Apolipoprotein D (apoD), a member of the lipocalin family, is a 29-kDa secreted glycoprotein that binds and transports small lipophilic molecules. Expressed in several tissues, apoD is up-regulated under different stress stimuli and in a variety of pathologies. Numerous studies have revealed that overexpression of apoD led to neuroprotection in various mouse models of acute stress and neurodegeneration. This multifunctional protein is internalized in several cells types, but the specific internalization mechanism remains unknown. In this study, we demonstrate that the internalization of apoD involves a specific cell surface receptor in 293T cells, identified as the transmembrane glycoprotein basigin (BSG, CD147); more particularly, its low glycosylated form. Our results show that internalized apoD colocalizes with BSG into vesicular compartments. Down-regulation of BSG disrupted the internalization of apoD in cells. In contrast, overexpression of basigin in SH-5YSY cells, which poorly express BSG, restored the uptake of apoD. Cyclophillin A, a known ligand of BSG, competitively reduced apoD internalization, confirming that BSG is a key player in the apoD internalization process. In summary, our results demonstrate that basigin is very likely the apoD receptor and provide additional clues on the mechanisms involved in apoD-mediated functions, including neuroprotection.

Significance: This is the first evidence that a specific receptor is involved in apoD internalization. Basigin, a transmembrane glycoprotein, is implicated in the internalization process of apoD.

Results: The internalization of apoD is mediated by a specific cell surface receptor.

Conclusion: Basigin, a transmembrane glycoprotein, is implicated in the internalization process of apoD.

Background: ApoD appears to be a protein with multiple functions that could influence inflammatory and oxidative pathways to prevent neurotoxicity.

Significance: This is the first evidence that a specific receptor is involved in apoD internalization.

Apolipoprotein D (apoD) was first described in 1963 as a protein associated with lipoproteins in human plasma; more specifically, with HDL (1). Since its discovery, apoD has been characterized as a 29-kDa secreted glycoprotein comprising eight-stranded antiparallel β barrels that form a hydrophobic, conically shaped cavity that is referred to as the pocket of apoD binding to its ligands (2). It is a member of the family of lipocalins responsible for the binding and transport of small lipophilic molecules (3–5). ApoD is known to bind more specifically to arachidonic acid (AA),2 progesterone, and sphingomyelin with high affinity but also to interact directly or indirectly with cholesterol, bilirubin, and estradiol (3, 6–8). In many species, apoD is widely expressed during embryonic, postnatal, and adult life. In humans, it is mainly expressed in the testes, brain, placenta, kidneys, spleen, lungs, ovaries, and pancreas (4, 9–14). However, unlike most apolipoproteins, apoD is poorly expressed in the human liver and intestine. In addition, it is found primarily in the central nervous system in rodents, suggesting an important role for this protein in this tissue (4).

At the cellular level, apoD is internalized in different cell types, including NIH/3T3 cells (15), vascular smooth muscle cells (16, 17), and murine astrocytes (18), by an unknown mechanism and is translocated to the nucleus in response to stress such as serum deprivation, oxidative molecules, and proinflammatory factors (15, 19–21). In addition, its expression is up-regulated during aging or in several pathological conditions such as atherosclerosis, different types of cancer, and neurological diseases, including Alzheimer and multiple sclerosis (22–24). Therefore, apoD may play a protective role in response to stressful stimuli. This hypothesis was confirmed by several in vivo studies. Indeed, neuronal overexpression of apoD in transgenic mice led to an increased resistance to oxidative stress (25) and inflammation (26). In contrast, apoD deletion in mice resulted in decreased resistance and survival in response to oxidative stress in the brain (25). Moreover, it has been reported that apoD could specifically prevent lipid peroxidation through a highly conserved methionine residue (Met-93), converting reactive to non-reactive lipid hydroxides (27, 28). Studies have also suggested that apoD could influence inflammatory pathways or prevent toxicity by interacting with its multiple ligands, such as the regulation of AA signaling and metabolism (26, 29, 30). Therefore, given its multiple partners and expression patterns, apoD has been proposed as a multiligand and multifunctional protein.

Although several studies have highlighted the potential protective role of apoD in neurological diseases, the exact molecular mechanisms involved in this process are still unclear. However, the potential protective role of apoD involves its uptake into cells (15, 18), possibly through a receptor-dependent mechanism. Therefore, we sought to determine how apoD was internalized into cells to better understand the function of...
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apoD under physiological and pathological conditions. We identified basigin as a cell surface receptor important for apoD internalization in 293T cells. Additionally, we demonstrated that its down-regulation impairs exogenous apoD internalization. Moreover, cyclophilin A, a natural ligand of basigin, blocked apoD uptake. Therefore, our findings clearly demonstrate that basigin can be proposed as the apoD receptor.

Experimental Procedures

**Cell Culture**—All cell lines (embryonic kidney cells, HEK293T, and human neuroblastoma cells, SH-SY5Y) were obtained from the ATCC. 293T cells and SH-SY5Y cells were maintained in Dulbecco’s modified Eagle’s medium (Wisent, St-Bruno, QC, Canada) and in RPMI medium (Wisent), respectively, supplemented with 10% inactivated fetal bovine serum, glutamine (2 mM), penicillin G (100 units/ml), and streptomycin (100 µg/ml). The cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere.

**Radiolabeling of HapoD**—Human apoD (HapoD), purified from breast cyst fluid, was iodinated according to the iodine monochloride method as described by Brodeur et al. (31). Briefly, HapoD (400 µg) was incubated with sodium 125 iodide (400 µCi) in 0.5 M glycine (pH 10). Free iodine was removed using gel filtration on Sephadex G-25, followed by dialysis in PBS. [125I]apoD concentration was assessed by protein assay (Bio-Rad). The specific activity ranged from 0.13–0.16 µCi/µg protein.

**HapoD Binding Assay**—293T cells were seeded at 2 × 10⁵ cells/well onto 24-well plates (Sarstedt, Montreal, QC, Canada). After 24 h, the cells were washed twice with 1 ml of PBS and incubated for 2 h at 4 °C with a range of concentrations of [125I]apoD (1–20 µg/ml) in a total volume of 250 µl of buffer (pH 7.4) containing 4% BSA, 25 mM HEPES, and 125 µl of Dulbecco’s modified Eagle’s medium (2×) for total binding. Non-specific binding was measured by addition of a 20-fold excess of unlabeled HapoD. The cells were washed once with PBS, followed by two washes with PBS containing 0.2% BSA. The cells were then solubilized in 750 µl of NaOH (0.1 N) and counted with a Cobra II counter (Canberra-Packard, Ramsey, MN). Protein concentration was assessed as above. Specific binding, defined as the difference between total binding and non-specific binding, was obtained with GraphPad Prism 4 software. Nonlinear saturation binding data were transformed into linear data (ratio of cell bound to free [125I]apoD versus cell-bound [125I]apoD plots), according to the Scatchard method (32). The equilibrium dissociation constant (K_d) and maximum binding capacities (B_max) were calculated using GraphPad Prism software.

**HapoD Biotinylation**—HapoD was biotinylated with N-hydroxysuccinidobiotin (NHS-d-Biotin, Sigma-Aldrich, St. Louis, MO). HapoD (10 mg/ml in 0.1 M sodium carbonate buffer (pH 9.5)) was incubated overnight at 4 °C with 10% NHS-d-Biotin (11 mg/ml). The reaction solution was then dialedyzed with PBS, and the biotinylated HapoD concentration was determined as above. The HapoD biotinylation was confirmed by Western blot analysis using HRP-conjugated streptavidin (GE Healthcare).

**293T Cell Membrane Preparation**—293T cell membranes were prepared according to the modified technique of Kawaguchi et al. (33). Briefly, 293T cells were lysed in PBS containing 8.6% sucrose in the presence of complete protease inhibitors (Roche Molecular Diagnostics) with a Polytron homogenizer (Fisher Scientific, Ottawa, ON, Canada) for 30 s on ice. The homogenate was layered onto 40% sucrose (diluted in PBS) and centrifuged at 25,000 × g for 30 min at 4 °C. The membrane fraction, collected at interphase, was diluted in PBS and then centrifuged at 25,000 × g for 30 min at 4 °C. The pellet (membrane fraction) was resuspended in PBS and sonicated briefly, and then protein concentration was determined.

**Biotinylated HapoD Binding to 293T Membrane Proteins**—

Biotinylated HapoD was used to pull down the protein complex composed of apoD and its putative receptor. 293T cell membranes (250 µg of protein) were incubated at room temperature for 30 min with 20 µg of biotinylated HapoD in a final reaction volume of 700 µl. The negative control (non-specific binding) was performed in the presence of a 10-fold excess of unlabeled HapoD. Because it was important to ensure a strong binding of this complex along the pulldown assay, we used the glutaraldehyde cross-linker, which offers the possibility to cross-link the ε amino group within a distance of 8 Å (34). Therefore, to cross-link apoD to its receptor, the reaction mixture, diluted in 20 mM HEPES buffer (pH 7.5), was treated with 0.11% glutaraldehyde for 5 min at 37 °C. The reaction was stopped by the addition of 0.1 M Tris-HCl (pH 8.0). The mixture was then centrifuged twice at 25,000 × g for 30 min at 4 °C, and the membrane pellet was resuspended in PBS.

**Purification of Biotinylated HapoD-Receptor Complex**—The apoD-membrane protein complex was purified using streptavidin magnetic beads (New England Biolabs). The membrane pellet was solubilized in 2% (v/v) Triton X-100 in PBS for 30 min at 4 °C, followed by removal of the detergent by gel filtration through a Sephadex G-50 column (New England Biolabs). Streptavidin beads (400 µg) were washed in PBS and incubated overnight at 4 °C with the solubilized membrane proteins containing the covalent apoD-receptor complex. The complex was further washed five times in PBS and eluted from the streptavidin beads with 40 µl of 0.1% (w/v) SDS in PBS. The eluate was mixed with SDS loading buffer, migrated on SDS-PAGE (12%), and visualized by silver staining. Finally, the protein bands corresponding to the apoD-membrane protein complex and absent in the negative control were defined as bands of interest. These were excised and analyzed by mass spectrometry (LC-MS/MS) at the Institute for Research in Immunology and Cancer proteomics platforms (Montreal, QC, Canada) for protein identification. To eliminate the false positives, the regions in the negative control corresponding to the bands of interest were also analyzed by mass spectrometry.

**Cell Transfections and apoD Internalization**—293T cells were plated at 5 × 10⁴ cells in 24-well plates (Sarstedt). After 24 h, the cells were transfected with predesigned siRNA (20 nM) used for human basigin (siBSG) (Ambion, Life Technologies) and negative control siRNA (siCtrl) (GFP-22 siRNA, Qiagen, Toronto, ON, Canada). Transfections with siRNA were carried out using Lipofectamine RNAiMAX transfection Reagent (Life Technologies) according to the instructions of the manufac-
turer. To overexpress basigin, SH-5YSY cells (8 × 10⁴) were plated in 24-well plates (Sarstedt). After 24 h, the cells were transfected with the pCMV6 plasmid containing the human basigin coding region (BSG (NM_198589) human cDNA ORF clone, OriGene, Rockville, MD) and the pCMV6 empty plasmid (for negative control) using Lipofectamine 2000™ reagent (Life Technologies) according to the instructions of the manufacturer. After 48 h, the cells were exposed to biotinylated HapoD (biot-HapoD) (250 ng/ml) for 24 h. BSG protein down-regulation/overexpression and biot-HapoD internalization were evaluated by Western blot analysis and immunocytochemistry as described in the following sections. To confirm the specificity of the BSG knockdown effect on apoD internalization, 293T cells were transfected with siBSG or siCtrl exposed to biot-HapoD for 24 h (as described above), incubated with tetramethylrhodamine-conjugated human transferrin (Tf) (Life Technologies) for 24 h, and analyzed by confocal microscopy.

**Protein Extraction and Western Blot Analysis**—Basigin protein expression and apoD internalization levels were evaluated by Western blot analysis. 293T cells (transfected by siBSG or siCtrl) exposed to biot-HapoD for 24 h were washed twice with ice-cold PBS and homogenized in cold lysis buffer (50 mM Tris-HCL (pH 7.3), 150 mM NaCl, 5 mM EDTA, and 0.2% (v/v) Triton X-100) complemented with complete protease inhibitors (Roche Molecular Diagnostics). Homogenates were incubated for 30 min at 4°C and sonicated, and then protein concentration was assessed. Proteins (30 µg/sample) were separated on 12% (w/v) SDS-polyacrylamide gels and transferred to PVDF (Millipore). Membranes were further incubated with HRP-conjugated streptavidin (1:10,000, GE Healthcare), primary human basigin mouse monoclonal antibody (1:10,000, Ancell), and β-Actin mouse monoclonal antibody (1:10,000, Sigma-Aldrich). Thereafter, the membranes were incubated with secondary antibody anti mouse-HRP antibody (1:10,000, GE Healthcare) (for basigin and β-actin antibodies) and visualized by chemiluminescence (ECL, GE Healthcare) using a Fusion FX7 system (Vilber Lourmat).

**Immunocytochemistry**—After incubation with biot-HapoD for 24 h, 293T and SH-SY5Y cells (transfected and non-transfected) were washed twice with ice-cold PBS and fixed for 15 min in 4% paraformaldehyde in PBS containing 2% sucrose, washed with PBS, and processed for immunocytochemistry. The cells were permeabilized and blocked for 1 h at room temperature in PBS containing 0.1% (w/v) Triton X-100 and 10% (w/v) BSA. After blocking, the cells were incubated overnight at 4°C in a humid atmosphere with FITC-conjugated anti-basigin (1:1000, Ancell) and Alexa Fluor 568 streptavidin conjugate (1:1000, Life Technologies). Thereafter, the cells were washed twice for 10 min with PBS and once with PBS containing 100 ng/ml of DAPI (Sigma-Aldrich) for nucleus staining. The cells were mounted on slides with Prolong Gold antifade (Life Technologies).

**Cyclophilin A/PPIA Competition Assay**—Cyclophilin A, a natural ligand of BSG, was used as a competitor to confirm the specific role of BSG in apoD internalization. 293T cells were incubated with HapoD (250 ng/ml) in the presence or absence of an equal amount or a 20-fold excess of recombinant human cyclophilin A (peptidyl-prolyl isomerase (PPIA)) for 4 h. The cells were then washed twice with PBS, fixed in 4% paraformaldehyde, and processed for immunocytochemistry as described previously. apoD internalization was detected using a specific antibody against HapoD (1:100, 2B9 mouse monoclonal antibody (24)) and a goat anti-mouse IgG-Alexa 488 antibody (1:1000, Life Technologies). HapoD and DAPI labeling was visualized by confocal microscopy. 293T cells were also incubated for 4 h with tetramethylrhodamine-conjugated human transferrin (250 ng/ml) by addition or in the absence of a 20-fold excess of PPIA. Transferrin internalization was analyzed by confocal microscopy.

**Results**

**Receptor-mediated Binding and Uptake of HapoD**—apoD is a secreted protein that can be internalized by various cell types (15–18). It has been reported that exogenous apoD can prevent the uptake of AA by 293T cells (30), but no information exists on its internalization. In this study, we demonstrated that, under normal conditions, endogenous expression of apoD was not detected by immunocytochemistry in 293T cells (Fig. 1A). However, when 293T cells were incubated for 24 h with HapoD, we observed apoD internalization, possibly through a receptor-dependent mechanism (35), into vesicular compartments in the perinuclear area (Fig. 1B). 293T cells were incubated for 2 h with increasing concentrations of [125I]apoD binding (total [125I]apoD binding) in the presence or absence of unlabeled apoD in excess (nonspecific [125I]apoD binding) to assess the specific binding of apoD. This binding assay (Fig. 1C) revealed that [125I]apoD binding to 293T cells was concentration-dependent and saturable. Moreover, Scatchard analyses of the saturation curve (Fig. 1D) showed a single binding site on the cell surface, with a Kd of 9.38 ± 2.45 µg/ml and a Bmax of 0.25 ± 0.02 ng/µg cell proteins. Overall, these data indicate that the binding and internalization of apoD in 293T cells is a receptor-dependent mechanism.

**Identification of the apoD Receptor**—To identify the apoD receptor, we designed a strategy to stabilize the interaction between the apoD protein and its receptor to purify the complex with magnetic beads. When purified, the apoD-receptor complex was visualized by SDS-PAGE and silver staining (Fig. 2A). The specific bands (Fig. 2A, I–5) that were present in the extract purified from cells incubated with biotinylated apoD only and absent in the negative control (in the presence of a 10-fold excess of unlabeled apoD) were analyzed by mass spectrometry. apoD was detected in bands 1 and 2 only (Fig. 2B), suggesting that the covalent complex comprising apoD and its receptor is present in these bands at ~60 and 120 kDa. Several proteins that could act as receptors were identified in bands 1 and 2 (Fig. 2B). The first one was the sodium/potassium-translocating ATPase (identified in bands 1 and 2, Fig. 2B), an integral membrane protein (110 kDa) that hydrolyzes ATP and transports sodium and potassium across the cell plasma membrane.
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To maintain ionic gradients (36). The other ones were latrophilin-2 (190 kDa) (37) and leucine-rich repeat neuronal protein 4 (79 kDa) (38). These three proteins exceed the possible size of the apoD receptor present in the covalent complex, with apoD observed either at about 60 kDa in band 1 or at about 120 kDa in band 2 (Fig. 2A). The last candidate, known as BSG, is a cell surface transmembrane glycoprotein that plays an important role in several cellular activities, including growth, differentiation, survival, and adhesion (39). BSG is widely expressed in many cell lines, including in 293T cells, and in multiple glycosylated forms, high (HG-BSG, at about 45–65 kDa) and low (LG-BSG, detected at about 32 kDa) glycoforms (40). Therefore, the protein linked to apoD in the 60-kDa complex (band 1, Fig. 2A) is very likely the low glycosylated form of basigin.

Impact of Basigin Concentrations on apoD Internalization in 293T and SH-SY5Y Cells—To investigate whether basigin is involved in the apoD internalization process, we assessed the impact of the BSG down-regulation (using specific RNAi) on apoD internalization. The BSG protein is mainly localized on the plasma membrane of 293T cells (Fig. 3Aa). However, in 293T cells treated with biotinylated apoD, BSG staining was detected on the plasma membrane as well but also appeared in vesicular compartments (Fig. 3, Ad–Af) along with apoD being internalized (Fig. 3, Ae and Af). These data suggest that exposure of 293T cells to exogenous apoD involves the redistribution of BSG. Interestingly, apoD internalization was strongly reduced in siBSG-treated cells (Fig. 3, Be and Bf) compared with cells transfected with siCtrl (Fig. 3, Bb and Bc). Indeed, apoD internalization was diminished by 60% following a significant 40% decrease of the expression of all glycosylated forms of BSG (HG-BSG and LG-BSG) (Fig. 3, C and D). Because siBSG-treated cells presented a residual apoD uptake, we evaluated the effect of BSG down-regulation on transferrin internalization. BSG down-regulation had no effect on transferrin internalization (Fig. 6). This process is known to be independent of BSG (41), confirming that the decreased internalization of apoD is a direct consequence of the down-regulated expression of BSG. Therefore, the residual apoD uptake in siBSG-treated cells most likely results from the residual BSG expression.

To confirm its role, the effect of BSG on apoD internalization was also evaluated in SH-SY5Y human neuroblastoma cells. These cells display low BSG expression levels compared with HeLa (42) and 293T cells (Fig. 4A). In addition, it has been reported that apoD can promote cell survival and differentiation in SH-SY5Y cells, but only in the presence of neuronal differentiation factors (7, 43). Indeed, we detected low apoD internalization levels in SH-SY5Y cells (Fig. 4, Ba–Bc). Interestingly, the overexpression of BSG in SH-SY5Y cells induced an important increase in intracellular exogenous apoD levels (Fig. 4, Bd–Bf) compared with SH-SY5Y cells transfected with the empty vector (Fig. 4, Bb and Bc). Overall, these results demonstrate that BSG is a key player in the apoD internalization process.

HapoD Internalization Is Blocked by Cyclophilin A—It has been reported previously that BSG binds cyclophilin A (also known as PPIA), a protein secreted in response to inflammatory stress that triggers signaling and chemotactic activities (44, 45). To further verify the specificity of the BSG effect on apoD internalization, we performed a competition assay between apoD and cyclophilin A. 293T cells were incubated for 4 h with purified human apoD in the presence or absence of cyclophilin A (Fig. 5). As described previously, exogenous apoD is internal-
ized into vesicular compartments of 293T cells (Fig. 5, A–C). Treatment with a concentration of cyclophilin A similar to that of exogenous apoD had no effect on apoD internalization (Fig. 5, D–F). However, the addition of cyclophilin A in excess (20×) had no effect on the endogenous expression of apoD (Fig. 5, J–L) but completely blocked exogenous apoD internalization (Fig. 5, G–I). Moreover, as a control, transferrin internalization was not affected by the presence of cyclophilin A in excess (Fig. 6). Overall, these results demonstrate that apoD internalization is blocked by cyclophilin A.

Discussion

ApoD is a secreted glycoprotein that can be internalized by various cell types (15–18). However, the quantity of apoD internalized depends on the cell type and the stressful conditions (15, 18), suggesting the involvement of a specific mechanism responsible for this internalization. In this study, we clearly demonstrate that the internalization of apoD is not a passive diffusion through the plasma membrane but, rather, a specific cell surface receptor-mediated uptake by BSG.

Also named CD147 and extracellular matrix metalloproteinase inducer, BSG is a multifunctional cell surface transmembrane glycoprotein that has two extracellular Ig domains (46, 47). It has been reported that BSG is widely expressed in many tissues, including in the brain (39, 48–50). In the brain, BSG is strongly expressed in the limbic system (including the hippocampus, olfactory system, amygdala, and enthorinal cortex), cortex, and cerebellum (51). A similar tissue distribution has been observed for apoD (12) in concordance with the interactions observed in this study between apoD and BSG. Interestingly, BSG-deficient mice have been shown to suffer from many disorders related to female fertility, spermatogenesis, retinal development, and, more importantly, the nervous system (48, 52–54). Indeed, these mice have learning and memory task def-
icits (54). Most interestingly, apoD knockout mice also have impaired learning, memory, and orientation-based and motor tasks (25). Therefore, BSG and apoD could have similar or related functions. Indeed, the expression of both BSG and apoD is increased in several inflammatory diseases, including atherosclerosis (55–57), ischemia (58, 59), multiple sclerosis, and experimental autoimmune encephalomyelitis (22, 23, 60–62), suggesting that apoD and BSG could very well be involved in common pathways in these pathologies.

ApoD is associated with reduced invasive and proliferative activity of several cancer cell types (21, 63, 64). The role of BSG in cancer progression has also been well demonstrated. BSG is
known to interact with several tumor- and inflammation-induced molecules, including monocarboxylate transporters, cyclophilins, integrins, and caveolin 1 (45, 65–67). In addition, in tumor cells, elevated BSG expression enhances tumor invasion by stimulating the secretion of multiple matrix metalloproteinases, this function being dependent upon glycosylation levels (68–70). Indeed, the tumorigenic properties of BSG are mainly due to the high glycosylated form of BSG, HG-BSG (40, 67, 68, 70). However, the low glycosylated form of BSG, LG-BSG, is presumed to be a precursor for HG-BSG formation in the endoplasmic reticulum (40, 71). Both HG-BSG and LG-BSG are expressed on the plasma membrane of many cell types, and the high HG:LG ratio is highly correlated with the lymphatic metastasis abilities of hepatocarcinoma cell lines (72). Moreover, overglycosylation of BSG is associated with multidrug resistance in human leukemia (68). In addition, BSG promotes MMP-2 and MMP-3 production in breast cancer cells, which display increased tumoral invasion and metastasis capacities (73, 74). This can, however, be abolished by the upregulation of caveolin 1 (67, 75), which can bind to LG-BSG and

![FIGURE 4. ApoD internalization in SH-SY5Y cells. A, BSG expression (green) in human neuroblastoma SH-SY5Y and 293T cells was assessed by confocal microscopy. Note that SH-SY5Y cells display low BSG protein expression compared with 293T cells. B, effect of biot-apoD internalization on SH-SY5Y cells with or without overexpression of BSG. SH-SY5Y cells, transfected with a plasmid expressing human basigin (pBSG, d–f) or with the empty plasmid (vehicle, a–c) for 48 h, were exposed to biot-HapoD for 24 h. BSG protein expression and biot-HapoD internalized were assessed by confocal microscopy with anti-BSG antibodies (green, a and d) and Alexa Fluor 568 streptavidin (red, b and e), respectively. Note that the low BSG expression (Ab) in SH-SY5Y cells resulted in a decrease of biot-HapoD internalization (Ab), which was improved (Be) by overexpression of BSG protein (Bd). Nuclei were labeled with DAPI (blue). Scale bars = 10 μm.](image-url)

![FIGURE 3. Basigin down-regulation affects apoD internalization. A, confocal analysis of 293T cells incubated with (d–f) or without biot-HapoD (a–c). The BSG protein and internalized exogenous apoD were stained with anti-BSG antibodies (green, a and d) and Alexa Fluor 568 streptavidin (red, b and e), respectively. The insets show a high magnification of the colocalization (yellow) between BSG and internalized apoD in vesicular compartments of biot-HapoD-treated cells (f) compared with non-treated cells (c). Note that exposure to apoD induces the redistribution of basigin localization (d) compared with non-treated 293T cells (a). B, confocal analysis of the down-regulation of BSG in 293T cells. 293T cells were transfected with siBSG (Bd–Bf) or siCtrl (negative control, Ba–Bc) for 48 h and thereafter incubated with biot-HapoD for 24 h and analyzed by confocal microscopy. The decreased BSG expression (d) was confirmed by staining with anti-BSG antibodies (green) compared with siCtrl-transfected cells (a). Biot-HapoD internalization was assessed using Alexa Fluor 568 streptavidin (red) in siBSG-transfected (e) compared with siCtrl-transfected (b) cells. Note that Biot-HapoD internalization is decreased in siBSG-transfected cells (Be) compared with siCtrl-transfected (Bb) and non-transfected 293T cells (Aa). Nuclei were labeled with DAPI (blue). Scale bars = 10 μm. C and D, Western blot analysis (C) and quantification (D) of basigin expression and biot-HapoD internalization in extracts of siBSG and siCtrl-transfected cells treated with biot-HapoD for 24 h. β-Actin was used as a loading control. Values were normalized on β-Actin protein expression and on siCtrl values, which were given an arbitrary value of 100. Normalized values are presented as mean ± S.E. (each experiment was performed in triplicate). Two-way analysis of variance following by Bonferroni post-test: ***, p < 0.001 compared with siCtrl-transfected cells.](image-url)
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FIGURE 5. ApoD internalization inhibition by cyclophilin A. 293T cells were incubated for 4 h with HapoD (250 ng/ml) supplemented with 250 ng/ml PPIA (1:1) (D and E) or a 20-fold excess of PPIA (1:20) (G and H). As a control, the cells were incubated with HapoD only (1:0) (B and C) or a 20-fold excess of PPIA only (0:20) (U and K). Internalized HapoD was immunostained with anti-HapoD (green) and analyzed by confocal microscopy. Nuclei were labeled with DAPI (blue). Scale bars = 10 μm.

FIGURE 6. Basigin inhibition does not alter transferrin internalization. A, 293T cells transfected with siBSG for 48 h (as describe above) were incubated with tetramethylrhodamine-conjugated human Tf for 24 h. The Tf staining (red, b and c) was revealed by confocal microscopy. BSG expression was immunostained with anti-BSG (green). B, confocal analysis of Tf internalization (red) in 293T cells incubated for 4 h, with tetramethylrhodamine-conjugated human Tf (250 ng/ml) by addition of a 20-fold excess of PPIA (1:20) (D and F). As a control, the cells were incubated with only Tf (1:0) (A and C). Nuclei were labeled with DAPI (blue). Scale bars = 10 μm. The results show that BSG down-regulation has no effect on transferrin uptake in 293T cells and that cyclophilin A does not compete with transferrin.

block its conversion to HG-BSG, resulting in an increase of LG-BSG on the cell surface and a decrease of the HG:LG ratio (40). Interestingly, our results suggest that apoD needs to form a complex with LG-BSG to be internalized. Therefore, the increase of LG-BSG on the cell surface, promoted by caveolin 1, could induce apoD internalization, thereby reducing the risk of tumorigenesis. Although a parallel induction of apoD and caveolin 1 by oxidative stress has been demonstrated in NIH/3T3 cells (15), the relation between apoD and caveolin 1 has not yet been established. However, the impairment of spatial memory tasks (76, 77) and the inflammation induced by traumatic brain injury (77) in caveolin 1-deficient mice suggest a tight relationship between apoD and caveolin 1.

Overexpression of neuronal human apoD in transgenic mice reduces as well T cell infiltration and production of proinflammatory cytokines following coronavirus-induced neurodegeneration (26). In this study, we demonstrated that an excess of cyclophilin A decreases apoD uptake into the cells. Interestingly, BSG has been identified as an essential signaling receptor for cyclophilins, which are secreted in response to inflammatory stress (44, 45, 78). Cyclophilin-mediated signaling results in chemotaxis of neutrophils, eosinophils, and T cells (45, 79–81). Moreover, it has been shown that cyclophilin A, secreted by prion-infected mouse brains, induces the release of cytokines by microglia and astrocytes in vitro (82). These studies suggest that both BSG and apoD might influence inflammatory pathways, possibly through the regulation of AA signaling and metabolism (26). Indeed, apoD could stabilize or sequester its preferential ligand, AA, into the cell membrane, reducing the availability of free AA and preventing its conversion into proinflammatory molecules (29, 30). Therefore, the binding of apoD to BSG could reduce its interaction with cyclophilin A and, as a result, attenuate the inflammatory process. In addition, our results show that apoD internalization is followed by the endocytosis of BSG, leading to the redistribution of BSG. The apoD endocytotic transport pathway remains unknown. Nevertheless, it has already been reported that BSG is internalized by a clathrin-independent endocytosis and recycling pathway (83, 84). The colocalization of exogenous apoD internalized and basigin suggest that apoD can follow the same endocytosis pathway as basigin for its internalization process.

In summary, this study is the first to identify BSG, more particularly LG-BSG, as a receptor essential for apoD internalization. Binding of apoD to LG-BSG probably involves a specific region of apoD, referred to as the spike, forming in the back of ligand binding pocket (2, 85). However, the exact mechanisms of interaction between apoD and BSG remain to be determined. Nonetheless, the strong relationship between BSG and apoD highlighted in this study provides additional clues on the mechanisms involved in apoD-mediated functions, including its neuroprotective effect.

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