Diabetes-associated SorCS1 regulates Alzheimer's amyloid-β metabolism: Evidence for involvement of SorL1 and the retromer complex (Journal of Neuroscience (2010) (13110-13115))

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SorCS1 and SorL1/SorLA/LR11 belong to the sortilin family of vacuolar protein sorting-10 (Vps10) domain-containing proteins. Both are genetically associated with Alzheimer’s disease (AD), and SORL1 expression is decreased in the brains of patients suffering from AD. SORCS1 is also genetically associated with types 1 and 2 diabetes mellitus (T1DM, T2DM). We have undertaken a study of the possible role(s) for SorCS1 in metabolism of the Alzheimer’s amyloid-β peptide (Aβ) and the Aβ precursor protein (APP), to test the hypothesis that Sorcs1 deficiency might be a common genetic risk factor underlying the predisposition to AD that is associated with T2DM. Overexpression of SorCS1c-β-myc in cultured cells caused a reduction ($p = 0.002$) in Aβ generation. Conversely, endogenous murine Aβ$_{40}$ and Aβ$_{42}$ levels were increased (Aβ$_{40}$, $p = 0.044$; Aβ$_{42}$, $p = 0.007$) in the brains of female Sorcs1 hypomorphic mice, possibly paralleling the sexual dimorphism that is characteristic of the genetic associations of SORCS1 with AD and DM. Since SorL1 directly interacts with Vps35 to modulate APP metabolism, we investigated the possibility that SorCS1c-β-myc interacts with APP, SorL1, and/or Vps35. We readily recovered SorCS1:APP, SorCS1:SorL1, and SorCS1:Vps35 complexes from nontransgenic mouse brain. Notably, total Vps35 protein levels were decreased by 49% ($p = 0.009$) and total SorL1 protein levels were decreased by 29% ($p = 0.003$) in the brains of female Sorcs1 hypomorphic mice. From these data, we propose that dysfunction of SorCS1 may contribute to both the APP/Aβ disturbance underlying AD and the insulin/glucose disturbance underlying DM.

Introduction

Rare, early-onset familial Alzheimer’s disease (EOFAD) is believed to begin with the accumulation of oligomeric forms of the 42 amino acid amyloid β peptide (Aβ$_{42}$) in the hippocampus and cerebral cortex (for review, see Lublin and Gandy, 2010). EOFAD is often caused by mutations in genes that directly influence Aβ metabolism, most commonly the amyloid β precursor protein (APP), presenilin 1 (PS1), or presenilin 2 (PS2) (for review, see Gandy, 2005). Genetic studies of late-onset Alzheimer’s disease (LOAD) point to a number of risk factor genes, including several that belong to one of three classes of molecules: (1) the apolipoprotein family, the most notable, apolipoprotein E (APOE) (Corder et al., 1993; Saunders et al., 1993); (2) the low-density lipoprotein receptor (LDLR) family (Kang et al., 1997; Lendon et al., 1997); and (3) the vacuolar protein sorting-10 (VPS10) domain-containing receptor family. Of note, SORL1 belongs to both the LDLR family and the VPS10-domain protein family and is genetically associated with AD (Rogaeva et al., 2007; Liang et al., 2009). A deficiency in SorL1 protein has been observed in the brains of patients suffering from LOAD and is believed to underlie the mechanism of the linkage of SORL1 with AD (Scherzer et al., 2004; Dodson et al., 2006; Sager et al., 2007). APP and SorL1 are frequently colocalized to the same subcellular compartments, and SorL1 has been demonstrated to modulate Aβ generation (Andersen et al., 2005; Offe et al., 2006; Nielsen et al., 2007; Schmidt et al., 2007) via an interaction with the core component of the retromer complex, Vps35 (Andersen et al., 2010), as proposed by Small and Gandy (2006). Human studies have shown that Vps35 and other components of the retromer complex are deficient in the brains of AD patients (Small et al., 2005), and animal
Figure 1. Overexpression of SorCS1cβ-myc decreases Aβ generation. A, Western blot analysis of APP metabolites in HEK293t cells transiently transfected with APP695 and SorCS1cβ or APP695 and empty vector control. Lysates and conditioned media were probed for holoAPP, APP695 or APPCTFs, and Aβ using pAb369 and 6E10, respectively. B, Protein levels were normalized to actin and expressed as percentage of empty vector control. APPCTFs and Aβ were additionally normalized to holoAPP levels to account for transfection variations. Data were collected in duplicate or triplicate from three independent experiments. Significant reductions (**p < 0.01) in cellular α/β CTF (p < 0.001) and secreted Aβ (p = 0.002) were observed upon overexpression of SorCS1cβ-myc, as compared to empty vector control.

Figure 2. Sorcs1 hypomorphic mouse brains accumulate APP metabolites, including Aβ42. A, Sorcs1 gene expression was quantified using real-time PCR. Sorcs1 mRNA was normalized to Actb mRNA. Significant reductions in Sorcs1 mRNA were observed in the brain in Sorcs1 hypomorphic mice (Sorcs1+/− n = 3) compared to wild-type mice (Sorcs1+/- n = 3). B, Western blot analysis of APP metabolites in Sorcs1+/− (n = 6) and Sorcs1−/- (n = 6) hemibrains. Membrane proteins were fractionated by differential solubilization and analyzed by SDS-PAGE and Western blotting. Endogenous holo-APP and APP α/β CTFs were visualized with pAb369. Protein levels were normalized to actin and presented as expression percentage of control. Sorcs1−/- mice exhibited significant increases (⁎p < 0.05, **p < 0.01) in α/β CTF compared to wild-type littermates (top band p = 0.026, bottom band p = 0.009). D, E, Aβ40 and Aβ42 levels were normalized to brain weight and presented as pmoles per gram. No difference was observed in total Aβ40 or Aβ42 levels when male and female mice were grouped for comparison of Sorcs1−/- mice (n = 6) to Sorcs1+/− mice (n = 6) (D); however, when Aβ40 and Aβ42 were compared in female Sorcs1+/− (n = 3) versus Sorcs1−/- (n = 3), increases in Aβ42 (p = 0.044) and Aβ42 (p = 0.007) were observed (E).

Materials and Methods

Antibodies. α-Myc (Cell Signaling Technology), α-GFP (Roche), α-Vps35 (Abcam), α-SorL1 (BD Biosciences), and anti-mouse, anti-rabbit, and anti-goat HRP conjugates (Vector Laboratories) were purchased. pAb369 (C-terminal APP antibody) was used to detect human and mouse holoAPP and C-terminal fragments (Buxbaum et al., 1990). Anti-Sorcs1/3 (this study) recognizes endogenous Sorcs1 but, under certain circumstances, also reacts with Sorcs3.

Cell culture studies. HEK293t cells were cultured at 37°C/5% CO2 in growth medium (DMEM, 10% FBS, 1% penicillin/streptomycin, 1% l-glutamine, Invitrogen). 293t cells were transfected with human APP695, GFF, and pcDNA4 (empty vector) or human APP695, GFF, and murine Sorcs1cβ-myc cDNA (Nielsen et al., 2008), using Lipofectamine 293 (Invitrogen) at a ratio of 1:4 cDNA:Lipofectamine, according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were collected in ice-cold PBS and centrifuged at 5500 × g at 4°C for 15 min, and the media were collected and snap frozen. Cells were subsequently harvested in RIPA buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% DT, 1% NP40, 0.2% SDS, 0.1% NaN3, 50 mM NaF, 10 mM Na2PO4 plus Roche complete EDTA-free protease inhibitor tablet) using 5 cycles of 20 s vortex/5 min ice incubation. Cell debris was removed by centrifugation at 4°C at 10,000 × g for 15 min. Protein concentrations from cell lysates and media were determined using the Bio-Rad Protein Determination Kit. Absorbance was read at 595 nm using a Bio-Rad Microplate Reader (680XR) and analyzed using Microplate Manager v5.2.1. Samples were subsequently prepared in 5× Laemmli buffer and boiled at 95°C for 5 min. Equal amounts of total protein were loaded onto 12% Bis-Tris SDS-PAGE gels for electrophoresis and transferred to PVDF membranes. The membrane was analyzed by Western blot using pAb369 (APP C-terminal) to detect APP holoprotein.
pregnant female mice. Chimeric offspring were bred to B6 mice to test for germline transmission of the targeting construct. Homozygous floxed (1) 2 beads (Santa Cruz Biotechnology) with either (1) 2 µg of the appropriate primary (cells); (2) 1:10 dilution of α-SorCS1/3 (brain); or (3) IgG control antibody according to the manufacturer’s instructions.

Indirect immunohistochemistry and microscopy. HEK293t cells were cultured in eight-well chamber slides for 24 h before transfection with low levels of APP and SorCS1β-myc cDNA. Forty-eight hours after transfection, cellular localization of proteins was detected by sequential scanning confocal immunofluorescence microscopy using Alexa 488- or Texas Red-conjugated secondary antibodies and the Leica TCS DMI 63× oil objective lens. Images were processed using ImageJ. Images shown are representative fields.

Construction of Sorcs1 hypomorphic mouse. Mouse genomic DNA for the Sorcs1 vector was cloned from C57BL/6j (B6) BAC DNA. The vector was generated by insertion of fragments with 5′ homology and 3′ homology on either side of genomic Sorcs1 DNA. LoxP sites and an SORCS1 promoter, all of exon 1 (containing the translational start site), and a portion of intron 1.

The targeting vector was linearized and electroporated into 129/SV embryonic stem cells. Following neomycin selection, the targeted cells were microinjected into B6 blastocysts and implanted into B6 pseudopregnant female mice. Chimeric offspring were bred to B6 mice to test for germline transmission of the targeting construct. Homozygous floxed 129/SV mice were bred with B6 Elisa-Cre mice (The Jackson Laboratory) to generate Sorcs1 hypomorphic mice.

Verification of Sorcs1 mRNAs levels in Sorcs1 hypomorphic mice. RNA was harvested and purified using RNeasy spin columns (Qiagen). cDNA was synthesized from 1 µg of total RNA using the SuperScript III first-strand cDNA synthesis kit and oligo-dT and random hexamer primers (Invitrogen). Sorcs1 gene expression was quantified using the Realplex real-time PCR system (Eppendorf) and TaqMan Universal PCR Master Mix (Applied Biosystems). A 50-fold reduction in Sorcs1 mRNA expression compared to wild-type brain was observed (TaqMan probe Mm00491259) and is therefore referred to as a Sorcs1 hypomorphic mouse rather than a knockout mouse. The housekeeping gene Actb was used as control (TaqMan probe Mm0060793).

Preparation of brains for analysis. Mice were killed with CO2 and brains in 1000 µL RIPA buffer using Fisher Scientific Proteinase Inhibitor Cocktail (Roche Applied Sciences) and homogenized in RIPA buffer using Fisher Scientific Power Gen 1000 homogenizer for 2 cycles of 10 s on ice. A 300 µg aliquot of cell lysate or a 1000 µg aliquot of brain protein was used for immunoprecipitation using A/G plus agarose beads (Santa Cruz Biotechnology) with either (1) 2 µg of the appropriate primary (cells); (2) 1:10 dilution of α-SorCS1/3 (brain); or (3) IgG control antibody according to the manufacturer’s instructions.

Statistical analysis. Densitometric analysis of Western blot bands (integrated density) was performed using Multi Gauge v3.1 software. Statistical analysis of Western blot bands (integrated density) was performed using Multi Gauge v3.1 software. Levels of holoAPP and α/BCTF were normalized to actin and expressed as percentage of control. Total Aβ levels were analyzed by Western blot, and bands were normalized to percentage of control (empty vector). Absolute Aβ40 and Aβ42 concentrations were quantitatively determined by sandwich ELISA (Wako), and Aβ40/Aβ42 ratios were calculated. Certain intergenotype and intragenotype comparisons were not biologically relevant, precluding the use of 2 × 2 matrices for statistical design (i.e., the comparison of Sorcs1−/− males vs Sorcs1−/− females). In all instances, Shapiro–Wilks test of normality and Levene’s test for homogeneity of variance were used for inclusion in parametric tests (p > 0.05 for Levene’s and Shapiro–Wilks tests). Independent-samples t tests (parametric design) or Mann–Whitney U tests (nonparametric design) were used to determine significant mean differences between groups. Significance for t tests are reported with a p < 0.05 using two-tailed tests with an α level of 0.05. All statistical analysis was performed using SPSS v18.0.

Results

Overexpression of Sorcs1β decreases Aβ generation

To determine whether Sorcs1β modulates APP metabolism and Aβ formation, we analyzed levels of exogenously expressed mouse Aβ (40 and 42) ELISA kit (WAKO), according to the manufacturer’s instructions. Absorbance was read at 450 nm using a Bio-Rad microplate reader. Results were normalized to wet brain weight and expressed as picograms per gram.
human APP and its metabolites in HEK293t cells upon co-overexpression of Sorcs1c-myc. Upon co-overexpression of APP and Sorcs1c-myc, levels of cellular α/β-C-terminal fragment (α/βCTF) were decreased by 54% (top band: \( t_{16} = 7.336, p = 0.001 \)) and 51% (bottom band: \( t_{16} = 7.214, p = 0.001 \)) when compared to empty vector control (pCDNA4) (Fig. 1A,B). Total secreted Aβ was decreased by 35% (\( t_{16} = 3.687, p = 0.002 \)) following correction for holoAPP levels.

Sorcs1 hypomorphic mouse brains accumulate APP metabolites, including Aβ_{42}

To determine whether Sorcs1 has a direct effect on Aβ generation in vivo, we next analyzed the levels of endogenous murine APP and its metabolites in the brains of Sorcs1 hypomorphic mice. These mice exhibited a 50% decrease in Sorcs1 mRNA transcripts in the brain (Fig. 2A). The cross-reaction of our anti-Sorcs1 antibody (anti-Sorcs1/3) with Sorcs3, however, precludes a more precise statement regarding the level of Sorcs1 protein. When compared to wild-type littermates, the levels of α/βCTF in the brain of Sorcs1 hypomorphs were increased by 20% (top band: \( t_{10} = -2.612, p = 0.026 \)) and 30% (bottom band: \( t_{10} = -3.201, p = 0.009 \)) (Fig. 2B,C). Analysis of total Aβ_{40} and Aβ_{42} levels by sandwich ELISA revealed that the levels of Aβ_{40} (\( t_{4} = -2.912, p = 0.044 \)) and Aβ_{42} (\( t_{4} = -5.113, p = 0.007 \)) were increased in the brains of female Sorcs1 hypomorphs in comparison to wild-type females (Fig. 2E). No differences in Aβ_{40} or Aβ_{42} levels were observed when Sorcs1 hypomorphs were compared to wild type (male and female grouped) (Fig. 2D).

**Sorcs1 associates with and colocalizes with APP**

SorL1 and Sorcs1 are both members of the VPS10-domain-containing family of proteins. SorL1 directly interacts with APP and modulates APP processing (Andersen et al., 2006; Spoelgen et al., 2007), raising the question as to whether a similar interaction might occur involving APP and Sorcs1. In immunoprecipitation/immunoblotting experiments using transfected 293t cell lines, we observed co-recovery of holo-Sorcs1c-myc together with holoAPP and the α/βCTFs (Fig. 3A,B). To further validate this interaction, we sought to determine whether APP coimmunoprecipitated with Sorcs1/3 in nontransgenic C57BL/6j brain tissue. Again, immunoprecipitation of endogenous Sorcs1/3 resulted in coimmunoprecipitation of holoAPP (Fig. 3C). Furthermore, indirect immunofluorescence microscopy for Sorcs1c-myc and APP revealed substantial physical codistribution throughout the cell, most especially in the perinuclear region (Fig. 3D). Together, these data suggest the existence of a physiological complex that includes APP and Sorcs1/3.

**Brains from female Sorcs1 hypomorphs contain decreased levels of Vps35**

Andersen et al. (2010) recently reported that the retromer component Vps35 is required for SorL1 to modulate APP metabolism (Andersen et al., 2005; Offe et al., 2006; Nielsen et al., 2007; Schmidt et al., 2007). We therefore tested the possibility that the Vps35 and/or SorL1 forms complexes with Sorcs1/3 and measured SorL1 and Vps35 levels in Sorcs1 hypomorphs. We were able to demonstrate coimmunoprecipitation of SorL1 and Vps35 with Sorcs1/3 from brain tissue (Fig. 4A). Differential solubilization of brain tissue in Triton X-100 and SDS was subsequently used to study the cellular localization of these proteins in Sorcs1 hypomorphs. While we found that both total SorL1 (\( t_{4} = 2.020, p = 0.003, 29\% \) decrease) and Vps35 (\( t_{4} = 4.708, p = 0.009 \)) protein levels were decreased in female Sorcs1 hypomorphs, differential solubilization revealed that only Vps35 protein levels in the SDS fraction (\( t_{4} = 6.400, p = 0.003, 71\% \) decrease) were reduced (Fig. 4C), indicating drastically reduced levels of Vps35 in subcellular fractions/compartments insoluble in Triton X-100 but soluble in SDS (Ali et al., 1989; Messier et al., 1993).

**Discussion**

Sorcs1 is the most recent member of the Vps10 family of proteins (Hermey et al., 2003) to be associated with AD (Liang et al., 2009). This has prompted us to hypothesize that Sorcs1 might play some of the same roles already established for SorL1 in the...
modulation of APP metabolism. To investigate that possibility, we performed cell-based assays that clearly demonstrated that APP α/βCTF (p < 0.001, ~50% decrease) and total Aβ (p = 0.002, 35% decrease) were decreased upon overexpression of Sorcs1ββ. To validate this observation in vivo, we turned to the Sorcs1 hypomorphic mouse, where, in the brains of Sorcs1 hypomorphs, we observed a 25–30% increase in APP α/βCTF (p = 0.026, p = 0.009 respectively), a 14% increase in Aβ40 (p = 0.004), and a 24% increase in Aβ42 (p = 0.007) in the brains of female, but not male, Sorcs1 hypomorphic mice. These changes in APP metabolism are highly reminiscent of those observed in Sorl1 knockout mice (Andersen et al., 2005; Dodson et al., 2008), except that sexual dimorphism in Aβ levels has not been reported for Sorl1 knockout mice. The sexual dimorphism is especially interesting in light of the observation that the genetic linkage to Sorcs1 is stronger for women in both T2DM (Goodarzi et al., 2007) and AD (Liang et al., 2009) populations. Similar observations were recently reported in abstract form by Reitz et al. (2010), although those investigators used siRNA in cultured cells as their Sorcs1 knockdown model and so were unable to assess the possibility of sexual dimorphism.

We next assessed the possibility that Sorcs1, like Sorl1, directly influences APP metabolism and Aβ generation through molecules known to modulate APP metabolism. Specifically, as suggested by Small and Gandy (2006), Sorl1 and other Vps10-containing proteins might modulate APP processing by mediating the interaction between the retromer complex and APP. We were able to detect APP, APP α/βCTFs, Sorl1, and Vps35 in the anti-Sorcs1 immunoprecipitates from both APP/Sorcs1ββ-myc-doubly transfected cells and nontransgenic mouse brain. Interestingly, both Sorl1 and Vps35 total protein levels were also decreased in the brains of Sorcs1 hypomorphs. Further study will be required to determine whether the protein–protein complexes and/or decreased expression of Sorl1 and Vps35 play roles in the elevation of brain Aβ42 that we have observed in female Sorcs1 hypomorphs. Based on these immunoprecipitation data, Sorcs1 is well positioned to modulate one or more steps in APP metabolism. While the focus of Sorl1-related and, in this study, Sorcs1-related effects on APP has been on protein trafficking, it is important to remember that many Vps10-domain proteins are γ-secretase substrates, and competition for access to the catalytic site of γ-secretase may also contribute to the mechanism (Nyborg et al., 2006). Further studies are required (1) to confirm the pathogenic importance of the Aβ42 changes in vivo by crossing Sorcs1 hypomorphic mice with human APP-overexpressing mice capable of forming Aβ oligomers and plaques; and (2) to elucidate the detailed mechanism through which Sorcs1 regulates APP/Aβ metabolism.

We propose that probing the molecular consequences of Sorcs1 dysfunction will lead to pathways that elucidate the link between DM and AD.

References


