Direct Binding of Cholesterol to the Amyloid Precursor Protein: An Important Interaction in Lipid-Alzheimer's Disease Relationships?

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Summary

It is generally believed that cholesterol homoeostasis in the brain is both linked to and impacted by Alzheimer's disease (AD). For example, elevated levels of cholesterol in neuronal plasma and endosome membranes appears to be a pro-amyloidogenic factor. The recent observation that the C-terminal transmembrane domain (C99, also known as the β-CTF) of the amyloid precursor protein (APP) specifically binds cholesterol helps to tie together previously loose ends in the web of our understanding of Alzheimer's-cholesterol relationships. In particular, binding of cholesterol to C99 appears to favor the amyloidogenic pathway in cells by promoting localization of C99 in lipid rafts. In turn, the products of this pathway—amyloid-β and the intracellular domain of the APP (AICD) —may down-regulate ApoE-mediated cholesterol uptake and cholesterol biosynthesis. If confirmed, this negative-feedback loop for membrane cholesterol levels has implications for understanding the function of the APP and for devising anti-amyloidogenic preventive strategies in AD.

Keywords

Alzheimer's disease; amyloid precursor protein; APP; cholesterol; NMR; lipid rafts; membranes; structure; trafficking

Introduction

An early-2010 PubMed search on "Cholesterol AND Alzheimer's" yielded 1500 hits, exemplifying the large amount of attention devoted to unraveling this lipid-disease relationship. However, there remain many challenges to attaining a definitive understanding. For example, the results of experiments designed to elucidate linkages between the many proteins involved in cholesterol homeostasis and processes believed to be central to the etiology of Alzheimer's disease (AD) are rarely unambiguous because of pleiotropy and because of difficulties in extrapolating results from experiments with cultured cells to physiological conditions. Another set of challenges relates to detecting and monitoring the AD-relevant pools of cholesterol in neurons and glial cells. While familial hypercholesterolemia and obesity are generally regarded as risk factors for AD(1), cholesterol metabolism in the brain is largely isolated by the blood-brain barrier from cholesterol metabolism in the rest of the body, with

nearly all brain cholesterol being synthesized *in situ*(2–6). Measurement of total cholesterol levels in the CNS is not informative because 70–80% of brain cholesterol is dedicated to a structural role in myelin membranes(7). Modest quantities of cholesterol leave the brain primarily in the form of 24S-hydroxycholesterol, but enters in very small amounts only—in the form of 27-hydroxycholesterol(2;8). While cholesterol's mean half-life in the brain has been estimated to be at least 5 years its turnover in metabolically-active neurons is believed to occur much more rapidly than in glia and in myelin (2;5;9). There is data that suggests cholesterol-lowering statins reduce the risk of AD(1;10), although this is not without controversy. It is clear that apolipoprotein E (ApoE) is responsible for transport of cholesterol between cells in the brain and that the ε4 isoform of the protein is a major risk factor for the common late-onset form of AD; however, the exact mechanisms by which different variants of ApoE and related lipoprotein receptors variously promote or suppress AD pathogenesis are not understood(11–13).

Despite such limitations, there is a large body of evidence suggesting that increased levels of plasma membrane cholesterol promote the amyloidogenic processing of the amyloid precursor protein (APP) and thereby contribute to the key series of molecular (proteolytic) events widely believed to underlay with the etiology of AD(1;14–22). A number of genes involved in cholesterol homeostasis have been identified as susceptibility loci for sporadic or late-onset Alzheimer's disease (13;23–25). There is also evidence that cholesterol and other specific lipids may enhance the propensity of the amyloid-β (Aβ) polypeptide to form neurotoxic aggregates(26–32), although these latter studies are outside of the scope of this contribution. Amyloid plaques found in the brains of Alzheimer's patients are highly enriched in cholesterol (33).

As referenced above there are a number of excellent recent reviews on various aspects of the cholesterol-AD relationship, such that a comprehensive survey is not here merited. Instead, this paper is focused on very recent results from our own lab that offer a mechanistic hypothesis that links together a number of previous observations regarding how cholesterol promotes amyloidogenesis and how processing of the amyloid precursor protein may in turn impact cholesterol homeostasis.

Structure of C99, the Transmembrane C-Terminal Domain of the Amyloid Precursor Protein (APP)

While the structures of the Aβ polypeptides in various states have been the subject of numerous biophysical studies, there have been few attempts to study the structure of the immediate precursor to Aβ: the 99 residue transmembrane C-terminal domain (C99) of APP. C99 is the product of β-secretase cleavage of APP and is the substrate of γ-secretase cleavage. We recently initiated structural studies of C99 using solution nuclear magnetic resonance (NMR) spectroscopy to examine this protein under conditions in which it is solubilized in membrane-mimetic lyso-myristoylphosphatidylglycerol micelles(34). While the use of detergent micelles in biophysical studies of membrane proteins is not without pitfalls, the lyso-phospholipids used in our study are structurally similar to true phospholipids and are generally thought to be "mild" in the sense that they are good at maintaining native-like membrane protein function and stability (see review in(35)). This study of full length C99 complements and extends results from studies of fragments of this protein (36–48), most of which did not include an intact transmembrane segment.

While the process of completing a high resolution structure for C99 is on-going, its secondary structure and membrane topology have been characterized as summarized in Figure 1. The transmembrane (TM) domain appears to be an unbroken helix(34;45). Calculations suggest that the stability of this helix in the vicinity of the γ-secretase cleavage sites may be lower than

normal for standard TM helices(49), such that it may be relatively easy to disrupt this helix when it binds to the active site of γ-secretase, as required for cleavage. The cytosolic distal C-terminus of C99 is an amphipathic helix that is associated with the membrane surface(34). This domain starts right after the important NPTY Tyr-phosphorylation motif, which is thought to be critical to protein-protein interactions that play a role in regulating intracellular trafficking of APP and derived C99(38;42;50;51). A second surface-associated segment was found in the extracellular domain, where a short hydrophobic sequence (LVFFA) dips into the membrane, followed by a short connecting loop to the beginning the TM domain(34). The site for non-amyloidogenic α-secretase cleavage is located just before this membrane-interactive motif. It is likely that the affinities of both the extracellular hydrophobic segment and the distal C-terminus for the membrane surface are not very high, such that the association of these segments with the membrane surface *in vivo* may be modulated by the local membrane lipid composition and/or (for the C-terminus) phosphorylation of Tyr762. One can also speculate that α-secretase cleavage of C99 and/or C99 trafficking may be coupled to the state of membrane association of one or both of these segments.

The NMR results were also consistent with a reported tendency for C99 to homodimerize (40;45;52–55), although the avidity of C99 dimers in the lyso-phospholipid micelles used in the NMR studies appears to be only modest(34), suggesting that the oligomeric state of C99 in cells may favor either monomer or dimer depending on C99 concentration, membrane composition, and state of interactions with other proteins.

Evidence that the C-terminal Transmembrane Domain of APP Binds Cholesterol

The binding of cholesterol and cholesterol analogs to C99 in lyso-phospholipid micelles was monitored using NMR(34). At concentrations that approached its (low) solubility limit cholesterol induced significant shifts in a sub-set of C99's backbone amide NMR resonances. The residues associated with the peaks that underwent the largest shifts in response to cholesterol were localized almost exclusively to the extracellular loop connecting the surface-associated hydrophobic segment in the extracellular domain and the start of the TMD, suggestive of a possible cholesterol binding site centered at this position (see Figure 1). However, because the solubility limit of cholesterol was reached before saturation of binding could be attained this result alone did not exclude the possibility that the changes in the NMR spectrum reflect non-specific interactions of cholesterol with C99 rather than formation of a stoichiometric complex. For this reason, we turned to the use of a recently developed water soluble cholesterol analog, "CHOBIMALT", in which the –OH cholesterol has been replaced by a tetrasaccharide (Figure 2). When C99 was titrated with CHOBIMALT, the peaks that underwent maximal shifts were seen to be the same as those that shifted the most in response to *bona fide* cholesterol (34). This indicates that CHOBIMALT's mode of interaction with C99 faithfully mimics that of cholesterol and is not significantly perturbed by the analog's tetrasaccharide head group. It was possible to complete titration of C99 with this compound. When the averaged chemical shift changes of the residues located in the extracellular connecting loop were plotted as function of the CHOBIMALT concentration, it was observed that the binding curve approaches a plateau, strongly suggestive of saturation of binding (Figure 2)—a hallmark for the formation of a stoichiometric complex. As shown in Figure 2 this data is well fit by a 1:1 binding model. K_d was determined to be a very modest 28 mol% ± 14 mol%. The mol% unit is used to express the concentrations of molecules in membranes and is defined as: 100 X moles of CHOBIMALT/(moles of CHOBIMALT + moles of micellar lyso-phospholipid).

It is probable that the binding of cholesterol to C99 in actual membranes would be somewhat more avid than the ca. 25 mol% K_d, both because cholesterol is likely to bind more tightly to

C99 than to CHOBIMALT and because the membrane should provide a more favorable environment for lipid-protein binding than detergent micelles. An important priority for future work will be to examine this matter experimentally. In any case it is clear that C99's avidity for cholesterol is modest, being weaker by orders of magnitude, for example, than the binding of a typical agonist to a typical G protein-coupled receptor(56). However, given that steady state cholesterol levels in the plasma membrane (PM) are often 40–50 mol% (57), C99's affinity for cholesterol appears to be well-matched for this protein to sense membrane cholesterol levels. At PM concentrations of cholesterol that are probably at the lower end of the physiological range, most C99 would be expected to be uncomplexed with cholesterol, while at the upper end it would be expected to be ≫50% saturated. The possible functional relevance of this observation is discussed later in this review.

While the structural details of the cholesterol-C99 complex await full elucidation, the NMR titration results clearly indicate that the loop between the extracellular membrane surface-associated segment and the start of the TMD is the epicenter for C99-cholesterol interactions (see Figure 1). This loop includes the sequence VGSNK. With its interfacial location and several residues capable of both accepting and donating hydrogen bonds, it is probable that this loop forms hydrogen bonds with cholesterol's –OH moiety. A cholesterol binding sequence found in a number of proteins has previously been described(58;59)— the CRAC motif: L/V-$X_{1–5}$-Y-$X_{1–5}$-R/K This motif is believed to drive cholesterol binding by specifically recognizing its –OH moiety. While C99 does not contain the canonical CRAC sequence, it has previously been noted that its VGSNK motif bears some resemblance(60), suggesting that C99 binds cholesterol through interactions akin to those previously documented for other cholesterol-binding proteins. Whether cholesterol binds with similar affinity to both C99 monomers and dimers is unclear, as is the question of whether a single cholesterol molecule makes contacts with 2 subunits in a dimer.

The fact that C99 binds cholesterol suggests that full length APP does so as well. However, it should be pointed out that while C99 is often observed to be found associated with the detergent-resistant membrane fractions of cell extracts, full length APP is often seen to fractionate with detergent-extractable membranes(61;62), suggesting either that these two molecules have very different affinities for cholesterol or that there are additional factors that differentially contribute to determining their membrane localization.

The sites in C99 believed to be central to binding of cholesterol are also present in the Aβ polypeptides. There have been a number of studies that indicate that Aβ interacts with cholesterol, typically in a manner that is believed to promote oligomerization, fibrillization, and/or cholesterol oxidation(26–32;63–66). It is possible that the cholesterol binding site we have documented in C99 may play an important role in cholesterol-Aβ interactions even though Aβ has significant aqueous solubility and interacts with membranes in ways that are generally different from C99.

Why Binding of Cholesterol to C99 is Expected to Promote Amyloidogenesis

The propensity of C99 and, possibly, full length APP to form stoichiometric complexes with cholesterol may represent a factor that favors the amyloidogenic pathway by promoting localization of APP/C99 to cholesterol-rich membrane domains and organelles where γ-secretase and possibly β-secretase reside. There is now a large body of data showing that significant amounts of both β-secretase and γ-secretase, but not α-secretase, reside within cholesterol-rich membrane microdomains, often referred to as "lipid rafts"—a term we will use herein for the sake of simplicity. Lipid rafts are found primarily in the trans-Golgi, plasma, and endosomal membranes(57;67;68). β-secretase cleavage of APP has been proposed to occur primarily in membrane rafts of the PM and endosomes (69–75), although this is not without

controversy(61;76) and significant quantities of β-secretase is sometimes found in the bulk membrane(61;62). γ-secretase cleavage of C99 appears to occur primarily in rafts located in the endosomes (16;22;75;77–81). On the other hand, non-amyloidogenic α-secretase is believed to reside primarily in the bulk (non-raft) phase of the plasma membrane(71;82–84) and to be inactivated when forced to associate with rafts(85).

Membrane cholesterol within the plasma or endosomal membranes is distributed between two pools: free cholesterol in the bulk membrane phase and cholesterol that is associated with lipid rafts or related ordered microdomains. Based on studies with model membranes of defined composition it is thought that for a given membrane lipid composition, the partitioning ratio between free cholesterol and raft-associated cholesterol will be determined partly by the total mol% of cholesterol in the membrane, with higher cholesterol concentrations resulting in higher ratios in raft domains(57;86). When considered in this conceptual framework, three populations of C99 or APP are predicted. First is C99/APP that is uncomplexed with cholesterol. This population of protein would be prominent in membranes that have a mol% cholesterol below the K_d for binding to C99/APP, which appears to be roughly 25 mol% based on our NMR studies(34). This population would be subject to proteolytic processing primarily via α-secretase. Second is C99/APP that is complexed with cholesterol but remains in the bulk membrane phase. This population of protein would predominate only if the mol% of cholesterol in the membrane is high enough to exceed K_d and yet is low enough so that the amount of free cholesterol relative to the population of raft-associated cholesterol is low. If indeed the ca. 25 mol% K_d measured for cholesterol/C99 interaction under micellar conditions approximates the K_d in real membranes we wonder if *non*-raft associated cholesterol-C99/APP complexes would ever dominate since this level of cholesterol is well above the cholesterol concentration at which rafts will begin to form, at least in typical model membranes. A third population of C99/APP would be complexed with cholesterol in rafts. This is the population of the protein that is widely thought to be subject to amyloidogenic processing by β- and γ-secretases. This population is expected to predominate when the total cholesterol concentrations are on the order of K_d or higher.

To summarize this model, at low plasma membrane/endosomal cholesterol concentrations, free C99 and APP will predominate in the plasma membrane and the protein will primarily be subject to α-secretase-initiated processing. However, as cholesterol levels in the membrane rise to the point where a majority of this lipid is found in rafts, most C99 and possibly APP will complex with cholesterol, most of which will be located in lipid rafts. Localization of a majority of APP to rafts is expected to result in a tip in the balance between α-secretase- and β-secretase-initiated processing towards β-secretase, with subsequent γ-secretase cleavage of C99 also being promoted by cholesterol complexation and raft localization (Figure 3). We regard the latter (γ-secretase/C99) part of this model as being more uniformly supported by the existing data, with the β-secretase/APP part of this model being more controversial.

The above model assumes both that (i) both C99 and full length APP have a similar affinity for cholesterol, (ii) that C99/APP has a similar affinity for cholesterol in both rafts and in the bulk membrane and (ii) that complexation of C99/APP with cholesterol does not grossly perturb cholesterol's partitioning between bulk membrane and rafts. These assumptions have yet to be tested. Moreover, this model should be regarded as a *nested model* in the sense that cholesterol binding to APP/C99 is likely to prove to be only one of a number of factors that collude to induce intracellular trafficking and membrane/raft localization of APP/C99 (c.f., (22;51;87;88)). This may help to explain, for example, why the distribution of C99 vs. full length APP between detergent-resistant membranes and the bulk membrane is not always seen to be the same (c.f.,(62)). It should also be recognized that other factors besides cholesterol levels—such as sphingolipid content—may also help determine lipid raft content *in vivo*. Despite these caveats, there appears to be good reasons to suppose that the propensity of C99

and possibly APP to bind cholesterol is an important factor that promotes the amyloidogenic pathway.

A second possible mechanism by which cholesterol complexation with APP/C99 could promote amyloidogenesis is by directly modulating substrate binding, catalysis, or product dissociation by β- or γ-secretase. These enzymes have been shown to exhibit much higher activities in the presence of cholesterol than in the absence(89;90), although there is contrary data in the case of γ-secretase(91). Whether cholesterol's promotion of the activities of these secretases is due to alterations in the membrane microenvironment surrounding these enzymes or could involve a more specific allosteric regulation, possibly involving direct substrate-cholesterol interactions is not yet clear. It is interesting to note that a number of NSAID-class drugs are known to be γ-secretase modulators (GSMs), which inhibit and/or perturb γ-secretase cleavage of C99 to alter the $A\beta_{42}/A\beta_{40}$ production ratio (92–94). However, NSAIDs are not regarded as cholesterol mimics, although they may promote ordering of lipid bilayers in a way that resembles cholesterol(95).

Therapeutic Implications of Complex Formation Between C99/APP and Cholesterol

A serious concern regarding general inhibitors of β- or γ-secretase as potential preventative or therapeutic agents for Alzheimer's disease is that these proteases cleave other proteins besides C99/APP in ways that are essential for normal health(49;96–98), leading to concerns about toxicity(99;100). While such inhibitors are, nevertheless, under clinical development(101–103), this concern has led to a search for strategies for *specifically* inhibiting or favorably modulating cleavage of C99/APP relative to other substrates. For example, small molecule "γ-secretase modulators" (GSMs) of C99 cleavage have been described (92–94) and have been proposed to act by binding directly to C99 to alter its substrate recognition by γ-secretase (93). However, that these compounds directly complex C99 has been disputed(104).

It has previously been proposed that specifically altering the trafficking of C99/APP away from lipid rafts is expected to be beneficial (c.f., (105;106)). That C99 forms a complex with cholesterol, which likely leads to its raft localization, suggests that drug-like molecules that can inhibit complex formation between C99/APP and cholesterol might provide a way of avoiding Aβ production. There is evidence that drug-like molecules can be developed that act by specifically-recognizing and binding to single-span membrane proteins(107;108), suggesting that this approach is feasible.

Possible Teleology for Cholesterol Binding to C99/APP

Though subject to much continuing inquiry, the functions of the APP remain only partially elucidated (109–113). While for the most part there are not obvious direct relationships between APP's proposed functions and cholesterol, there is evidence that the *products* of the amyloidogenic pathway—both Aβ and the intracellular domain of the APP (AICD)—can play a direct role in regulating cholesterol homeostasis, acting to lower cellular cholesterol levels (114–116).

It has been shown that Aβ stimulates the release of cholesterol and some other lipids from cells in the form of lipoproteins(117) and also that fibrillar Aβ down-regulates cholesterol biosynthesis(118). Another study suggested that Aβ reduces biosynthesis of cholesterol and other lipids under conditions of ischemia(115;119). It has also been proposed that cytosolic Aβ acts as a (possibly indirect) inhibitor of HMG-CoA reductase (HMGR), the rate-limiting enzyme in the biosynthetic pathway for cholesterol(120). The same study suggested that Aβ production also stimulates sphingomyelinase activity, which would coordinate with cholesterol

reduction to decrease lipid raft content in the cell membrane. These observations suggest that Aβ production can down-regulate cellular cholesterol and raft content. It should be noted that while one normally thinks of Aβ as being released primarily into the extracellular milieu, an appreciable fraction of γ-secretase cleavage of C99 appears to occur in endosomes, where Aβ is released into the lumen (reviews in (22;80;81;121–123)). While some C99 in this luminal pool would be expected to be exocytosed from the cell when endosome-derived vesicles recycle to the plasma membrane there is also evidence that significant amounts of Aβ traffic to the secretory pathway, to mitochondria, and even to the cytosol(122;124–127). Some Aβ that is secreted into the ectoplasm may also be taken up again by cells(122). Thus, the impact of Aβ on cholesterol homeostasis could derive from both intra- and extracellular peptide pools.

The AICD that is released upon γ-secretase cleavage of C99 translocates to the cell nucleus in concert with Fe65 and Tip60, where it then acts as a transcriptional suppressor to the gene that encodes the LRP1 protein, a major apolipoprotein E receptor in the brain that mediates cellular cholesterol uptake via endocytosis. This suggests that AICD production down-regulates cellular cholesterol uptake(128), although this has been questioned(129).

Given the above considerations, a possible function of the amyloidogenic pathway may be to reduce total cellular cholesterol levels, as previously proposed (114–116) and as illustrated in Figure 3. The fact that APP now appears to be a cholesterol binding protein suggests that one of its functions may be to act as a cellular cholesterol sensor/receptor. When membrane cholesterol levels are elevated APP forms a complex with cholesterol, which promotes the amyloidogenic pathway. The peptide products of this pathway ultimately reduce both cholesterol uptake and biosynthesis, completing a negative feedback loop.

If indeed APP does turn out to be involved in regulating cellular cholesterol levels, why is this only now being uncovered? One possibility is that APP's role as a cellular cholesterol sensor is as a minor niche or backup player relative to the normally preeminent primary cellular cholesterol sensors(130;131). It is well known that that most genes do not appear to be essential to the organisms they support, in part because of functional redundancy(132–135)—for any given protein and its associated function, there is usually at least one other protein in the cell that can fulfill that function if the first protein is knocked out. If this is the case for APP, then it is easy to imagine how a role as a cellular cholesterol sensor would not be easily detected.

Many Unanswered Questions

As described in this review, the recent documentation that C99 and cholesterol form a complex under micellar conditions offers new insight into the nature of the relationships between cholesterol, APP processing, and Alzheimer's disease. This raises many new questions, which include: Does APP/C99 bind to other cholesterol-related compounds such as 24S-hydroxycholesterol and the products of oxidative damage of cholesterol? Do any of the mutations in the APP that lead to familial early-onset AD impact C99/APP-cholesterol interactions? What is the relationship of C99/APP homodimerization to cholesterol binding and how does this relate to protein trafficking and interactions with the secretases? Does binding of cholesterol to C99/APP directly alter substrate association, cleavage, and/or product release by the β- and γ-secretases? Finally, to what degree is cholesterol binding to APP a *decisive* event that commits APP to the amyloidogenic pathway, as opposed to merely being a factor in a network of APP-protein or APP-lipid interactions that collectively determine this fate of APP in the cell? It is hoped that this review will spur interest in searching for answers to these questions.

Finally, it should be pointed out that for cholesterol to serve as a regulatory mechanism for proteolytic processing of APP/C99 the physiological concentrations of cholesterol in the plasma and endosomal membranes must vary enough throughout the lifetime of a cell to

generate different outcomes at different points in time with regard to the rate of amyloidogenic processing and to the partitioning of APP/C99 between the amyloidogenic and non-amyloidogenic processing pathways. For model cell lines it has been shown that the overall cholesterol content of a cell can change by factors in the range of 2–4 at different phases of cell growth(136;137). However, even these relatively simple measurements do not appear to have been made for neurons in culture, much less under physiological conditions. Moreover, even for model cell lines almost nothing is known about the dynamic variability of cholesterol and lipid raft content in plasma or organellar membranes as a function of cellular physiological state. Quantitation of the dynamics of organelle-specific membrane cholesterol content in the neurons of living (and aging) organisms is a daunting goal, but one that may ultimately need to be surmounted as a key step toward elucidating the complex relationship between cholesterol and Alzheimer's disease.

Acknowledgments

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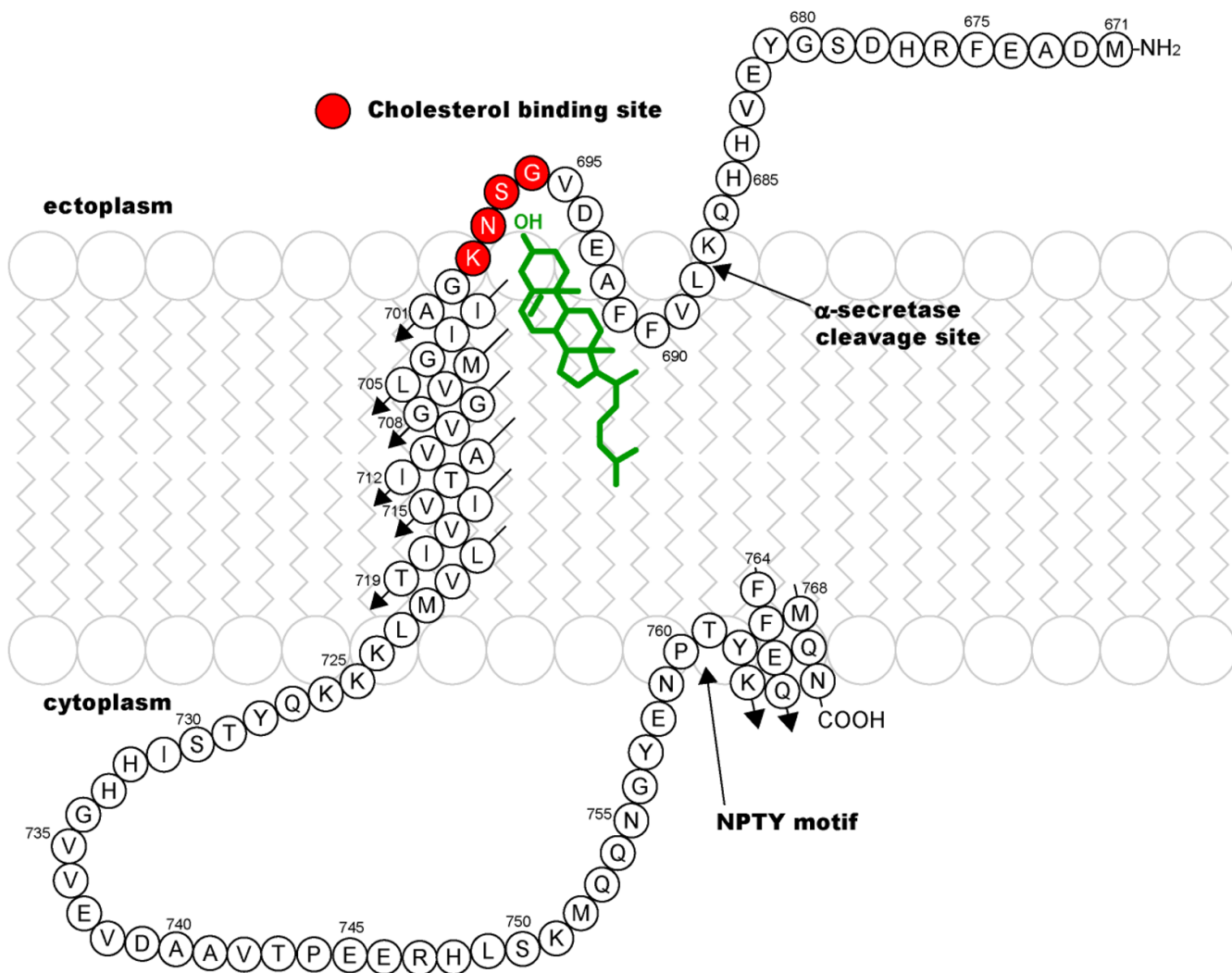
Figure 1.
Secondary structure and membrane topology of C99 and location of the focal point for its recognition and binding of cholesterol (red-highlighted sites), as derived from NMR studies of this protein in LMPG micelles(34).
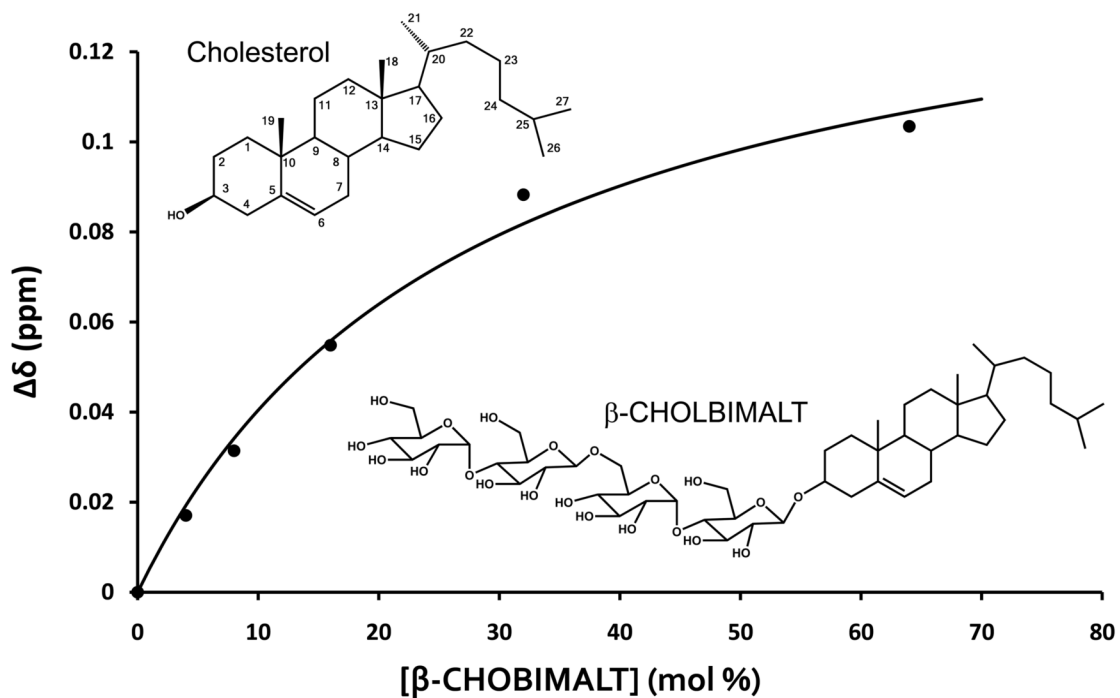
Figure 2.
NMR titration data for binding of a cholesterol analog (CHOBIMALT) to C99. A 1:1 binding model has been fit to the data, which is taken from(34) and represents the averaged values of the changes of chemical shifts for the backbone amide resonances of the residues located at the focal point for cholesterol binding to the C99 (the residues highlighted in red in Figure 1).
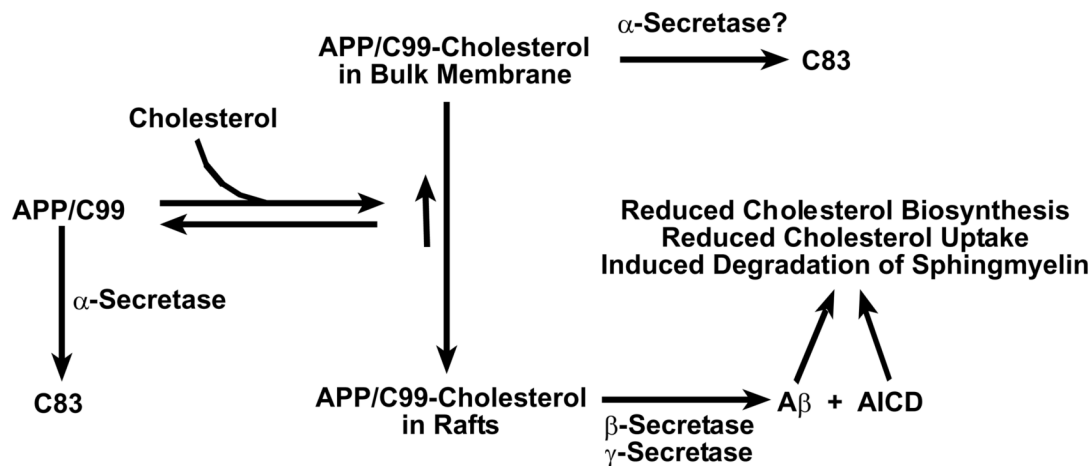
Figure 3.
Working model for how cholesterol binding to C99/APP is related to the amyloidogenic and non-amyloidogenic pathways for processing APP and for how the amyloidogenic pathway may be linked to cellular cholesterol reduction. Given that the transmembrane product of α-secretase cleavage of APP (C83) contains the sequence motif believed to be central to cholesterol binding by C99/APP (see Figure 1), C83 probably also binds cholesterol. In this case, elevated cholesterol levels would also result in raft localization of C83, subsequent γ-secretease cleavage of which would then release AICD and an apparently harmless peptide known as p3. Whether C99/APP-cholesterol complexes in the bulk (non-raft) membranes can be cleaved by α-secretase is unclear. It is known that both full length APP and C99 can serve as substrates for α-secretase cleavage in the bulk membrane(138).