Intracellular Retention of Caveolin 1 in Presenilin-deficient Cells

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Mutations in genes encoding presenilins (PS1 and PS2) are responsible for the majority of early onset familial Alzheimer’s disease. PS1, a critical component of γ-secretase, is responsible for the intramembranous cleavage of amyloid precursor protein and Notch. Other physiological functions have been assigned to PS without any clear identification of the mechanisms underlying these multiple biological roles. The early embryonic lethality of PS1 and PS2 double knock-out (PS1/2 null) mice prevents the evaluation of physiological roles of PS. To investigate new functions for presenilins, we performed a proteomic approach by using cells derived from PS1/2 null blastocysts and wild type controls. We identified a presenilin-dependent cell-surface binding of albumin. Binding of albumin depends on intact caveolae on the cellular surface. Abnormal caveolin 1 localization in PS1/2 null cells was associated with a loss of caveolae and an absence of caveolin 1 expression within lipid rafts. Expressing PS1 or PS2 but not the intracellular form of Notch1 in PS1/2 null cells restored normal caveolin 1 localization, demonstrating that presenilins are required for the subcellular trafficking of caveolin 1 independently from Notch activity. Despite an expression of both caveolin 1 and PS1 within lipid raft-enriched fractions after sucrose density centrifugation in wild type cells, no direct interaction between these two proteins was detected, implying that presenilins affect caveolin 1 trafficking in an indirect manner. We conclude that presenilins are required for caveola formation by controlling transport of intracellular caveolin 1 to the plasma membrane.

γ-Secretase activity is responsible for the cleavage of the transmembrane domain of the amyloid precursor protein (APP),1 releasing the amyloid peptide Aβ and the APP intracellular domain. Aβ is a major component of amyloid plaques characteristic of Alzheimer’s disease (AD). Presenilins (PSs) are the active core of γ-secretase (1, 2). Different molecular components of the γ-secretase complex were identified and include, in addition to PSs, Nicastrin, Aph1, and Pen-2 (3).

The PS-dependent proteolytic process is involved in the release of the cytoplasmic domains of an increasing range of type I integral membrane receptors such as Notch (4–6), ErbB-4 (7), E-cadherin (8), and others. PSs, in addition to their role as γ-secretase catalytic subunits, have an important function in subcellular trafficking of a selected group of proteins such as APP, Trk receptor (9), Nicastrin (10), and Pen-2 (11). PSs mediate additional physiological functions, including roles in calcium homeostasis, neurite outgrowth, apoptosis, and synaptic plasticity (12). Because deleting ps1 and ps2 genes in mice leads to an early embryonic lethality (13), the role of presenilins cannot be fully examined in double knock-out mice.

Therefore, we used cells derived from PS1 and PS2 double null (termed PS1/2 null) blastocysts and their WT controls (14) to perform a differential proteomic approach. We identified a presenilin-dependent defect in albumin binding reflecting an abnormal caveola-dependent pathway. We showed that caveolin 1 was mislocalized in PS1/2 null cells leading to abnormal caveola formation and an absence of caveolin1 in lipid rafts. Overexpression of PSs in PS1/2 null cells was sufficient to restore normal caveolin localization and function. We could not detect any physical interaction between presenilins and caveolin 1. These results imply that presenilins are responsible, in an indirect manner, for caveolin 1 transport to the cell surface to form caveola.

**EXPERIMENTAL PROCEDURES**

Materials—On embryonic day E3.5, blastocysts were flushed from wild type or PS1+/+ or PS2+/+ intercrossed mice (13) into M2 medium and plated on mitotically inhibited feeder cells. Five days later, the inner cell mass was removed, trypsinized, and plated onto new feeder cells. After 12 days, the cells were replated onto gelatin-coated dishes, grown until confluent, and then maintained in embryonic stem cell media (high glucose Dulbecco’s modified Eagle’s medium, 15% fetal bovine serum, 2 mM l-glutamine, 1% penicillin/streptomycin, 1× non-essential amino acids, sodium pyruvate, recombinant leukaemia-inhibitory factor) on uncoated plates. For each cell line, PCR genotyping was performed as described previously (13). These cells were named blastocyst-derived cells. SV40 transformation was used to generate rapidly growing cell lines as described previously (33). These cells were named SV40-transformed blastocyst-derived cells.

NIDC-myc6, PS1, and PS2 cDNA constructs were described previously (14). GFP-Cav1 cDNA construct was a generous gift from the laboratory of Dr. Van Deurs (Sweden).

Two-dimensional Gel Electrophoresis—Total protein extracts from mesenchymal cells were fractionated by two-dimensional gel electrophoresis followed by staining with Coomassie Blue. Proteins were excised from the gel, digested with trypsin, and the resulting peptides were analyzed by mass spectrometry.

**RESULTS**

Differential Proteomic Analysis—To our surprise, we were unable to evidenced any clear identification of the mechanisms underlying these multiple biological roles. The early embryonic lethality of PS1 and PS2 double knock-out (PS1/2 null) mice prevents the evaluation of physiological roles of PS. To investigate new functions for presenilins, we performed a proteomic approach by using cells derived from PS1/2 null blastocysts and wild type controls. We identified a presenilin-dependent cell-surface binding of albumin. Binding of albumin depends on intact caveolae on the cellular surface. Abnormal caveolin 1 localization in PS1/2 null cells was associated with a loss of caveolae and an absence of caveolin 1 expression within lipid rafts. Expressing PS1 or PS2 but not the intracellular form of Notch1 in PS1/2 null cells restored normal caveolin 1 localization, demonstrating that presenilins are required for the subcellular trafficking of caveolin 1 independently from Notch activity. Despite an expression of both caveolin 1 and PS1 within lipid raft-enriched fractions after sucrose density centrifugation in wild type cells, no direct interaction between these two proteins was detected, implying that presenilins affect caveolin 1 trafficking in an indirect manner. We conclude that presenilins are required for caveola formation by controlling transport of intracellular caveolin 1 to the plasma membrane.

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WT, PS1/2 null, and PS1/2 null cells transiently transfected with the presenilin 1 cDNA (blastocyst-derived and SV40-transformed blastocyst-derived cells) were analyzed by two-dimensional electrophoresis by the method of O'Farrell. The ampholine mixture used for the first dimension electrofocusing gel was composed of 60% (w/v) of ampholine, pH 5–7, and 40% of ampholine, pH 3–10. The second dimension was performed on SDS-12% polyacrylamide gels. Gels were then stained by Coomassie Blue and BSA-sequenced with a MALDI-TOF mass spectrometer (Carol Beach, University of Kentucky).

Endocytosis Assays—Blastocyst-derived PS1/2 null cells and WT controls were grown in complete medium on 25-mm glass coverslips. When cells were 75–80% confluent, the medium was replaced by 2 ml of complete medium containing FITC-conjugated albumin (Sigma) at a final concentration of 10μg/ml. Cells were incubated at 37°C for 5 and 30 min, washed in PBS, and fixed in 3.7% formaldehyde for 10 min. The cells were washed for 20 min in PBS and mounted on a slide. Virtually identical experiments were carried out with FITC-conjugated transferrin (10μg/ml, sigma). PS1/2 null cells were grown on 60-mm tissue culture dishes and transiently transfected with the presenilin 1 or presenilin 2 cDNA using Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions. The transfected cells were plated on 25-mm coverslips in 6-well plates after 24 h. FITC-albumin uptake was then examined in transfected cells 36 h after the initial transfection.

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RESULTS

In order to identify new functions for presenilins, we used a differential proteomic approach (Fig. 1). We compared the twodimensional profile of total extracts from WT and PS1/2 null cells. Proteins of interest were those expressed in WT cells and absent in PS1/2 null cells. We performed the differential proteomic approach with SV40-transformed blastocyst-derived cells (Fig. 1A) and blastocyst-derived cells (Fig. 1B). Several proteins corresponded to the chosen criteria, and among them a strongly expressed protein was common to blastocyst-derived cells and their SV40 counterpart. This protein was sequenced with a MALDI-TOF mass spectrometer and identified as bovine serum albumin (BSA). This result shows that there is a
defect in the endocytosis and/or binding of albumin from the fetal bovine serum in the absence of presenilins.

Caveolae-deficient endothelial cells show defects in the uptake and transport of albumin in vivo (15). Caveolin 1 null mouse embryonic fibroblasts show defects in the endocytosis of albumin but not transferrin (16). Caveolin-mediated endocytosis in endothelial cells is stimulated by the binding of albumin to a specific albumin-binding protein localized in caveolae (17). Therefore, the defect of endocytosis and/or binding of albumin observed in PS1/2 null cells (Fig. 1) could be linked to the abnormal caveolae-dependent pathway.

We next examined the ability of WT and PS1/2 null cells to bind/endocytose the fluorescent tracer FITC-albumin. Fig. 2 shows that PS1/2 null cells (left panel) failed to accumulate FITC-albumin even after 30 min of continuous incubation in contrast to WT cells showing cell surface labeling with FITC-albumin within 5 min of incubation. To ensure that the lack of expression from the presenilins did not globally affect ligand binding/endocytosis, we also examined the intracellular uptake of a second fluorescent tracer, FITC-transferrin, known to be endocytosed via clathrin-coated pits. As shown in Fig. 2 (right panel), FITC-transferrin was rapidly endocytosed in both WT and PS1/2 null cells. Thus, PS1/2 null cells show a selective defect in caveolae-mediated ligand binding reminiscent of that observed in caveolin 1-deficient cells (16).

To ensure that the defect in caveolae-dependent endocytosis observed in PS1/2 null cells was specifically because of a lack of PS proteins, we examined whether the transient expression of the presenilin cDNA was sufficient to restore albumin binding in PS1/2 null cells. As shown in Fig. 3A, the two-dimensional gel profile of proteins in PS1/2 null cells re-expressing PS1 was comparable with that of the corresponding WT cells (see Fig. 1). The presence of BSA was detected on Coomassie-stained two-dimensional gels of either SV40-transformed blastocyst-derived PS1/2 null cells (top) or blastocyst-derived PS1/2 null cells (bottom) overexpressing PS1. We confirmed this result by restoring efficient FITC-albumin uptake in blastocyst-derived PS1/2 null cells expressing either PS1 (Fig. 3B, top panel) or PS2 (Fig. 3B, bottom panel). These results show that caveolin functionality is dependent upon either PS1 or PS2 expression.

Because the phenotype observed in PS1/2 null cells (Figs. 1–3) is reminiscent to the one observed in caveolin 1-deficient cells (16), we wondered if caveolin 1 was expressed in PS1/2 null cells. By Western blotting, we were able to detect caveolin 1 expression in PS1/2 null cells (Fig. 3C). We decided to further investigate how caveolin 1 was regulated in PS1/2 null cells in comparison to WT cells. We employed the following three distinct methods to address this issue. We first explored caveolin 1 localization by immunofluorescence. We then observed caveolae formation by EM. Finally, we looked at the association of caveolin 1 with lipid rafts by sucrose density centrifugation.

In order to evaluate caveolin 1 (Cav1) distribution between WT and PS1/2 null cells, we performed indirect immunofluorescence experiments (Fig. 4). In most of WT cells, endogenous caveolin 1 was predominantly localized at the plasma membrane with typical caveola-associated punctated staining (Fig. 4A, I), whereas in PS1/2 null cells, endogenous caveolin 1 showed pronounced punctated perinuclear localization (Fig. 4A, II). Altered localization of caveolin 1 in PS1/2 null cells was also observed after forced expression of GFP-Cav1 (Fig. 4A, IV) and with endogenous caveolin 2. This result shows that there is an intracellular retention of caveolin 1 in PS1/2 null cells. To evaluate if caveolin 1 membrane localization was PS-dependent, we examined if transient expression of presenilin 2 cDNA was sufficient to induce a change in caveolin 1 localization. As shown in Fig. 4B, transient expression of FLAG-presenilin 2 (V) in PS1/2 null cells induced redistribution of endogenous caveolin 1 from its perinuclear localization (VI). Micro-injection of PS1 cDNA in GFP-Cav1 positive PS1/2 null cells (Fig. 4B, VII) allowed GFP-Cav1 to be relocalized as in WT cells (VIII). In contrast, no noticeable changes in GFP-Cav1 localization was observed after the microinjection of the Notch NICD (Fig. 4B, IX) construct in GFP-Cav1-positive PS1/2 null cells (Fig. 4B, X). These results show that caveolin 1 is mislocalized in PS1/2 null cells and that even though most of the PS1/2 null mice phenotypes are attributable to a loss of Notch 1 function (13), presenilins are involved in the subcellular localization of caveolin 1 independently from Notch activity.

Expression of caveolin 1 in cells lacking caveolae causes de novo formation of caveolae, whereas ablation or mislocalization of caveolin 1 expression causes a loss of caveolae (18–20). We wondered if PS1/2 null cells showing mislocalized caveolin 1 were still able to form caveolae. Caveolae are well defined organelles, forming flask-shaped necks at the plasma membrane easily distinguishable by electron microscopy. The presence of caveolae in PS1/2 null cells was investigated by EM. We found a marked reduced number of caveolae at the plasma membrane of presenilin-deficient cells (Fig. 5B) in comparison to WT cells (Fig. 5A). In addition, only 20% of PS1/2 null cells showed caveolae at the plasma membrane compared with 100% of WT cells. These data show that presenilins are involved in caveolae formation.

Caveolae are a subset of specialized liquid-ordered domains, referred to as lipid rafts. Caveolae are isolated by their insolubility in Triton X-100 and their sedimentation in low density fractions over sucrose gradients (21). We performed isolation of CEM of WT and PS1/2 null cell proteins (see "Experimental

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Procedures). It has been shown previously that caveolae-enriched fractions isolated in this manner exclude markers for caveolar proteins such as the Golgi apparatus, mitochondria, and the endoplasmic reticulum (22, 23). Distribution of BIP and γ-adaptin, non-raft proteins localized in the ER and the trans-Golgi, respectively, was also examined to monitor the contamination of CEM with ER and trans-Golgi membranes (Fig. 6). Flotillins have been described as a new family of proteins associated with caveolae (24). As expected (Fig. 6A), flotillin 1 expression was restricted to fractions 4 and 5 (CEM), implying that extraction with Triton X-100 yielded good separation of lipid rafts from BIP and γ-adaptin not detected in CEM fractions. Caveolin 1 expression was not restricted to CEM and was also found in other fractions in WT cells. As shown previously (25–27), PS1 was also concentrated in rafts fractions. Caveolin 1 expression was detected in fraction 5 in WT cells (Fig. 6A) but not in PS1/2 null cells (Fig. 6B). In PS1/2 null cells, caveolin 1 was present only in fraction 7 and in high density fractions (fractions 9–12) with Triton X-100-soluble proteins. This result shows that in the absence of presenilins, caveolin 1 was not detected in the low density fractions normally enriched in caveolae (fractions 4 and 5), further supporting the fact that caveolae do not form. Most surprisingly, flotillin 1 expression was no longer associated with lipid rafts in PS1/2 null cells, implying that not only caveolae but also other lipid rafts may be affected by a deficiency in presenilins expression.

In order to determine the molecular mechanism responsible for caveolin 1 mislocalization in PS1/2 null cells, we tested if presenilin 1 and caveolin 1 were able to interact with one another. Despite a co-expression of caveolin 1 and PS1 in lipid rafts (Fig. 6A), we were not able to detect any interaction between endogenous presenilin 1 and caveolin 1 by immunoprecipitation in WT cells either from total extract or from CEM fractions.

Altogether these results show that PS1 or PS2 expression is required for a correct localization of caveolin 1 in caveolae at the plasma membrane but that the absence of presenilins may affect other protein(s) directly implicated in caveolin 1 sorting.

![Fig. 3. PS expression restores albumin uptake in PS1/2 null cells.](image)

![Fig. 4. Presenilin-dependent localization of caveolin 1.](image)

![Fig. 5. Lack of caveolae in PS1/2 null cells.](image)
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Fig. 6. Raft dissociation of Cav1 and flotillin 1 in PS1/2 null cells. Blastocyst-derived WT (A) and PS1/2 null (B) cells were lysed in 1% Triton and subjected to flotation sucrose density gradient centrifugation. Equal volumes of each precipitated fraction were analyzed by Western blotting with antibodies against BIP, γ-adaptin (ada), Cav1, PS1, and flotillin 1. Caveolin1 and flotillin 1 mark low buoyant density lipid raft fractions. Non-raft proteins resident in the ER (BIP) and the trans-Golgi (ada, γ-adaptin) are recovered in heavier fractions.

DISCUSSION

In this paper we performed a differential proteomic approach using blastocyst-derived WT and PS1/2 null cells in order to identify new roles of presenilins beyond Alzheimer’s disease. By using this approach, we identified a presenilin-dependent caveolin 1 localization leading to an absence of caveolae when presenilins are missing. Caveolin 1 can be now added to the list of proteins with a presenilin-dependent trafficking.

We observed an intracellular retention of caveolin 1 in PS1/2 null cells (Fig. 4). The perinuclear localization of caveolin 1 in PS1/2 null cells partially co-localized with γ-adaptin by immunofluorescence. In these experiments, caveolin 1 was more broadly distributed than γ-adaptin. The precise localization of caveolin 1 in the absence of presenilins needs further investigation.

The mechanism by which presenilins, such as chaperone proteins, will allow the correct caveolin 1 localization and caveolae formation does not involve a complex between caveolin 1 and presenilin and is independent from Notch 1 signaling (Fig. 4). Flotillin 1 always targets to the lipid raft/caveolae fractions independent of the presence of caveolin 1. Thus, the absence of flotillin 1 from lipid raft fractions in PS1/2 null cells is not because of caveolin 1 mislocalization, implying that the mechanism by which presenilins transport raft proteins to lipid rafts is more global and may directly affect a common protein involved in the targeting of raft-associated proteins to lipid rafts. Lipid rafts are multifunctional organelles playing important roles in a variety of cellular processes, and in this work, we pointed to an important new role for presenilins.

Neurons do not form caveolae but form lipid rafts with caveola-like properties. APP was found in membrane fractions with caveola-like properties, suggesting that caveola-like structures might be important in the manifestation of AD. Moreover, targeting β-secretase to lipid rafts was shown to up-regulate β-site processing of APP, and presenilins were found highly enriched in caveolin-3-positive caveolae in astrocytes surrounding senile plaques. Recently, γ-secretase complex was shown to be localized in lipid raft microdomains of post-Golgi and endosomes. We can thus postulate that in neurons, presenilins may be implicated in the trafficking of proteins such as flotillin 1, which is responsible for lipid raft formation. These lipid rafts would form the best environment for γ-secretase activity. A better understanding of how γ-secretase complex is formed and transported to the cell membrane may lead to the development of new pharmacological strategies to specifically target the cleavage of APP and delay the progress of AD.

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