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The amphipathic helix of an enzyme that regulates phosphatidylcholine synthesis remodels membranes into highly curved nanotubules

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ABSTRACT

CTP:phosphocholine cytidylyltransferase (CCT) is an amphitropic protein regulating phosphatidylcholine synthesis. Lipid-induced folding of its amphipathic helical (AH) membrane-binding domain activates the enzyme. In this study we examined the membrane deforming property of CCT *in vitro* by monitoring conversion of vesicles to tubules, using transmission electron microscopy. Vesicle tubulation was proportional to the membrane density of CCT and proceeded either as growth from a pre-formed surface bud, or as a global transformation of roughly spherical vesicles into progressively thinner tubules. The tubulation pathway depended on the lipid compositional heterogeneity of the vesicles, with heterogeneous mixtures supporting the bud-extension pathway. Co-existence of vesicles alongside thick and thin tubules suggested that CCT can discriminate between flat membrane surfaces and those with emerging curvature, binding preferentially to the latter. Thin tubules had a limiting diameter of ~12 nm, likely representing bilayer cylinders with a very high density of 1 CCT/50 lipids. The AH segment was necessary and sufficient for tubulation. AH regions from diverse CCT sources, including *C. elegans*, had tubulation activity that correlated with α-helical length. The AH motifs in CCT and the Parkinson's-related protein, α-synuclein, have similar features, however the CCT AH was more effective in its membrane remodeling function. That CCT can deform vesicles of physiologically relevant composition suggests that CCT binding to membranes may initiate deformations required for organelle morphogenesis and at the same time stimulate synthesis of the PC required for the development of these regions.

1. Introduction

The amphipathic helix (AH) is a membrane binding motif utilized by many peripheral or amphitropic proteins [1]. Aside from their function in anchoring proteins to membranes, there is a growing appreciation of an additional feature of AH segments: they bend bilayers upon insertion [2]. When the AH segment partitions into the bilayer it occupies only the proximal monolayer, usually at a position centered within the interfacial zone of the proximal leaflet. This asymmetric location of the bound AH impacts on the lipid packing pressure profile across the bilayer resulting in positive curvature induction [3]. In an elastic anisotropic medium such as a phospholipid bilayer the expansion of a monolayer by the inserted AH will be related to surface density of the AH, the elasticity of the lipid being expanded, and the degree of displacement of lipids by the AH [3]. Curling the proximal monolayer will also curl the distal monolayer (in the opposite manner) because there is tight transverse coupling due to the necessity of maintaining the connection where the acyl chains meet. Although the basic physics of AH-induced membrane bending has been well described, some determinants of curvature induction require further investigation, such as how curvature induction is impacted by the lipid composition, or by properties of the AH such as its length, hydrophobicity, amphipathy, or its embedded position in the bilayer.

One of the clearest examples of the function of membrane remodeling by an AH motif is in the process of endocytosis. Proteins like epsin, amphiphysin, and endophilin contain one or more AH motifs which induce plasma membrane invagination in a process orchestrated with scaffolding domains; e.g. BAR domain, dynamin or clathrin oligomers [4–7]. Another cell function involving AH induction of

Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; AH, amphipathic helix; NLS, nuclear localization sequence; DMPC, 1,2-dimyristoyl-*sn*-glycerophosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycerophosphoglycerol (sodium salt); DAG, diacylglycerol; POPS, 1-palmytoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] (sodium salt); POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; PI, bovine liver phosphatidyl inositol; SM, egg sphingomyelin; DTT, 1,4-dithio-D-threitol; SUVs, sonicated unilamellar vesicles; MLVs, multilamellar vesicles; LUVs, large unilamellar vesicles; SLVs, sucrose-loaded vesicles; NE, nuclear envelope; PM, phosphomimic; NE mix, LUVs composed of egg PC/POPE/PI /POPS/SM (55/20/10/10/5), PBS, phosphate buffered saline; TEM, transmission electron microscopy

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curvature is in vesicular transport between the ER and Golgi. Here the AH segment of a protein such as Arf or Sar-1 is responsible for initiating a bud that will later become coated with a scaffold protein complex, such as COP-1[4]. These AH elements can tubulate vesicles *in vitro* when added at a high protein/lipid ratio (e.g. 1 AH motif/20 lipids). This process is typically monitored by electron microscopy, where the AH causes long, thin protrusions from vesicles composed of lipid mixtures from biological sources [4–7].

CTP:phosphocholine cytidylyltransferase (CCT), the rate-limiting enzyme in PC synthesis, contains one of the longest membrane-binding AH motifs that has been described [1,8]. CCT is subject to regulation at many levels including regulation by amphitropism, i.e. reversible membrane interactions [9]. In its inactive soluble form CCT is silenced by a weak interaction between its catalytic domain (C) and an auto-inhibitory segment (domain M) within its regulatory tail region (Fig. 1A) [10]. In its active, membrane bound form domain M adopts an AH structure (Fig. 1B) [8] and inserts into the bilayer of membranes with PC-deficient compositions. Activation of the membrane-bound CCT rebalances the membrane PC content. PC homeostatic control by CCT is observed in cells as an acceleration of the CCT-catalyzed step and the overall rate of PC synthesis in response to changes in cell membrane lipid compositions; for example fatty acid [11] or diacylglycerol enrichment [12]. *In vitro* the activation of the pure enzyme can be monitored in response to lipid vesicles enriched in anionic lipid, diacylglycerol, or PE [13–16]. The AH of CCTα from rat is localized between residues ~234–300 [17]. Its polar face is rich in charged residues, both basic and acidic side chains form the interface between polar and non-polar helical faces [18], and its non-polar face contains many bulky hydrophobic residues that create a ~120° hydrophobic wedge for membrane intercalation (Fig. 1B). Its helical hydrophobic moment (peak $\mu_H = 0.69$) is one of the highest among characterized membrane-interacting AH motifs [1]. The CCT β_2 form also contains a conserved AH region, and diverges from the CCTα form only at the N and C-terminal ~50 residues. The AH of CCT resembles that of α-synuclein, the misfolding of which is associated with the pathology of Parkinson's Disease. Both are very long (70–90 residues [8,19]), contain a repeated 11mer sequence motif [20], and their binding mechanisms are reliant on both electrostatic and hydrophobic driving forces, [1,13,18,19,21,22]. Synucleins have been shown to both induce and sense membrane curvature [23–25]. We sought to compare the membrane remodeling activities of these two proteins in parallel.

Lagace and Ridgway [26] first showed that over-expression of CCTα can remodel membranes in cells. In that paper they reported that not only the full-length CCTα but a variant missing the amphipathic helical M domain was effective at inducing lipidic tubules in CHO cell nuclei, where the alpha isoform was localized. Tubulation by the constitutively active truncated construct was independent of oleic acid treatment, which triggers membrane translocation of the full-length CCT, and was suggested to be a result of massive phospholipids synthesis driving membrane expansion. Somewhat incongruously, expression of a catalytically dead full-length CCT induced a proliferation of nuclear tubules, suggesting a curvature driving force distinct from excessive synthesis and build-up of PC [26]. Mutations in domain M which either increased or decreased membrane affinity were accompanied by large changes in membrane tubulation proclivity in cells [27], implicating domain M in curvature induction. Lagace and Ridgway [26] also showed that CCTα transforms vesicles into tubules *in vitro*. Incubation of LUVs prepared from a complex synthetic lipid mixture or total brain lipid extract with CCTα promoted the formation of non-branching tubules with diameters in the range of 20–50 nm extending from the vesicles. The role of the M domain in curvature promotion *in vitro* was not tested.

In the present work we used EM to study in detail the process by which vesicular bilayers of various lipid compositions transform into very thin tubules as the concentration of bound CCT increases. We probed both lipid and protein determinants for this activity, and compared the efficiency of different CCT forms and α-synuclein.

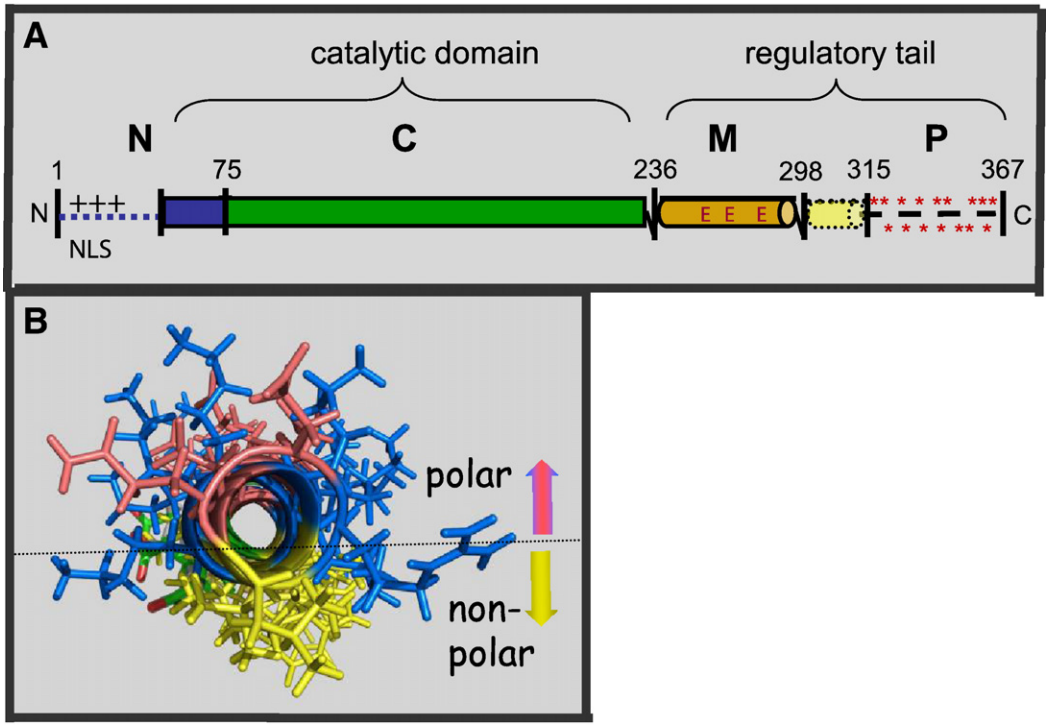

Fig. 1. (A) Domain structure of CCTα subunit indicating NLS (nuclear localization signal), the three glutamates (E) mutated to glutamine in domain M, the 16 phosphoserine sites (*) in region P substituted with glutamate in the PM variant. CCTα and β2 are highly conserved except for the N- and C-terminal regions indicated by the dashed lines. CCTβ2 lacks the NLS motif. (B). Domain M amphipathic helix (modeled from PDB 1pei and 1peh), with stick rendering of side chains, residues 242–288 viewed down helix axis. Yellow, hydrophobic sides chains; blue, basic; pink, acidic. The 3 glutamates are colored green (carbon) and red (oxygen).