed and the extent of cell aggregation was quantified as the ratio of the number of cells at the indicated time (Nt) to that at the initiation of incubation (No) as described in Materials and Methods. Data are means ± s.e.m. from three separate experiments. *P<0.05, **P<0.01 versus the corresponding value for CHO–SHPS-1 cells (B) or DMSO-pretreated cells (C).

Fig. 8. Trans-endocytosis of CD47 from cultured hippocampal neurons to neighboring glial cells or to HEK293T cells expressing SHPS-1. (A-C) Mouse hippocampal neurons were cocultured for 1 hour with HEK–SHPS-1 cells that had been further transfected with an expression vector for GFP to allow visualization of cell shape. The cells were then fixed, stained in the presence of 0.1% Triton X-100 with a mAb to CD47 (red; A), and examined for GFP fluorescence (green; B). A merged image is shown in C. Arrows indicate CD47-positive vesicles in a HEK–SHPS-1 cell. Bar, 20 μm. (D-I) Mouse hippocampal neurons (and glial cells) were cocultured for 3 hour with HEK293T cells that had been transfected with an expression vector for CD47-YFP. Expression of CD47-YFP was confirmed by detection of YFP fluorescence (green; D, G). Merged images are shown in F and I. Arrows indicate CD47-positive vesicles in neighboring GFAP-positive cells. Bar, 20 μm. (J-L) Glial cells in mouse hippocampal neuron cultures were fixed and stained with mAbs to SHPS-1 (green; J) and to GFAP (red; K). A merged image is shown in L. Bar, 20 μm. (M-O) Hippocampal neurons (and glial cells) were isolated, cultured for 24 hours, and then transfected with an expression vector for CD47-YFP. Twelve hours after transfection, the cells were fixed and stained with a mAb to GFAP (red; N). Expression of CD47-YFP was confirmed by detection of YFP fluorescence (green; M). A merged image is shown in O. Enlarged images of the boxed regions are shown in the insets. Arrows indicate CD47-positive vesicles in a GFAP-positive astrocyte. Bar, 20 μm.
(Murata et al., 2006). Moreover, several CD47-positive vesicle-like structures were observed in the HEK–SHPS-1 cells that made contact with neurites via the CD47-positive filopodia (Fig. 8A-C). Again, these CD47-positive vesicle-like structures were not observed when neurons were cocultured with HEK293T cells expressing GFP alone (data not shown). These data suggested that trans-endocytosis of CD47 indeed occurs from primary cultured neurons to neighboring cells expressing SHPS-1. Forced expression of the DN mutants dynamin 1(K44A) or Rac(T17N) in HEK–SHPS-1 cells resulted in a marked decrease in the number of CD47-positive vesicles detected in these cells [17.83±1.43 (n=3) for control cells, 11.18±1.35 (n=3; P<0.05) for dynamin 1(K44A), 10.17±0.61 (n=3; P<0.05) for Rac(T17N)]. Forced expression of dynamin 1(K44A) resulted in the accumulation of CD47 immunoreactivity at the sites of contact between neurites and HEK–SHPS-1 cells (supplementary material Fig. S7). These results thus suggested that dynamin and Rac participate in CD47 trans-endocytosis from neurons to HEK–SHPS-1 cells.

We next examined whether CD47 was trans-endocytosed from CD47-expressing HEK293T cells to neurons. However, CD47-positive vesicle-like structures were rarely observed in microtubule-associated protein 2 (MAP2)-positive neurites or cell bodies adjacent to CD47-expressing HEK293T cells (Fig. 8D-F). Similar results were obtained when neuronal axons were examined with antibodies to the axon marker Tau-1 (data not shown). By contrast, CD47-positive vesicles were frequently detected in glial fibrillary acidic protein (GFAP)-positive astrocytes located adjacent to CD47-expressing HEK293T cells (Fig. 8G-I). We found that GFAP-positive astrocytes indeed expressed SHPS-1 endogenously (Fig. 8J-L). We therefore finally examined whether trans-endocytosis of CD47 might occur from neurons to astrocytes. Cultured neurons were transfected with a vector for CD47-YFP and cocultured with glial cells, CD47-YFP-positive vesicles were indeed detected in the GFAP-positive astrocytes that made contact with the transfected neurons (Fig. 8M-O). We further analyzed trans-endocytosis of CD47 from neurons to neighboring glial cells by time-lapse imaging. CD47-YFP-positive vesicles were generated at sites of cell-cell contact between neurons and neighboring glia-like nonneural cells, and they subsequently moved into the interior of the latter cells (see Movie 3 in supplementary material).

Discussion

We have here shown that the trans-interaction of CD47 with SHPS-1 initiates the transfer of CD47 from CD47-expressing cells to neighboring SHPS-1-expressing cells and the subsequent internalization of the ligand-receptor complex into the latter cells. Conversely, SHPS-1 was found to undergo trans-endocytosis from SHPS-1-expressing cells to neighboring CD47-expressing cells, suggesting that trans-endocytosis of CD47 and SHPS-1 occurs bidirectionally. The trans-endocytosis of other transmembrane-type ligand-receptor partners, including BOSS-Sevenless, Notch-Delta (or -Serrate), and EphB-ephrinB, has been described previously (Cagan et al., 1992; Klueg and Muskavitch, 1999; Marston et al., 2003; Zimmer et al., 2003). The latter two ligand-receptor partners also undergo trans-endocytosis in a bidirectional manner.

Our extensive characterization of the mechanism by which the complex of CD47 with SHPS-1 is internalized into SHPS-1-expressing cells revealed that this process occurs via a clathrin-mediated pathway and is dependent, at least in part, on dynamin. We also showed that a juxtamembrane region (amino acids 405-424), but neither of the tyrosine phosphorylation sites in the cytoplasmic portion of SHPS-1, is required for efficient internalization of CD47 into neighboring SHPS-1-expressing cells, consistent with the role of a clathrin-dependent pathway in this process. Although tyrosine- or leucine-based motifs have been thought to mediate binding of the AP2 complex during clathrin-dependent endocytosis of receptors (Sorkin, 2004), no such motifs are present in the juxtamembrane portion of SHPS-1 implicated in CD47 trans-endocytosis. By contrast, we found that epsin promotes CD47 trans-endocytosis. Epsin contains a ubiquitin recognition domain and is thought to couple ubiquitinated proteins to clathrin (Sorkin, 2004). Indeed, SHPS-1 was previously shown to be ubiquitinated (Murai-Takebe et al., 2004), and the juxtamembrane portion (amino acids 405-424) contains potential ubiquitination sites. This juxtamembrane region of SHPS-1 may thus contribute to CD47 trans-endocytosis by serving as a site for clathrin linkage via epsin.

We also showed that trans-endocytosis of CD47 was markedly inhibited by interference with the activity of Rac or Cdc42 or with the polymerization of actin. Small GTP-binding proteins of the Rho family are implicated in the regulation of endocytosis of receptors such as those for transferrin (clathrin-dependent) and interleukin 2 (clathrin-independent) (Lamaze et al., 1996; Lamaze et al., 2001). Moreover, Rac was previously shown to regulate trans-endocytosis of the EphB4-ephrinB2 complex (Marston et al., 2003). Reorganization of the actin cytoskeleton is also important for multiple steps in endocytosis, including membrane invagination and subsequent fission as well as movement of endocytotic vesicles (Engqvist-Goldstein and Drubin, 2003). It is therefore feasible that proper regulation of reorganization of the actin cytoskeleton by Rac and Cdc42 is required for efficient endocytosis of the CD47–SHPS-1 complex. We also found that Rab5 regulates the trafficking of vesicles containing the CD47–SHPS-1 complex.

Our present study also implicates CD47 trans-endocytosis in regulation of the function of the CD47–SHPS-1 system. Time-lapse imaging showed that trans-endocytosis of CD47 occurred at contact sites between cells expressing CD47-YFP and those expressing SHPS-1–CFP, and that such contact was disrupted after completion of trans-endocytosis. By contrast, expression of a DN mutant of dynamin prevented trans-endocytosis of CD47 as well as stabilized cell-cell contacts. We also showed that cocultured CHO-CD47 and CHO–SHPS-1 cells initially underwent pronounced aggregation but subsequently dissociated from each other. Such dissociation was accompanied by marked down-regulation of both CD47 and SHPS-1. By contrast, the aggregation of CHO-CD47 cells with CHO–SHPS–1–ΔC404 cells, the latter of which do not mediate trans-endocytosis of CD47, was sustained. Moreover, treatment of CHO–SHPS-1 cells with cytochalasin D, which also prevents trans-endocytosis of CD47, resulted in sustained aggregation of these cells with CHO-Cd42 cells. Together, these results suggest that trans-endocytosis of CD47 is responsible, at least in part, for the efficient removal of the CD47–SHPS-1 complex from the cell surface after the trans-interaction of CD47 with SHPS-1.

We found that trans-endocytosis of CD47 occurred from cultured hippocampal neurons to neighboring HEK293T cells expressing SHPS-1. Moreover, it occurred from cultured hippocampal neurons to neighboring GFAP-positive astrocytes, which express endogenous SHPS-1. Conversely, trans-endocytosis of CD47 from nonneural cells to neurons was rarely observed. The complex of EphB2 and ephrin was also recently shown to undergo trans-endocytosis from neurons to glial cells (Lauterbach and Klein, 2006). The engagement of CD47 expressed on neurons by SHPS-1 markedly promotes formation of filopodia and neurites (Miyashita
et al., 2004; Murata et al., 2006). We have now shown that cultured hippocampal neurons extended multiple filopodium-like protrusions toward HEK293T cells expressing SHPS-1. Trans-endocytosis of CD47 may thus be responsible for the efficient removal of the CD47–SHPS-1 complex from the surface of neurons by glial cells, thereby terminating the positive regulation of filopodium or neurite formation in neurons. Astrocytes have also been shown to phagocytose degenerating axonal boutons (Deller et al., 1997). In addition, the synaptic membrane of dendrites is trans-endocytosed into neighboring astrocytes as double-membrane vesicle-like structures, termed spinules, in the adult rat hippocampus (Spacek and Harris, 2004). Trans-endocytosis of the CD47–SHPS-1 complex from neurons to glial cells might therefore also contribute to the formation of such phagosomes or spinules, and thereby play a role in formation or maintenance of synaptic structure.

It remains unknown how a membrane-bound ligand fixed on the surface of one cell is transferred into a neighboring receptor-expressing cell. Our results indicate that the full-length CD47 molecule is trans-endocytosed into SHPS-1-expressing cells, suggesting that cleavage of the extracellular region of CD47 (ectodomain shedding) is not responsible for the transfer process. We therefore propose the following model for the transfer of CD47 to a neighboring SHPS-1-expressing cell and its subsequent internalization (Fig. 9). The interaction of CD47 with SHPS-1 at a site of cell–cell contact results in invagination of the cell membrane of the SHPS-1-expressing cell to form a coated pit that contains the CD47–SHPS-1 complex still attached to the membrane of the CD47-expressing cell. The coated pit is then pinched off to form a vesicle that contains the CD47–SHPS-1 complex and another vesicle derived from the CD47-expressing cell (as shown at the bottom left of Fig. 9). Alternatively, the coated vesicle containing the CD47–SHPS-1 complex might be derived from the membrane of both types of cell (as shown at the bottom right of Fig. 9). The electron microscopic analysis showed that MVBs in typical late endosomes/lysosomes were CD47-labeled in CHO–SHPS-1 cells but not in CHO-Ras cells, that were in contact with CHO-CD47 cells, as shown in Fig. 2A,B. In addition, staining of cathepsin D, a late endosome/lysosome marker, is frequently localized at the CD47-positive vesicles in CHO–SHPS-1 cells, that are in contact with CHO-CD47 cells (Fig. 2C,D). Thus, these results suggest that CD47-positive vesicles, those found by immunofluorescence microscopy, contain MVBs and trans-endocytosed CD47 that are sorted into the general endocytic pathway from early endosome to lysosome (van der Goot and Gruenberg, 2006). By contrast, it was suggested that interaction of CD47 with SHPS-1 induces degradation of SHPS-1 by the proteasome-mediated pathway (Ogura et al., 2004). Thus, the lysosome and/or proteasome-mediated pathways may regulate the degradation of the CD47–SHPS-1 complex after its trans-endocytosis.

So far, we failed to show the ultrastructure of the CD47-positive vesicles at a very early stage of vesicle formation. However, the electron microscopic images indicated that the CD47-positive inner vesicles appear to be composed of a single membrane, supporting the second model shown at the bottom right of Fig. 9.

Materials and Methods

**Primary antibodies and reagents**

A rat mAb to mouse SHPS-1 (p84) and that to mouse CD47 (miap301) were kindly provided by C. F. Lagenaur (University of Pittsburgh, PA) and P.-A. Oldenborg (Umeå University, Sweden), respectively. Rabbit pAbs to SHPS-1 were from Upstate Biotechnology. A mouse mAb to β-tubulin was obtained from Affinity Bioreagents; mouse mAb to GMAP and to MAP2 were from NeoMarkers; and a mouse mAb to Tau-1 was from Chemicon. Rhodamine-conjugated phallolidin was obtained from Molecular Probes. A mouse mAb (12CA5) to the hemagglutinin (HA) epitope was from Roche, and a mouse mAb (9E10) to the Myc epitope was purified from the culture supernatants of hybridoma cells. A mouse mAb to β-tubulin (TUB 2.1) was from ICN Biomedicals. Cytochalasin D was from Sigma, and Y-27632 was from Calbiochem. Rabbit pAbs to cathepsin D were kindly provided by Y. Uchiyama (Osaka University, Japan).

**Plasmids**

Expression vectors for mouse CD47, Myc-tagged mouse SHPS-1, Myc-tagged mouse CD47, mouse SHPS-1 and GFP were described previously (Sato et al., 2003; Miyashita et al., 2004; Ohnishi et al., 2005). To construct expression vectors for the SHPS-1 mutants, ΔC474, ΔC424 and ΔC399, DNA fragments corresponding to amino acids 1–474, 1–424 and 1–399 of mouse SHPS-1 were amplified by the polymerase chain reaction (PCR) and subcloned into pTracer-CMV. To construct expression vectors for SHPS-1–CFP and CD47–YFP, DNA fragments corresponding to amino acids 1–509 of mouse SHPS-1 and amino acids 1–321 of mouse CD47 were amplified by PCR and subcloned into pECFP-N1 for SHPS-1–CFP or pVenus-N1 for CD47–YFP. The vectors pECFP-N1 and pVenus-N1 (Matsueka et al., 2004) were kindly provided by M. Okada (Osaka University, Japan) with the permission of A. Miyawaki (RIKEN, Saitama, Japan). Plasmids encoding Myc-Rac(T17N) and Myc-NWASP-CRIB were kindly provided by Y. Takai (Osaka University, Japan); those encoding Myc-tagged epsin(1-205) and epsin(204-458) were kindly provided by A. Kikuchi (Hiroshima University, Japan); and those encoding HA-tagged Rab5(S34N) and Rab5(Q79L) were kindly provided by T. Sasaki (Tokushima University, Japan). A plasmid encoding HA-tagged dynamin 1(K44A) was kindly provided by S. L. Schmid (Scripps Research Institute, La Jolla, CA).

**Cell culture and transfection**

CHO-Ras cells were kindly provided by S. Shirahata (Kyushu University, Fukuoka, Japan). CHO-Ras cells stably expressing mouse SHPS-1 (CHO–SHPS-1 cells) or mouse CD47 (CHO–CD47 cells) were kindly provided by N. Honma (Kirin Brewery, Gunma, Japan). CHO-Ras cells stably expressing SHPS-1–CFP or SHPS-1–4F were described previously (Sato et al., 2003). CHO-Ras cells stably expressing SHPS-1–ΔC474, SHPS-1–ΔC424 or SHPS-1–ΔC399 were established by transfection with corresponding expression vectors and selection with Zeocin (500 μg/ml; Invitrogen). HEK293T–TrkB cells, which express the brain-derived neurotrophic factor receptor TrkB, were kindly provided by H. Nawa (Niigata University, Japan). HEK293T cells stably expressing SHPS-1 (HEK–SHPS-1 cells) were established by transfection of HEK293T–TrkB cells with pTracer-CMV/mSHPS-1 and selected as described above. Hippocampal neurons were isolated from newborn mice and cultured as described previously (Miyashita et al., 2004). They were cultured alone for 14–16 days before coculture with transfected HEK293T cells.
Immunofluorescence analysis and quantification of trans-endocytosis

CHO–SHPS-1 and CHO-CD47 cells were detached from culture dishes by exposure to 0.1% EDTA and suspended in culture medium. Equal numbers (5 × 10^5) of CHO-CD47 and CHO–SHPS-1 cells were mixed in 200–400 μl of culture medium, and then plated on a new 35-mm culture dish containing 2 ml of fresh culture medium, cultured for 1 hour, and then fixed with 4% paraformaldehyde. To determine the extent of trans-endocytosis in CHO-CD47 or CHO–SHPS-1, we developed two assays to quantify the numbers of endocytosed vesicles. In assay A, the fixed cells were incubated for 1 hour with buffer G [0.5% goat serum in phosphate-buffered saline (PBS)] in the absence or presence of 0.1% Triton X-100 and were then subjected to immunostaining with rat mAbs to mouse CD47 or to mouse SHPS-1 in the same buffer. The cells were then washed with Fluo-880-conjugated goat anti-rat IgG and then stained with mAbs to CD47 or to SHPS-1 together with Cy3-conjugated goat pAbs to rat IgG (Jackson ImmunoResearch Laboratory). The numbers of surface (Alexa Fluor 488-positive) and internalized (Cy3-positive) CD47 (or SHPS-1)-positive vesicles were counted for 20 CHO–SHPS-1 (or CHO-CD47) cells that formed contacts with CHO–SHPS-1 (or CHO-CD47) cells and were averaged.

To examine the effects of DN mutants of epsin, dynamin or Rac or of NWASP-CRIB on trans-endocytosis, we transfected CHO-Ras cells with expression vectors for each construct (or the corresponding empty vector) and for SHPS-1. Twenty-four hours after transfection, the cells were fixed and stained with antibody to SHPS-1 or CD47 and then incubated with Alexa Fluor 488-conjugated goat pAbs to rat IgG in the same buffer, and fixed again. The cells were then blocked with unlabeled goat pAbs to rat IgG (50 μg/ml; Jackson ImmunoResearch Laboratory), fixed, permeabilized for 1 hour with buffer G containing 0.25% saponin, and stained with mAbs to CD47 or to SHPS-1 together with Cy3-conjugated goat pAbs to rat IgG (Jackson ImmunoResearch Laboratory). The numbers of surface (Alexa Fluor 488-positive) and internalized (Cy3-positive) CD47 (or SHPS-1)-positive vesicles were counted for 20 cells adjacent to CHO–CD47 cells and were averaged.

To determine trans-endocytosis in cocultures of HEK–SHPS-1 cells and hippocampal neurons, we transfected the HEK–SHPS-1 cells with expression vectors for GFP, dynamin 1(K44A), or Rac(17N). Twenty-four hours after transfection, the cells were detached and transferred to culture dishes in which neurons had been cultured for 14-16 days. After coculture for 1 hour, the cells were fixed and stained in the presence of 0.1% Triton X-100 with mAbs to CD47 and to the Myc or HA epitope tags and with amonunomethyl coumarin (AMCA)-conjugated goat pAbs to mouse IgG (Jackson ImmunoResearch Laboratory) to confirm the expression of each mutant or NWASP-CRIB. The numbers of surface and internalized CD47-positive vesicles were counted for 20 cells adjacent to CHO–CD47 cells and were averaged.

To examine trans-endocytosis in cocultures of HEK–SHPS-1 cells and hippocampal neurons, we transfected the HEK–SHPS-1 cells with expression vectors for GFP, dynamin 1(K44A), or Rac(17N). Twenty-four hours after transfection, the cells were detached and transferred to culture dishes in which neurons had been cultured for 14-16 days. After coculture for 1 hour, the cells were fixed and stained in the presence of 0.1% Triton X-100 with mAbs to CD47 and to the Myc or HA epitope tags (to confirm expression of the mutant proteins). The total number of CD47-positive vesicles was determined for each of 20 HEK–SHPS-1 cells that formed contacts with neurons and was averaged.

To examine trans-endocytosis in cocultures of HEK293T cells and hippocampal neurons, we transfected HEK293T cells and hippocampal neurons, we transfected HEK293T cells with an expression vector for CD47-YFP. Twelve hours after transfection, the cells were fixed and stained with antibody to CD47 and incubated with Alexa Fluor 488-conjugated goat pAbs to rat IgG, and then incubated with buffer G containing 0.1% saponin for blocking, and then subjected to immunostaining with rat mAbs to mouse CD47 and to the 1:1 mixture of propylene oxide and 1:1 mixture of 100 CHO–SHPS-1 (or CHO-CD47) cells that formed contacts with CHO–SHPS-1 (or CHO-CD47) cells and were averaged. The total number of CD47-positive vesicles was determined for each of 20 HEK–SHPS-1 cells that formed contacts with neurons and was averaged.

Primary embryos were embedded in 1:1 mixture of propylene oxide and ethanol, and with 100% propylene oxide sequentially for 10 minutes each, and then incubated in a 1:1 mixture of propylene oxide and Quetol-812 (NISshin EM Co Ltd, Tokyo, Japan) overnight. Finally, cells were embedded in 100% Quetol-812 and embedded. Ultrathin sections of the resulting blocks were prepared using a diamond knife on a microtome (Ultratome-E, Reichert- Jung) and placed onto 200-mesh nickel grids. The samples were stained by incubation with 2.5% (wt/vol) uranyl acetate in 47.5% ethanol for 8 minutes, and then incubated in 2.7% (wt/vol) lead citrate for 5 minutes, followed by several washes with water to remove excess stain. The samples were examined on a transmission electron microscope (JEM-1010, JOEL).

Cell aggregation assay

The cell aggregation assay was performed as described previously (Takeichi, 1977) with slight modifications. Equal volumes of CHO–CD47 and CHO–SHPS-1 cell suspensions (1 × 10^5/ml) in Ca^2+ - and Mg^2+-free Hank’s balanced salt solution were mixed together and maintained at 37°C with gentle shaking for the indicated times before fixation with 2% glutaraldehyde. Aggregates of three or more cells are considered equivalent to single cells in this assay.

Time-lapse imaging

CHO-Ras cells were transfected with expression plasmids for SHPS-1–CFP or CD47-YFP in a separate experiment. The expression plasmid for SHPS-1–CFP was cotransfected with a vector for dynamin 1(K44A) or the corresponding empty vector. Twenty-four hours after transfection, two different cells were mixed and plated on a glass-bottom dish. Images were acquired from 30 minutes after plating.

Immunoblot analysis

Immunoblot analysis was performed as described previously (Fujikawa et al., 1996; Miyashita et al., 2004).

RNA interference

Two 19-nucleotide sequences, corresponding to nucleotides 1065-1083 and 4820-4838 of human CHC mRNA (GenBank accession number NM004859), were selected for construction of the siRNA vectors. Two pairs of 64-nucleotide sequences, each of which contains a targeting sequence and its reverse complementary sequence (pair no. 1, 5'-GATCCCCGAATGCAAAATATCTTGAATTGATTCTTCTGCATTTCCGGG-3' and 5'-AGCTTTTCCAAAAAGGAAA-TGAGAAGAATCAATCTCTTGAATTGATTCTTCTGCATTTCCGGG-3'; pair no. 2, 5'-GATCCCCCAAAACTCTGTAGAAATCGAGATTCTTCTGCATTTCCGGG-3' and 5'-AGCTTTTCTCCAAAAACACTGAGATTCTTCTGCATTTCCGGG-3'), were synthesized. After annealing, each pair of oligonucleotides was inserted into the pSUPER vector (Brunmelkamp et al., 2002). The resulting vectors, pSUPER-CHC-1 and pSUPER-CHC-2, were digested with Kpn I and Sal I, and the released DNA fragments containing the H1 promoter and a downstream short hairpin sequence were rendered blunt-ended and subcloned into the blunted SalI site of the mouse SHPS-1 expression vector pCAGGS-mSHPS-1. Each of the resulting vector, siRNAchc-1 and siRNAchc-2, encodes mouse SHPS-1 and an siRNA specific for CHC mRNA.

Statistical analysis

Quantitative data were analyzed using Student’s t-test. A P value of <0.05 was considered statistically significant.

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References


**Supplementary Fig. S1**
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### (CTR) Triton

A: SHPS-1
B: Phalloidin
C: Merge

### (-Triton)

D: SHPS-1 (surface)
E: Phalloidin
F: Merge

### Cells with SHPS-1+ vesicles (%)

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<th>(+Triton)</th>
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* Significant difference

### SHPS-1+ vesicles/cell

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* Significant difference

**I**

- Miap301 (CD47-extracellular)
- SHPS-1

**J**

- 9E10 (Myc) (CD47-intracellular)
- Merge

**K**

- Merge

**L**

- SHPS-1
- CD47-Myc

**M**

- Merge
Supplementary Fig. S2
Kusakari et al.

A

SHPS-1-Myc
Blot:
9E10
36912 (µg protein / lane)
p84

Ratio of signal intensity
0.2 0.4 0.6 0.8 1.2

B

CD47-Myc
Blot:
9E10
miap

Ratio of signal intensity
0.2 0.4 0.6 0.8 1.2

C

CHO-SHPS-1
Blot:
p84

Ratio of signal intensity
0.2 0.4 0.6 0.8 1.2

CHO-CD47
Blot:
mia
Supplementary Fig. S3
Kusakari et al.

Fluorescence intensity (a.u./pixel)

WT ΔC474 ΔC424 ΔC404 ΔC399 4F
Supplementary Fig. S4

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A

SHPS-1

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B

CD47+ vesicles/cell

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</table>

SHPS-1

C

N_{int}/N_{total} or N_{sur}/N_{total} (%)

<table>
<thead>
<tr>
<th></th>
<th>Surface</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SHPS-1
Fluorescence intensity (a.u./pixel)
**Supplementary Fig. S6**

Kusakari et al.

**CHO-SHPS-1 + CHO (control) CHO-CD47**

Blot: p84

Blot: β-Tubulin

SHPS-1

**CHO-CD47 + CHO (control) CHO-SHPS-1**

Blot: miap301

Blot: β-Tubulin

CD47
Supplementary Fig. S7
Kusakari et al.

HEK-SHPS-1

CD47
Dynamin-1(K44A)
Merge

Ratio of fluorescence intensity (contact/free)

Control (GFP)  Dynamin-1 (K44A)

*