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Authors

Zhou, Shuxia
Zhou, Hua
Walian, Peter J.
et al.

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Title: CD147 is a regulatory subunit of the γ-secretase complex in Alzheimer's disease amyloid β-peptide production

Author: Shuxia Zhou, Hua Zhou, Peter J. Walian, and Bing K. Jap*

Author affiliation: Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720

Corresponding author: Bing K. Jap; Lawrence Berkeley National Laboratory, Mail Stop: Donner, 1 Cyclotron Road, Berkeley, CA 94720; telephone: 510-486-7104; Fax: 510-486-6488; e-mail: bkjap@lbl.gov

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid β-peptide; Psn-1, presenilin-1; NTF, N-terminal fragment; CTF, C-terminal fragment; Nct, nicastrin.

Abstract γ-Secretase is a membrane protein complex that cleaves the β-amyloid precursor protein (APP) within the transmembrane region, following prior processing by β-secretase, producing amyloid β-peptides ($A\beta_{40}$ and $A\beta_{42}$). Errant production of Aβ-peptides that substantially increases $A\beta_{42}$ production has been associated with the formation of amyloid plaques in Alzheimer's disease patients. Biophysical and genetic studies indicate that presenilin-1 (Psn-1), which contains the proteolytic active site, and three other membrane proteins, nicastrin (Nct), APH-1, and PEN-2 are required to form the core of the active γ-secretase complex. Here, we report the purification of the native γ-secretase complexes from HeLa cell membranes and the identification of an additional γ-secretase complex subunit, CD147, a transmembrane glycoprotein with two immunoglobulin-like domains. The presence of this subunit as an integral part of the complex itself was confirmed through co-immunoprecipitation studies of the purified protein from HeLa cells and solubilized complexes from other cell lines such as neural cell HCN-1A and HEK293. Depletion of CD147 by RNA interference was found to increase the production of Aβ peptides without changing the expression level of the other γ-secretase components or APP substrates while CD147 overexpression had no statistically significant effect on amyloid β-peptide production, other γ-secretase components or APP substrates, indicating that the presence of the CD147 subunit within the γ-secretase complex directly down-modulates the production of Aβ-peptides.

γ-secretase was first recognized through its role in the production of the Aβ peptides that are pathogenic in Alzheimer's disease (AD) (1). γ-Secretase is a membrane protein complex with unusual aspartyl protease activity that cleaves a variety of type I membrane proteins, such as APP, CD44, DCC, ErbB4, E-cadherin, LRP, N-cadherin, Nectin-1, and Notch, within their transmembranous regions (2-11); therefore, in addition to its role in AD, γ-secretase has been found to participate in other important biological functions, such as intracellular signaling. γ-Secretase processing of APP requires prior removal of a major fragment of the APP extracellular domain (sAPP$_\beta$) by β-secretase to yield a membrane bound fragment (APP CTF$_\beta$). Subsequent cleavage of this membrane bound fragment by γ-secretase results in the release of the Alzheimer's disease (AD) associated amyloid β-peptides (12). The proteolytic activity of γ-secretase is found not to be critically dependent on the specific sequence, but instead on the size of the extracellular domain (13); such sequence independent characteristics of the substrate are reminiscent of those of the 26S proteasome complex that cleaves substrates in a non-sequence specific manner. γ-secretase is present in almost all animal species, vertebrates and invertebrates; it is expressed in many human organs and tissues.

Activation of γ-secretase is highly regulated. Upon formation of the core complex (Psn-1, Nct, APH-1 and PEN-2), the proteolytic component, Psn-1, is autocatalytically processed to form an active core complex. This processing entails cleavage of Psn-1 to yield an N-terminal fragment (Psn-1 NTF) and a C-terminal fragment (Psn-1 CTF), which remain stably within the complex. Psn-1 contains eight putative transmembrane (TM) domains,

with the N-terminal and C-terminal ends as well as the region of autocatalytic processing on the hydrophilic loop between TM 6 and TM7 located at the cytoplasmic face of the plasma membrane, according to a widely acknowledged topological model (14-15), though alternative models have been proposed (16). Nicastrin is a type I transmembrane glycoprotein with a large N-terminal extracellular domain and a short (20 residues) intracellular domain. The first evidence that Nct is an essential component of the γ-secretase complex came from RNA interference experiments in *C. elegans*, where deletion of the Nct gene produced an embryonic lethal phenotype highly reminiscent of those produced by reduction of the activity of genes involved in the Notch signaling pathway or by reduction of presenilin expression (11). The genetic screen in *C. elegans* designed to search for mutations that cause Notch signaling defects led to the identification of APH-1 (anterior pharynx defective) and PEN-2 (presenilin enhancer) membrane proteins that play critical roles in γ-secretase activity (17). APH-1 and PEN-2 were subsequently proven to be integral components of γ-secretase complex through co-immunoprecipitation and RNA interference experiments, which indicated that they are physically and functionally associated with γ-secretase (18,19). There is overwhelming evidence, both *in vivo* and *in vitro*, that the expression of four components, Psn-1, Nct, APH-1 and PEN-2, are sufficient to produce a complex with γ-secretase activity (20-23).

Although the complexes made up of the four components have been found to be enzymatically active, it is not yet clear whether such complexes constitute the native form of the γ-secretase complex. Interestingly, experiments in which these four subunits were over-expressed in yeast (21), Drosophila cells (20), mammalian cells (22) and budded

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virus particles from Sf9 cells (24) produced differing levels of γ-secretase activity with the budding virus particles having by far the highest level of activity. It has been suggested that such different levels of activity might be due to the presence of unknown co-factors or integral components that modulate the activities of the γ-secretase complex *in vivo* (24,25). In the present study, we identified a new regulatory subunit of γ-secretase, CD147, through the purification of native γ-secretase complex from detergent-solubilized HeLa cell membranes. We confirmed that CD147 is an integral component of γ-secretase by co-immunoprecipitation experiments conducted on both the purified complex and cell membranes from various cell lines. Through RNA interference and over-expression investigations in CHO-APP$_{695}$ cells, we demonstrated that the presence of CD147 in the γ-secretase complex directly down-modulates the production of Aβ-peptides.

Materials and Methods

Purification of γ-secretase complex and identification of CD147 subunit. Cells from 50-liters of suspension HeLa cell culture were homogenized using a glass homogenizer; unbroken cells and cell debris were removed by low speed centrifugation followed by high-speed centrifugation to collect the membranes. The membranes were solubilized with FOS-CHOLINE-12 detergent (Anatrace) and subjected to an additional high-speed centrifugation step to remove the insoluble material. The solubilized membranes were then applied to a Q-Sepharose HP column (Amersham Biosciences). The bound proteins were eluted with a NaCl step gradient (100mM, 200mM, 300mM, 400mM, 500mM and 1M). Fractions were evaluated by western blot with antibodies against Nct and Psn-1

CTF. Nct and Psn-1 were found in fractions that eluted at a salt concentration of 200mM. These fractions were pooled and loaded onto a lentil lectin column (Amersham Biosciences). The bound proteins eluted at 200mM methyl α-D-mannopyranoside. The eluted fractions were pooled, concentrated and applied to a molecular sieve column (Pharmacia Superdex 200). The peak fractions centered at a molecular weight of about 250–300KD, based on a column calibration using various molecular weight standards, were pooled and concentrated. An aliquot of the concentrated sample was analyzed by SDS-PAGE (4-20% Tris-HCl, Bio-Rad) and the gel was stained with Coomassie blue. The concentrated sample was also analyzed for the presence of Psn-1 (NTF and CTF), Nct, PEN-2 and APH-1 by western blot. After in-gel trypsin digestion, peptides generated from the unidentified 50KD band were extracted and analyzed to obtain their amino acid sequences (UC Davis, Molecular Structure Facility). A GenBank BLAST search revealed the homology of the peptides with corresponding fragments of the CD147 sequence.

Antibodies, quantitative western blot and co-immunoprecipitation. Anti-presenilin-1 CTF (MAB5232), anti-human CD147 (CBL535), anti-APP N-terminal 66-81aa (MAB348) and anti-Aβ 1-17aa (MAB1560) monoclonal antibodies were purchased from Chemicon; rabbit anti-nicastrin (N1660) and goat anti-CD147 (E4029) polyclonal antibodies were purchased from Sigma; anti-presenilin-1 NTF (SC-1245) and goat anti-mouse CD147 antibody (T-18, SC9756) were purchased from Santa Cruz Biotechnology; anti-APH-1 antibody H-2D and anti-PEN-2 antibodies were kindly provided by G. Yu and T.W. Kim respectively. Antibody 192 that specifically recognizes sAPP$_\beta$ was a gift from P. Schubert (Elan Pharmaceuticals Inc.).

Quantitative western blots were performed as previously described (26). Briefly, the blots were developed with the ECL-Plus detection system (Amersham Biosciences), after which the labeled protein was visualized on a FluorChem8900 digital imaging system (Alpha Innotech Corporation). Band intensities were measured densitometrically using the AlphaEaseFC^{TM} software package (Alpha Innotech Corporation).

HeLa, HEK293 and neural cell membrane samples for co-immunoprecipitation experiments were prepared by solubilization with FOS-CHOLINE-12 detergent at 4^0C for 30 minutes. After ultracentrifugation at 120,000g for 60 minutes at 4^0C, the supernatants were collected as the solubilized membrane protein. The co-immunoprecipitation was performed as previously described (18,19,27). Briefly, the purified proteins and solubilized cell membranes were incubated with specific antibodies and protein G beads (Amersham Biosciences) at 4^0C overnight. Control experiments were carried out using non-immunized animal serum from the same species as each specific antibody was developed from. Immunoprecipitates were washed, eluted with reduced 2 X SDS Laemmli sample buffer and then applied to a 4-20% SDS-PAGE gel (Bio-Rad) for immunoblotting.

Cell lines. Suspension HeLa cells were obtained from the National Cell Culture Center, HCN-1A cells were purchased from ATCC and cultured using the protocols provided. HEK293 cells were cultured in DMEM medium containing 10% FBS. CHO-APP$_{695}$ cells

(kindly provided by S. Sisodia) were maintained in F-12K culture medium (Gibco 21127-022) containing 10% FBS, supplemented with 200μg/ml G418.

The full-length of CHO CD147 cDNA was cloned into pcDNA3.1/zeo(-) expression vector (Invitrogen) and transfected to CHO-APP$_{695}$ cells using lipofectamine2000 (Invitrogen). Cell lines with stably over-expressed CD147 were selected and cloned by using the antibiotic zeocin. The stably CD147 over-expressed CHO-APP$_{695}$ cell lines were maintained in F-12K culture medium (Gibco 21127-022) containing 10% FBS, supplemented with 200μg/ml G418 and 150μg/ml zeocin.

RNA interference. Three Stealth™ siRNA duplex oligoribonucleotides against CHO cell CD147 (GenBank # AF 320819) were synthesized by Invitrogen. The sequences were: 1. sense 5'-UAUGUCAAGGUUGCUGAUGGUCAGC-3', anti-sense 5'-GCUGACCAUCAGCAACCUUGACAUA-3'; 2. sense 5'-AAAGAGCAGGUAAGGUGUCUUGG-3', anti-sense 5' CCAAGACACACCUUACCUGCUCUUU-3'; 3. sense 5'-UUGUCGUUCAUGUGAUGACCACUGC-3', anti-sense 5'-GCAGUGGUCAUCACAUGAACGACAA-3'. siRNA oligos were transfected into CHO-APP$_{695}$ cells using the Block-iT™ transfection kit (Invitrogen) according to the manufacturer's protocol. The Block-iT™ Fluorescent Oligo that is not homologous to any known genes was used as transfection efficiency detector and a negative control to ensure against induction of non-specific cellular events caused by introduction of the oligo into cells. Among the three siRNA oligo duplexes against CD147, we selected the

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one which required the smallest concentration to achieve the desired knock-down effect to perform the titration experiments. Titration of the CD147 siRNA duplex was performed using graded concentrations as described previously (28-30). Culture media were replaced with fresh media after 64 hours; cells and conditioned media were harvested 8 hours later. CD147 and other protein expression levels were examined by quantitative western blot. $sAPP_{\alpha}$, $sAPP_{\beta}$ and total sAPP levels in culture media were also examined by quantitative western blot, while $A\beta_{40}$ and $A\beta_{42}$ were measured by ELISA.

$A\beta_{40}$ and $A\beta_{42}$ Quantitative Assay. $A\beta_{40}$ and $A\beta_{42}$ levels in the cell culture media were quantitatively measured by ELISA (Signet Laboratories, Inc. cat: 8940 & 8942) according to the protocol provided by the manufacturer. For measuring $A\beta_{40}$, the culture media were diluted 1:5 with diluents supplied in the kit because of its high concentration. Undiluted culture media were used for detecting $A\beta_{42}$. The data were statistically analyzed with one-way ANOVA and the student *t*-test.

Results

Identification of CD147 in the purified native γ-secretase complex. For the purification of endogenous γ-secretase complex, we developed a multi-step chromatographic protocol to obtain purified native γ-secretase complex from detergent-solubilized HeLa cell membranes. The purified sample was analyzed by SDS-PAGE (Fig. 1A) and yielded six strong bands, five of which had apparent molecular weights expected for the previously identified components of the γ-secretase complex (Psn-1 NTF, Psn-1 CTF, Nct, APH-1 and PEN-2) and one unexpected at a molecular weight of about 50KD.

The presence of the known components of the γ-secretase complex was confirmed by western blot (Fig. 1B). The unexpected 50KD band was initially identified through amino acid sequencing of proteolytic fragments as CD147, a highly glycosylated membrane protein with an apparent molecular weight of about 50KD. The identity of this band as CD147 was confirmed by polyclonal goat anti-human CD147 antibody and monoclonal mouse anti-human CD147 antibody (Fig. 1C).

To assess whether or not CD147 is a co-purifying contaminant, we tested for the presence of both CD147 and known members of the complex during the entire course of the purification. CD147 was found to co-elute with the previously identified γ-secretase components Nct and Psn-1 CTF throughout the purification process as monitored by immunoblot analysis. In the Q-Sepharose HP chromatographic run, Nct, Psn-1 CTF and CD147 were found in fractions eluting at a salt concentration of 200mM (Fig. 2A); in molecular sieve chromatography, these proteins were found to co-elute at an apparent molecular weight of 250-300KD (Fig. 2B). This molecular weight is consistent with a complex consisting of CD147, Nct, Psn-1, PEN-2 and APH-1.

CD147 is an integral component of the active γ-secretase complex in native membranes. The association of CD147 with γ-secretase complex was further explored through co-immunoprecipitation experiments on the purified protein with antibodies to CD147, Psn-1 CTF and Nct. As anticipated, we found that CD147 antibody immunoprecipitated Psn-1 CTF and Nct (Fig. 3A). Conversely, Psn-1 CTF and Nct antibodies were found to immunoprecipitate each other, as well as CD147 (Fig. 3B and

3C). Only the processed form of Psn-1 (Psn-1 cut to form Psn-1 NTF and Psn-1 CTF) was found to co-immunoprecipitate with CD147 antibody. The results of the co-immunoprecipitation experiments strongly indicate that CD147 is an integral component of the γ-secretase complex, not merely a transiently interacting binding partner.

In addition to purified protein, we conducted similar co-immunoprecipitation experiments with human neural cells (HCN-1A cell line) (Fig. 4A) and HEK293 cells (Fig. 4B); HeLa cell membranes were used as a reference (Fig. 4C). It was found that CD147 antibodies could also co-immunoprecipitate Psn-1 and Nct in neural and HEK293 cells. These experiments yielded results similar to that observed for purified protein and showed that CD147 is an integral component of the γ-secretase complex and whose presence is not limited to complexes obtained from HeLa cells.

Depletion of CD147 in the CHO-APP$_{695}$ cell line increases the production of amyloid β-peptides. The effect of CD147 deletion on γ−secretase activity was examined through silencing studies of the CD147 gene; silencing was accomplished through the transfection of Stealth™ siRNA against CD147 into CHO-APP$_{695}$ cells that stably over-expresses human APP$_{695}$. Titration experiments with graded concentrations of Stealth™ siRNA duplexes showed that siRNA mediates the down-regulation of CD147 expression in a dose-dependent manner (Fig. 5A and 5B) without changing the expression level of other components of γ-secretase, such as Psn-1 CTF and Nct, or the level of soluble APP$_\alpha$(sAPP$_\alpha$), sAPP$_\beta$ and total sAPP (Fig. 5A). The maximal reduction in CD147 expression was observed at 2 nM of siRNA duplexes. Higher concentrations of CD147

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siRNA duplex did not further detectably decrease CD147 expression (Fig. 6). Stealth™ siRNA at a concentration of 2 nM produced about a 90% reduction in CD147 expression; 0.5 nM caused about a 75% reduction and 0.125 nM yielded about a 60% reduction (Fig. 5B). Reduction of 90% CD147 expression surprisingly increased $A\beta_{40}$ and $A\beta_{42}$ levels by 52% and 87% respectively, as measured by ELISA; reduction of 75% CD147 expression increased $A\beta_{40}$ and $A\beta_{42}$ levels by 25% and 38% respectively (Fig. 5C and 5D); reduction of 60% CD147 expression did not significantly increase the levels of $A\beta_{40}$ and $A\beta_{42}$ as compared to controls using transfection reagents without siRNA (Fig. 5C and 5D). BLOCK-iT™ Fluorescent Oligo was used as the detector of transfection efficiency and another negative control; application of up to 80nM did not change the expression level of CD147 or the production of $A\beta_{40}$ and $A\beta_{42}$.

Over-expression of CD147 does not affect the production of amyloid β-peptide in CHO-APP$_{695}$ cells. To complement the RNA interference studies, we conducted experiments to over-express CD147 in the same cell line, CHO-APP$_{695}$, used for the silencing studies. We established stable CD147 over-expression cell lines by transfecting the full-length CD147 pcDNA 3.1/zeo(-) construct plasmid DNA into CHO-APP$_{695}$ cells. We compared the production of $A\beta_{40}$ and $A\beta_{42}$ in CD147 over-expressing and non CD147 over-expressing CHO-APP$_{695}$ cell lines. From these experiments it was found that CD147 over-expression had no statistically significant effect on amyloid β-peptide production (Fig. 7B and 7C) and caused no significant change in the expression levels of APP or γ-secretase components Psn-1 CTF and Nct (Fig. 7A).

Discussion

Our efforts to purify endogenous γ-secretase from detergent-solubilized HeLa cell membranes led to the discovery of an additional component of the native complex. Samples of the purified complex were found to contain the previously identified components of γ-secretase (Psn-1 NTF, Psn-1 CTF, Nct, APH-1 and PEN-2) and an additional membrane protein, CD147. The finding that CD147 is an integral subunit of the γ-secretase complex and not a transient binding partner (or co-purifying contaminant) was confirmed through co-immunoprecipitation experiments conducted on the purified complex with antibodies to CD147, Psn-1 CTF and Nct. Co-immunoprecipitation experiments with solubilized membranes from human neural cells (HCN-1A) and HEK293 cells also demonstrated that CD147 is an integral component of γ-secretase complexes in cells other than HeLa cells.

The role of CD147 within the γ-secretase complex was examined through silencing experiments. Suppression of CD147 expression resulted in specific dosage-dependent increased levels of amyloid β-peptide production without changing the expression level of the other γ-secretase components or APP available substrate. The results of the silencing experiments also suggested that a fraction of the CD147 population is not integrally associated with γ-secretase; this notion is supported by reports that CD147 participates in a range of biological responses (31-37). The presence of such forms of CD147 is also apparent in the results of the co-immunoprecipitation studies conducted for this effort where, after multiple immunoprecipitation cycles for the γ-secretase complex

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using Psn-1 CTF or Nct antibodies, a substantially higher amount of CD147 compared to Psn-1 or Nct remained in detergent solubilized membrane preparations (data not shown).

Could the increase in $A\beta_{40}$ and $A\beta_{42}$ production attributable to CD147 silencing be due to reduction of potential non-γ-secretase associated CD147? This question was addressed by experiments in which CD147 was overexpressed in the same cell type used for the silencing studies (CHO-APP$_{695}$). Overexpression of CD147 in these cells did not lead to statistically significant alterations in the production of $A\beta_{40}$ and $A\beta_{42}$, the expression of the other γ-secretase subunits or available APP substrates, indicating that the effect of silencing CD147 on amyloid β-peptide production is through those molecules of CD147 that are part of the γ-secretase complex. Taken together, the results of the co-immunoprecipitation studies, and the silencing and overexpession experiments, demonstrate that CD147 is an integral regulatory subunit of the native γ-secretase complex whose deletion increases the production of amyloid β-peptides.

The presence of an integral member of γ-secretase membrane protein with a regulatory effect on γ-secretase activity was not completely unexpected. Although the overexpresed complexes made up of Psn-1, Nct, APH-1 and PEN-2 have been demonstrated to be enzymatically active, it has not, however been shown that four subunits alone constituted the complete native form of the γ-secretase complex. Experiments in which these four subunits were over-expressed in different eukaryotic cell types (yeast, Drosophila and mammalian cells (20-22)) yielded significantly varied levels of γ-secretase activity.

From such observations it has been suggested that unknown co-factors may be modulating the activities of the γ-secretase complex *in vivo* (25).

The molecular mechanism by which CD147 exerts its effect on the activity of the γ-secretase complex remains to be elucidated. CD147, also referred to as basigin and EMMPRIN, is ubiquitously expressed in a variety of cells and tissues (31). It is predicted to have a short cytoplasmic domain consisting of about 40 amino acids, a putative transmembrane region about 25 amino acids long and a large extracellular region that contains two Ig-like domains (32). In addition, the amino acid sequence of the putative transmembrane region and its neighboring residues are highly conserved. Noteworthily, a charged residue, glutamic acid, is present in the middle of the putative transmembrane domain and is not found in any known γ-secretase substrates. Such charged residues are not commonly found in proteins that span the membrane only once; the placement of a charged residue in the middle of the lipid bilayer would be highly energetically unfavorable. This structural feature suggests that CD147 associates with other membrane proteins to exist in an energetically stable state. It should be noted that Psn-1 contains two negatively charged residues, aspartic acids, within its putative transmembrane region; how the charged residues of Psn-1 and CD147 are accommodated within the context of the lipid bilayer is not clear and the answer must await the high-resolution structure determination of the complex. CD147 is known to be an antigen or antigen carrier (32,33) and is also believed to be involved in neural-glial cell interactions (34). It has been reported to be a membrane protein with multiple roles supporting a diverse range of biological activities such as reproduction, neural function, inflammation, protein

trafficking and tumor invasion (35-39); such multiple functional characteristics attributed to a single protein is not unusual and has been observed in other protein complexes, for example the 26S proteasome, where a subcomplex of the regulatory particle has also been found to participate in markedly different biological processes (40). The connection of many of the previously described functional characteristics of CD147 to its role in γ-secretase activity is not clear; however, the results of the CD147 silencing and overexpression studies on Aβ-peptide production presented here do not appear to support a mechanism of CD147 regulation of γ-secretase activity involving protein trafficking.

CD147 has been found to interact with several proteins including members of the cyclophilin family, monocarboxylate transporters and MMP1 (37,41,42). For example cyclophilin A, a peptidyl prolyl cis/trans isomerase, binds to a proline in the second Ig-like domain of CD147. Determination of whether, and if so how, the interactions of these proteins with CD147 can affect the nature of CD147's structural conformation and/or interaction with the other members of the γ-secretase complex (Psn-1, Nct, APH-1 and PEN-2) is an obvious direction for future research.

An increase in the production of Aβ-peptides, especially Aβ_{42}, by removal of CD147 or selected mutations of Psn-1 is expected to increase the formation of Aβ-peptide plaque, a hallmark of Alzheimer's disease. Interestingly, deletion of CD147 in mice was found to result in various neurological abnormalities, including severe defects in nervous system development, pronounced spatial learning deficits in the Morris water maze testing and working memory deficits (43), a behavior phenotype similar to those observed in

transgenic mouse models of Alzheimer's disease (44,45). With the discovery of CD147 as an integral subunit of the native γ-secretase complex, obtaining details of the molecular mechanism of CD147 through atomic structure determination of the complex and subsequent functional studies of selected structure-directed mutants will be a crucial step in understanding the molecular processes involved in multiple-site cleavage of a constellation of substrates and in the design of Alzheimer's disease therapeutics.

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Figure Legends

Fig. 1 CD147 is found in the purified γ-secretase complex. (A) Purified sample was analyzed by SDS-PAGE (4-20% Tris-HCl gel) where the gel was stained with Coomassie blue. (B) The presence of the previously identified components of the γ-secretase complex was confirmed by western blot. (C) The 50KD band, initially identified as CD147 through amino acid sequencing, was confirmed by western blot using two different anti-CD147 antibodies.

Fig. 2 CD147 co-elutes with γ-secretase components during chromatographic purification. (A) In the Q-Sepharose HP chromatographic step, CD147 together with Psn-1 CTF and Nct, were found only on fractions eluting at a salt concentration of 200mM. (Elution steps shown in red: 100mM, 200mM, 300mM, 400mM, 500mM and 1M) (B) In molecular sieve chromatography, CD147, Psn-1 CTF and Nct co-eluted at a molecular weight of 250-300 kDa.

Fig.3 Co-immunoprecipitation experiments using purified proteins indicate that CD147 is an integral member of the γ-secretase complex. (A) In purified γ-secretase complex, antibody to human CD147 co-immunoprecipitates Psn-1 CTF and Nct. (B) Anti-Psn-1

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CTF antibody co-immunoprecipitates CD147 and Nct. (C) Anti-Nct antibody co-immunoprecipitates CD147 and Psn-1 CTF. The negative controls are shown in lane 3 - asterisks correspond to immunoglobulins.

Fig. 4 Anti-CD147 antibody co-immunopreciptates previously identified γ-secretase components in detergent solubilized neural cells, HEK293 and HeLa cell membranes. (A) In neural cells, HCN-1A cell line, anti-CD147 antibody co-immunoprecipitates Nct and Psn-1 CTF. (B) In HEK 293 cells, anti-CD147 antibody co-immunoprecipitates Nct and Psn-1 CTF. (C) In HeLa cell membranes, anti-CD147 antibody co-immunoprecipitates Nct and Psn-1 CTF. The negative controls (goat IgG) showed no binding with Nct or Psn-1 CTF. Asterisks correspond to immunoglobulins.

Fig. 5 CD147 as a member of the γ-secretase complex regulates the γ-secretase proteolytic activity in the production of $A\beta_{40}$ and $A\beta_{42}$ peptides. (A) CD147 RNAi specifically reduces CD147 expression in CHO-APP_{695} cells without changing the expression of other γ-secretase components. The expression levels were measured by quantitative western blots. CD147 RNAi does not change the amount of $sAPP_{\alpha}$, $sAPP_{\beta}$ and sAPP in culture media. (B) Graded concentrations of Stealth™ CD147 siRNA duplexes show a dose-dependent reduction in CD147 expression. The levels of $A\beta_{40}$ (C) and $A\beta_{42}$ (D) peptides in culture media were measured by ELISA, showing that the depletion of CD147 increased the production of amyloid β-peptides. Error bars represent standard deviation. Asterisks indicate a significant difference from control (in absence of

siRNA), as determined by one-way ANOVA (P<0.01). The results in (B), (C) and (D) were from six repeated experiments.

Fig. 6 Silencing of CD147 expression in CHO-APP$_{695}$ cells. (A) Immunofluorescence staining of CD147 on cells transfected with CD147 siRNA duplex (A-2 and A-4) and with transfection reagents only (A-1and A-3). Nuclei were double stained with DAPI (A-1 and A-2). Scale bars represent 10 μm. (B) Western blot of SDS solubilized cell lysates from CHO-APP$_{695}$ cells transfected with a graded dosage of CD147 siRNA duplex. Higher concentrations (>2nM) of CD147 siRNA duplex did not further reduce CD147 expression. Equal amounts of total proteins were applied to each lane.

Fig. 7 Overexpression of CD147 on CHO-APP$_{695}$ cells does not lead to significant changes in the production of Aβ_{40} and Aβ_{42} or in the expression of other γ-secretase components and APP. (A) The expression levels of CD147, APP, Nct and Psn-1 CTF were measured by western blots. The expression level of CD147 was substantially increased in CD147 overexpressed CHO-APP$_{695}$ (2) compared to CHO-APP$_{695}$ (1) cell line; the expression levels of APP, Nct and Psn-1 CTF in the CHO-APP$_{695}$ (1) and CD147 overexpressed CHO-APP$_{695}$ cell lines (2) were comparable. The levels of Aβ_{40} and Aβ_{42} are shown in (B) and (C) respectively. Error bars represent standard deviation. There are no statistically significant differences in the production levels of Aβ_{40} and Aβ_{42} by the CHO-APP$_{695}$ and CD147 overexpressed CHO-APP$_{695}$ cell lines, based on student t-test analysis (P>0.05).

Fig. 1

A
4-20% SDS-PAGE gel
stained with Commassie blue

250KD — Nct
150KD
100KD
75KD
50KD — CD147
37KD — Psn-1 NTF
APH-1
25KD
20KD — Psn-1 CTF
15KD — PEN-2
10KD

B
Western Blot

Nct
Psn-1 NTF
APH-1
Psn-1 CTF
PEN-2

C
1   2

CD147

1. mouse anti-CD147 Ab
2. goat anti-CD147 Ab

Fig. 2

A

1   2   3   4   5

Nct
Psn-1 CTF
CD147

B

300-250KD

Fractions   21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

Nct
Psn-1 CTF
CD147

25

Fig. 3

A
anti-CD147 co-IP

Psn-1 CTF   Nct
1   2   3   1   2   3

*   *

1.input 2.anti-CD147 3.goat serum

B
anti-Psn-1 CTF co-IP

CD147   Nct
1   2   3   1   2   3

*   *

1.input 2.anti-Psn-1 CTF 3.mouse serum

C
anti-Nct co-IP

CD147   Psn-1 CTF
1   2   3   1   2   3

*   *

1.input 2.anti-Nct 3.rabbit serum

Fig. 4

A
Neural cells

input anti-CD147

Nct →

Psn-1
CTF →   *

B
HEK293 cells

input anti-CD147 goat IgG

Nct →

Psn-1
CTF →   *

C
HeLa cells

input anti-CD147 goat IgG

Nct →

Psn-1
CTF →   *

Fig. 5

A

	0	0.125	0.5	2 (nM)
CD147				
Psn-1 CTF				
Nct				
APP				
sAPP$_\alpha$				
sAPP$_\beta$				
sAPP				

Fig. 6

A

B

40   20   10   5   2   0   siRNA(nM)

← CD147

Fig. 7

A

1   2

CD147

APP

Nct

Psn-1 CTF

B

☐ Aβ40

ng/ml

5
4
3
2
1

CHO-APP₆₉₅   CD147 overexpressed CHO-APP₆₉₅

C

■ Aβ42

ng/ml

0.25
0.2
0.15
0.1
0.05

CHO-APP₆₉₅   CD147 overexpressed CHO-APP₆₉₅