Amphipathic Helices as Mediators of the Membrane Interaction of Amphitropic Proteins, and as Modulators of Bilayer Physical Properties

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Abstract: The amphipathic helix (AH) motif is used by a subset of amphitropic proteins to accomplish reversible and controlled association with the interfacial zone of membranes. Functioning as more than mere membrane anchoring domains, amphipathic helices can serve as autoinhibitory domains to suppress the protein activity in its soluble form, and as sensors or modulators of membrane curvature. Thus amphipathic helices can both respond to and modulate membrane physical properties. These and other features are illustrated by the behavior of CTP: phosphocholine cytidylyltransferase (CCT), a key regulatory enzyme in PC synthesis. A comparison of the physico-chemical features of CCT's AH motif and 10 others reveals similarities and several differences. The importance of these parameters to the particulars of the membrane interaction and to functional consequences requires more systematic exploration. The membrane partitioning of amphitropic proteins with AH motifs can be regulated by various strategies including changes in membrane lipid composition, phosphorylation, ligand-induced conformational changes, and membrane curvature. Several amphitropic proteins that control budding or tubule formation in cells have AH motifs. The insertion of the hydrophobic face of these amphipathic helices generates an asymmetry in the lateral pressure of the two leaflets resulting in an induction of positive curvature. Curvature induction or stabilization may be a universal property of AHA proteins, not just those involved in budding, but this possibility requires further demonstration.

INTRODUCTION

The amphipathic helix, one of the most common secondary structure motifs in proteins, is used by a certain set of proteins to mediate weak, reversible binding to cell membrane surfaces. These proteins are a subclass of proteins often referred to as amphitropic, based on their regulated interconversion between two environments, aqueous and lipidic. Functionality is associated only with the latter environment [1-4]. Not all amphitropic proteins utilize amphipathic helices to mediate membrane binding. Most use lipid clamp motifs designed for binding a unique lipid monomer. PH, PX, and FYVE domains for binding PIPs fall into this category, as do C1 domains for diacylglycerol [4]. Furthermore, not all proteins that use amphipathic helices to bind membranes are amphitropic. For example, prostaglandin H2 synthase-1 [5], Hepatitis C virus NP5A protein [6,7], and myelin basic protein [8], contain stable AH membrane anchors, that do not appear to interconvert with non-helical, non-membrane bound conformations. The amphipathic helix motif is well-suited for membrane binding that lacks strict lipid selectivity, that involves penetration to approximately the level of the glycerol backbone of the lipids, and that depends mainly on hydrophobic forces to drive binding. Moreover, this type of protein-lipid interaction is well-suited for either responding to or inducing surface curvature.

Amphitropic proteins containing amphipathic helices (**AHA proteins**) have a variety of functions. For example, CCT regulates PC biosynthesis, ARF and Sar1 regulate formation of coated vesicles involved in traffic between the ER and Golgi, and vinculin mediates attachment of actin and focal adhesion proteins to the plasma membrane. However, a common feature is their ability to sense changes in membrane properties and/or to act on the membrane to change its properties. Thus, some bind membranes in response to physical properties created by shifts in membrane lipid composition; others bind in response to other conformational triggers and their binding modulates membrane physical properties. We will discuss the properties of amphipathic helices in a set of amphitropic proteins with the criteria that: (1) The AH segment has been shown unequivocally to mediate the membrane interaction; (2) The AH motif has been structurally characterized as helical; and (3) A regulatory mechanism for the reversible interaction of the AH motif has been described. Many very interesting proteins have proposed AH motifs for mediating binding, but do not strictly meet these criteria. These include DnaA [9,10], GRK5 [11], and huntingtin [12]. We also do not discuss the amphipathic helices of exchangeable apolipoproteins or perilipins, which interact with lipid particles, rather than cell membranes.

One of the most well-understood AH motifs mediates membrane interactions of CTP: phosphocholine cytidylyltransferase (CCT). This review highlights the mechanism of lipid sensing by CCT's AH, the role of the AH domain in autoinhibition of the enzyme, and the structural consequences to the membrane resulting from CCT binding. Four lessons on the function and regulation of AH motifs emerge from the study of CCT, and are also emerging in studies of the behavior of other AHA proteins, which we will discuss. (1) Amphipathic helices sense membrane physical properties; (2) Amphipathic helices bind membranes weakly, and their binding is thus subject to regulation; (3) Amphipathic helices can function as autoinhibitory domains; and (4)

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Membrane insertion of amphipathic helices modulates bilayer physical properties.

The fourth aspect of AHA proteins has garnered much attention recently with the elucidation of the structure of several proteins containing amphipathic helices that induce or respond to membrane curvature [13,14]. This includes endocytosis-associated proteins containing an **N-BAR domain** [15], such as amphiphysin, and proteins containing an **ENTH motif** [16], such as epsin. Amphipathic helices contribute to both these motifs. MinD, a bacterial protein that functions in cell division site selection, can also induce curvature [17]. Other AHA proteins *recognize* curved membranes but do not induce curvature per se. Examples in this subset include Arf-GAP [18], a GTPase Activating Protein for ARF, and α-synuclein [19,20], a protein whose folding disorder is linked to neurodegenerative diseases such as Parkinson's.

LESSONS FROM THE AMPHIPATHIC HELIX OF CCT AND APPLICATION TO OTHER AHA PROTEINS

INTRODUCTION TO CCT'S STRUCTURE AND FUNCTION IN CELLS

CCT controls membrane PC content in its capacity as the key regulatory enzyme in the CDP-choline pathway for PC synthesis [21,22]. The conserved catalytic domain of CCTs from several plant, invertebrate, and vertebrate species is followed by a domain whose sequence conservation is relatively poor across phyla, but is a predicted amphipathic helix. All major isoforms of mammalian CCT (α, β1, β2, and β3) contain highly homologous amphipathic helices following the catalytic domain. Mutagenesis [23-26], proteolytic protection [26-28], lipid photolabeling [29], and analysis of the membrane binding of domain M peptides [30-33] have shown that domain M is the principle membrane binding domain of CCTα. The exact boundaries of the membrane-insertion domain are uncertain. At the N-terminal border Tyr- 240 relocalizes to a more non-polar environment in the presence of lipid vesicles as assessed by fluorescence blue shifts [32]. This tyrosine, along with Phe-234, is protected from proteolysis with chymotrypsin upon membrane binding [28]. At the C-terminal boundary a turn is predicted between residues 294-298. Although the putative amphipathic helix between 299-312 is not required for membrane association [25,34] there is no evidence that it does not contribute.

Domain M is thought to sense physical properties of membranes arising from a deficiency in PC – properties such as negative charge, loose head group packing, acyl chain disorder, and negative curvature strain (see below). Binding of domain M as an α-helix to such membranes relieves an as yet unidentified inhibitory constraint in the catalytic domain, thereby accelerating the catalytic efficiency, and restoring the PC content of the membrane. Dissociation of CCT from PC-rich membranes results in domain M helix → disorder transition and inter-domain interactions that regenerate the inhibition at the active site. This simple cycle would explain how CCT can respond to fluctuations in membrane lipid composition to control membrane PC homeostasis.

Properties of the Amphipathic Helix of CCTα

Domain M peptides spanning residues 236-299 undergo disordered → helix transitions in the presence of anionic vesicles, as detected by circular dichroism (CD) [30,31,35]. An increase in α-helix content for ~58 amino acids is also seen in CD analyses of full-length CCT, but not a truncated CCT missing domain M [35]. NMR-derived structures of two overlapping domain M peptides bound to SDS revealed an α-helix between residues 240-288 [33]. This unbroken helix is flanked at its N-terminus by a bend and a loose 4-residue coil, which may represent part of the linker to the catalytic domain. The structure of amino acids 243 – 287 is shown in Fig. (**1A**). It is strikingly amphipathic. The non-polar face carves out a ~120 degree hydrophobic wedge. Acidic residues dominate the centre of the polar face. The interfacial region between the polar and non-polar faces contains a strip of basic residues on one side, and a mix of basic and acidic on the other. The N-terminal one-third of domain M houses most of the basic amino acids. These contribute to the electrostatic binding component (see below). The binding is also hydrophobic in nature. The degree of penetration of the hydrophobic helical face has been estimated by comparing the fluorescence quenching of the tryptophan residue by brominated PCs. The greatest quenching was obtained with 9,10-dibromo PC, suggesting penetration to the middle of the outer leaflet of the vesicles [30]. The C-terminal third of domain M is relatively rich in aromatic amino acids, which would stabilize its interfacial localization [36].

Comparison with the Features of Other AH Motifs

Fig. (**1B**) illustrates AH motifs of many of the AHA proteins discussed in this review. None other than α-synuclein [37] have any sequence homology with the CCT helix. These two proteins contain an 11mer motif, repeated (imperfectly) 3 times in CCT and 7 times in α-synuclein. The consensus sequence is **V**EE**KSKE**F**I**QK for CCT, and **V**AX**K TKE**G**V**XX for α-synuclein (the bold residues show homology between CCT and α-synuclein). The AH motifs are displayed in Fig. (**1B**) as *ideal* α*-helices*, however this is not necessarily accurate. For example, CD, neutron diffraction, and molecular dynamics simulation based on the experimental data suggest that the ARF1 amphipathic helix extends only between residues 2-13 [38]. The CCT helix (in complex with SDS), structurally determined by NMR methods, is not ideal throughout its length [33]. α-Synuclein is a 3-11 helix, with 11 rather than the 10.8 residues per 3 turns characteristic of an α-helix. This periodicity has emerged from EPR analysis of the accessibility of a set of 41 single-site nitroxide-labeled α-synucleins [39], and from the effect of membrane-embedded spin labels on the ^{13}C-NMR backbone resonances [40]. This uncertainty in the radian between residues will influence the calculated hydrophobic moment [41] and the hydrophobic sector. The other uncertainty is the length of the helices in their membrane-bound form. We used structural information where available, but the boundaries of these AH domains are somewhat arbitrary. With these limitations, we compared the parameters shown in Table **1**. The AH motifs show large variation in length (14 – 52 amino acids) and hydophobicity of the non-polar face (1.65 –

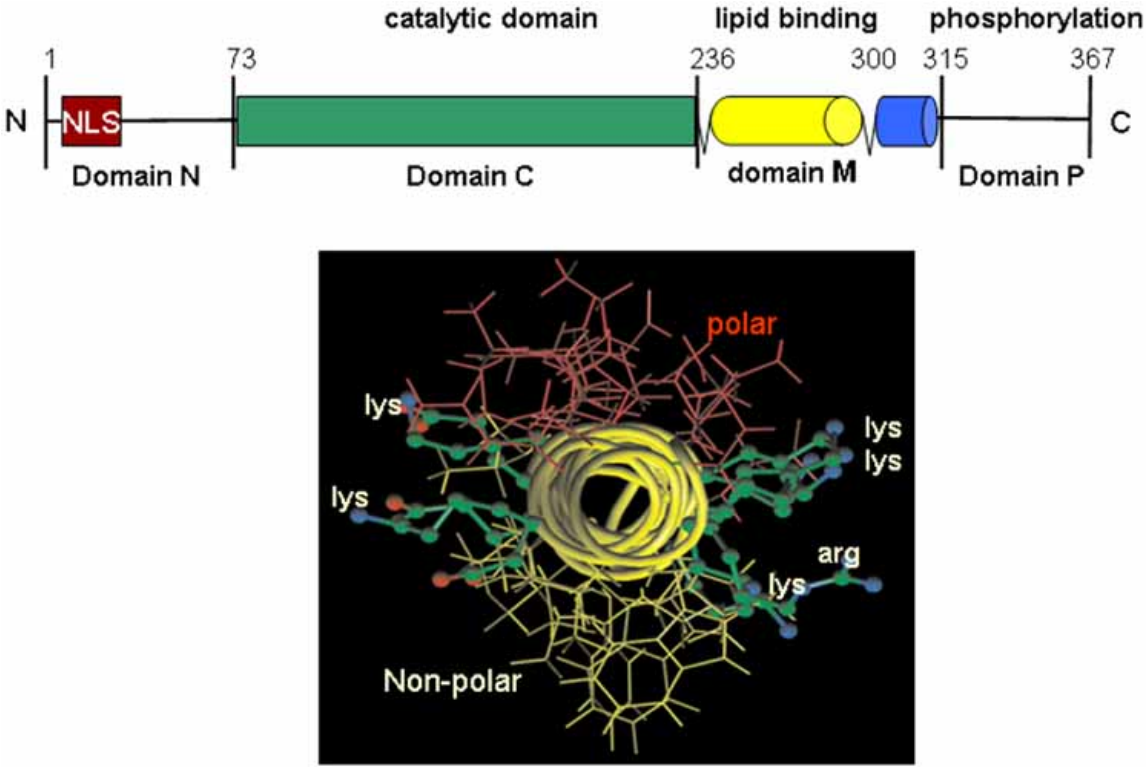

Fig. (1A). Domain Structure of CCTα (top) with an image of domain M (residues 243-287) displayed (bottom). The image is based on the atomic coordinates of two overlapping peptides solved by NMR [33]. The polar face is highlighted in red, the non-polar face in yellow, with the interfacial basic and acidic residues highlighted in ball and stick (carbon = green, oxygen = red, nitrogen = blue). The helical image in Fig. 1A is reprinted from Trends Biochem. Sci., Vol 25,Cornell, R.B. & Northwood, I.C., Regulation of CTP: phosphocholine cytidylyltransferase by amphitropism and relocalization, 441-447, 2000, with permission from Elsevier.

9.48 kcal/mol; Eisenberg consensus scale [42]). They vary on the content of aromatics (0 –5), and the character of the polar face. For example, the polar face is rich in basic residues in some, but a mix of acidic and basic in others. Arf-GAP has an unusually high serine content and α-synuclein has a high glycine content.

There are some common features. All have net positive charge, with the exception of Arf-GAP (-1). While the segment of CCT displayed (242-293) has a net charge of zero, the non-helical N-terminal flank (for which there is evidence of bilayer insertion [28,32]) contributes at least two more positive charges and upon docking on the membrane surface, three glutamates are protonated, as we discuss below. The charge of CCTs domain M as it docks onto the membrane surface is therefore +5. The AH motifs of amphiphysin, the related endophilin, and epsin are strongly basic with net charges of +7, +5, and +4, respectively. All peptides have oil → water partitioning free energies near zero, consistent with their amphipathic nature. The peak hydrophobic moments range between 0.4 – 0.8 on a scale where mellitin, a prototype amphipathic helix, has a value of 0.47. When the peak hydrophobic moment values for each peptide are plotted against their mean hydropathy, the coordinates for the 13 peptides in (Table **1**) cluster near the boundary defined by Eisenberg [42] for surface seeking helices. The one exception is MinD (*B. subtilis*), whose $<\mu_H>_{max}$ and <H> classify it as globular. The hydrophobic sectors are typically ~180°, but in the endocytosis-related proteins, amphiphysin, endophilin, and epsin, the hydrophobic sector is narrower, each 100° – 110°. In relation to an earlier classification scheme of amphipathic helices [43] the AH motif of CCT and α-synuclein resembles class A, and the AH motif of endophilin resembles class K in terms of the hydrophobic sector and charge distribution, but the others do not fit into designated categories.

In comparison to this set of amphipathic helices, CCT's domain M is the longest unbroken helix (52 residues). α-Synuclein is longer if the two consecutive helices are considered a membrane binding unit. CCT also has the highest concentration of charged residues (50%); the closest competitors for high charge density are Vinculin H3 and endophilin (each with ~38%), and amphiphysin and α-synuclein(1-36), each with ~33%. Several proteins share CCT's feature of the placement of one or more acidic residues at the boundary of the non-polar face: MinD, α-synuclein, Sar1, Arf-GAP, and vinculin H3. The hydrophobic sector of CCT more closely resembles that of the shorter endocytosis-related AH motifs found in epsin, amphiphysin and endophilin (~120° hydrophobic wedge).

LESSON #1. AMPHIPATHIC HELICES SENSE MEMBRANE PHYSICAL PROPERTIES

Response to Negative Surface Potential

Most amphitropic proteins using AH motifs bind selectively to lipid vesicles with anionic lipids. The particular

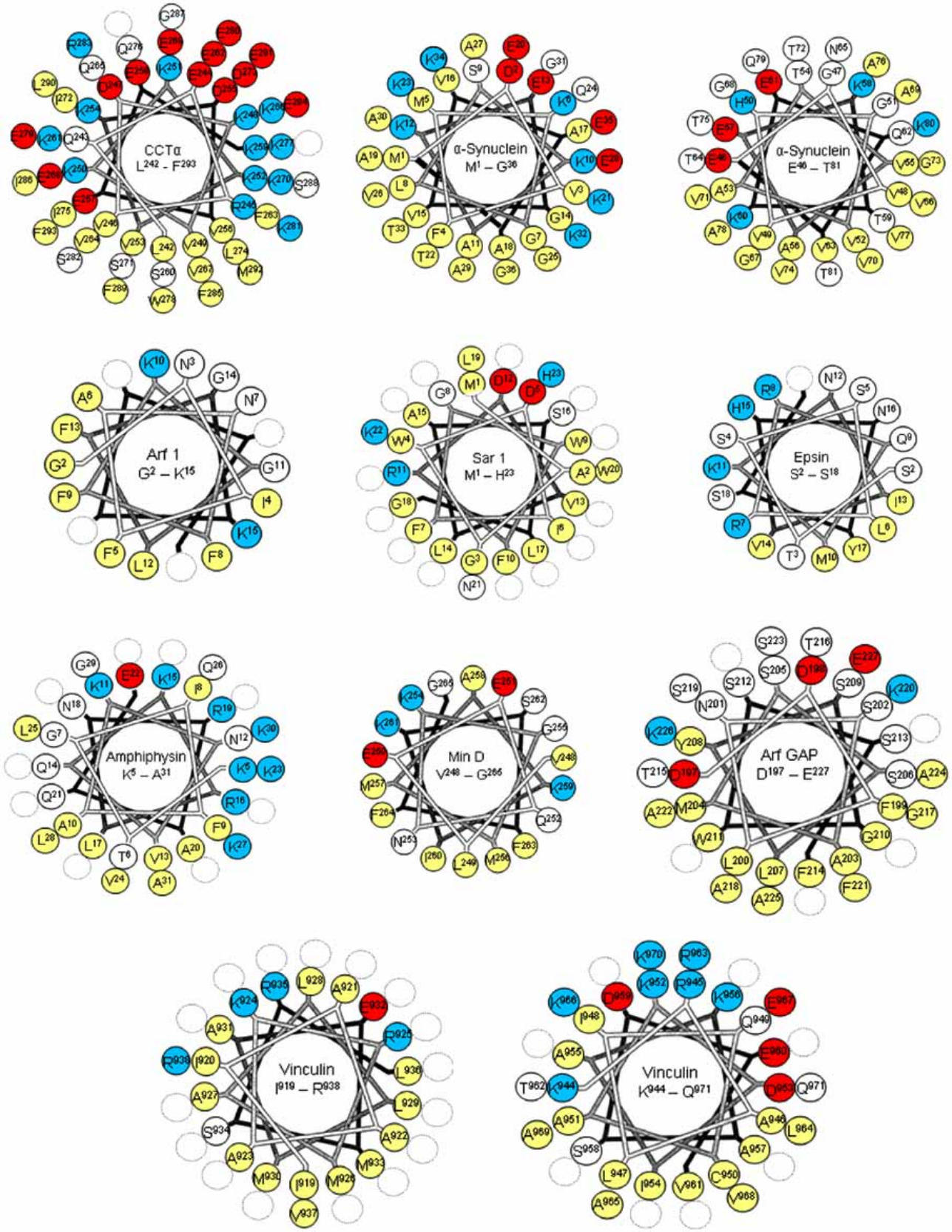

Fig. (1B). Helical wheel plots of 11 amphipathic helices in AHA proteins. The sequences displayed are from rat CCTα, human α-synuclein, bovine ARF1, S. *cerevisiae* Sar1, human epsin, rat amphiphysin, B. *subtilis* MinD, rat Arf-GAP, and human vinculin. Glycine is colored yellow (indicating hydrophobic), only when found in the non-polar helical face.

Table 1. Comparison of the Properties of AH Motifs of Selected AHA Proteins

Protein (residues)	Number of amino acid residues	Net charge	Positive charges K, R, H	Negative charges	Aromatic W, Y, F	Peak hydrophobic moment $<\mu_H>_{max}$ [b]	Mean hydropathy <H> (kcal/mol)[c]	Non-polar sector[d]	Mean hydrophobicity of non-polar face (kcal/mol)[e]	Hydrophobicity of non-polar face (kcal/mol)[f]
CCT (242-293)	52	0[a]	13	13	5	0.686	-0.29	120°	0.45	9.48
α - Synuclein (1-36)	36	+2	7	5	1	0.563	-0.02	180°	0.27	4.81
α - Synuclein (46-82)	37	+1	4	3	0	0.458	0.16	180°	0.28	5.01
Arf 1 (2-15)	14	+2	2	0	3	0.573	0.16	180°	0.55	3.87
Sar 1 (1-23)	23	+1	3	2	5	0.497	0.22	190°	0.35	4.22
Epsin (2-18)	17	+4	4	0	1	0.672	-0.26	110°	0.32	1.90
Endophilin (3-21)	19	+5	6	1	1	0.626	-0.15	100°	0.50	3.01
Amphiphysin (5-31)	27	+7	8	1	1	0.734	-0.29	110°	0.37	3.32
Min D (248-266)	19	+1	3	2	2	0.403	-0.014	140°	0.15	1.65
Arf GAP (197-227)	31	-1	2	3	5	0.468	0.15	180°	0.36	5.09
RGS4 (1-24)	24	+5	6	1	0	0.588	-0.066	170°	0.17	2.21
Vinculin H2 (919-938)	20	+3	4	1	0	0.620	-0.087	210°	0.36	4.33
Vinculin H3 (944-971)	28	+3	7	4	0	0.799	-0.121	160°	0.33	3.90

[a] see text for explanation;
[b] helical hydrophobic moment calculated using Eisenberg method [122] with the program EMBOSS using a sliding window of 11 residues and an angle of rotation of 100 degrees; value is reported for the peak $<\mu_H>_{max}$ of the sequence.
[c] hydropathy averaged over entire peptide calculated with the program EMBOSS using Eisenberg consensus scale, where the limits are +0.73 kcal/mol to -1.8 kcal/mol [42].
[d] angle subtended by the hydrophobic face of helix (estimated, +/- 10°);
[e] Eisenberg hydropathy of the residues in the hydrophobic face ÷ number of residues in hydrophobic face;
[f] sum of the Eisenberg hydropathy of the residues in the hydrophobic face.

head group is secondary; it is the net charge at the surface that dictates the strength of the binding. CCT binding and activation can be promoted by fatty acids >12 carbons in length, or by anionic phospholipids included in a zwitterionic PC vesicle [44-46]. The surface potential for half-maximal binding of CCT to anionic vesicles is -21 mV [47], equivalent to ~10 mol% singly charged anionic phospholipid at 0.125 M ionic strength. Despite their negative charge, PI and especially PIPs are poor activators; the reason for this is not known [48].

Since it is a response to the surface potential, binding can be reduced by raising the ionic strength. In addition, mutation to glutamine of the basic amino acids flanking the hydrophobic face in CCT's amphipathic helix led to progres-

sive reduction of binding that was sensitive to ionic strength and the anionic lipid content of the vesicles [32]. From this work the contribution of each lysine to the binding was estimated at only ~0.2 kcal/mol. The electrostatic energetic term for simple unfolded peptides that are adsorbed electrostatically at anionic surfaces has been estimated at 0.7 to 1 kcal/mol [49]. Because CCT peptides are not only adsorbed but inserted into the bilayer, an energetic cost of dehydrating the charged lysines decreases the net electrostatic component [50].

Domain M of CCT contains a cluster of basic residues in the N-terminal half. The VEEKS repeat region contains a mixture of basic and acidic residues and has a net charge of –2. Despite the net charge, peptides from this region *require* anionic lipids for membrane binding [30-32]. Their NMR-derived structures show that the lysines populate the interface between the polar and non-polar faces, whereas most of the acidic residues are positioned in the middle of the polar face (see Fig. **1A**). Thus the positioning of positively charged residues is more important than their number.

On the other hand, three glutamates, Glu-257, Glu-268, and Glu-279 are positioned at the border between the polar and non-polar helical faces (see Fig. **1A**). A negative charge on the peptide at these positions would clash electrostatically upon docking onto an anionic surface. The proposal that these glutamates serve as additional negative charge sensors stemmed from original work with a short unstructured palmitoylated hexapeptide containing a single carboxyl group, which inserts into lipid vesicles at low pH. Leenhouts *et al.* [51] showed that the carboxyl group dictated selection of PG/PC vesicles *vs.* PC vesicles as the pH was lowered due to selective protonation at the surface of the anionic vesicles, where the surface pH is effectively lower than bulk pH due to attraction of protons. Protonation of interfacial glutamates was also suggested for the anionic lipid selectivity of an apolipoprotein-derived peptide [52]. The binding of CCT [48] as well as domain M peptides [32] to anionic vesicles was pH-sensitive, and the apparent pK_a for binding varied with the mol% anionic lipid [32]. Substitution of the 3 interfacial glutamates with glutamine as a mimic for the protonated form eliminated the pH dependence of binding and greatly reduced anionic lipid selectivity. The glutamine-substituted peptide bound vesicles with much lower anionic lipid content. The membrane partitioning of full-length CCT expressed in COS cells increased by more than 5-fold upon substituting the three glutamates with glutamine [32]. Thus the glutamates serve to lower the hydrophobicity and affinity of domain M, preventing its association with membranes that have low anionic lipid content. This work also highlights a novel function of anionic lipids: to regulate protein-membrane interactions by modifying the charge and hydrophobicity of amphitropic proteins containing acidic residues.

Electrostatic and Hydrophobic Contributions in Other AHA Proteins

There is only a scattering of data reported concerning the electrostatic *vs.* hydrophobic contributions to the binding of other AHA proteins. In all cases except one (Arf-GAP) the AH motifs are net positively charged (See Fig. **1B** and Table **1**), and their binding is enhanced by increasing the acidic lipid content of vesicles, and diminished by raising the medium ionic strength [19,53-58] . This implies an electrostatic component to the binding, but does not evaluate the relative contribution of electrostatics *vs.* hydrophobic driving forces. Effects of mutations that reduce the positive charge or hydrophobicity on membrane binding have been evaluated for several AH motifs. Substitution of hydrophobic residues with negatively charged residues in the AH motif of MinD resulted in cytoplasmic rather than plasma membrane localization [59]. The binding of α-synuclein to acidic vesicles was disrupted by substitution of 6 threonines in the hydrophobic face with lysines [60]. Reduction of positive charge on the lysines at the boundary between the polar/non-polar faces by chemical modification also inhibits α-synuclein binding to acidic vesicles, albeit more weakly [60]. Mutation of four basic residues to alanine in the AH motif of RGS4 resulted in partial loss of helical induction and binding to anionic vesicles, whereas glutamine substitution of 3 leucines in the hydrophobic face more severely affected helix induction and completely abrogated binding [54]. Alanine substitution of Arg-11 at the border of the non-polar face of the Sar1 AH motif partially reduced vesicle binding and tubulation as well as budding efficiency, but much stronger effects were obtained upon alanine substitution of the aromatic residues Trp-4, Ile-6 + Phe-7, or Trp-9 [61]. Alanine substitution of leucine and phenylalanine residues on the non-polar face of Arf1 also reduced membrane binding in spite of the fact that this motif is linked at the N-terminal residue to a myristoyl chain [57]. These studies emphasize that while electrostatic attraction contributes to the binding, the hydrophobic character of the amphipathic helix is the key determinant of the binding strength.

The Arg and Lys residues in the very basic N-terminal helix of N-BAR domains or of ENTH domains have not been systematically explored for their role in binding. Phe →Glu substitution at positions 9 or 10 of amphiphysin's or endophilin's N-BAR reduced membrane interaction and tubulation activity [15,62]. However this substitution affects both charge and hydrophobicity, thus does not evaluate the respective contributions. High ionic strength lowered the binding affinity of epsin's ENTH domain ~10-fold, from 10^8 to 10^7 M^{-1} [56], but this domain includes the PIP_2 binding motif as well as the amphipathic helix "0". Other work has evaluated the role of some of the non-polar residues in the AH motif. Glutamine substitutions at Leu-6 or Met-10 of helix "0" resulted in 10-fold to 40-fold weaker binding [56] and poorer tubulation activity, whereas substitution to Trp, enhanced vesicle tubulation [16]. The ENTH domain containing the AH motif, but not an analogous domain (the ANTH domain) which lacks the AH motif penetrated monolayers containing 3% PIP_2. The mutants with weakened hydrophobic character were compromised in their penetration power [56]. Since the AH motifs in the N-BAR and ENTH proteins couple with other membrane-binding elements which have determinants for electrostatic and/or PIP_2-specific binding, the hydrophobic component of the amphipathic helix binding may dominate the interaction.

Several AH domains resemble CCT in that their polar faces are a mix of acidic and basic residues. Several studies indicate that lipid-bound α-synuclein's structure is an extended alpha helix with a break between residues 37-45

[37,63-65]. The acidic residues are mainly positioned opposite the hydrophobic face of the helices, like CCT. Acidic residues flanking the AH motif in the bacterial nucleotide exchange protein MinD have been postulated to function as membrane negative charge sensors, just as with CCT [66], but proof of this hypothesis is yet to emerge.

Response to Packing Disorder or Surface Defects at Phase Boundaries

CCT activation in liposomes containing increasing content of oxidized 1-palmitoyl, 2-arachidonoyl PC was correlated with acyl chain disordering, as demonstrated by ^{2}H-NMR-derived order parameters [67]. The effects on CCT activity and on acyl chain order were reversed by saturated sphingomyelin [67]. Selective activation of CCT was also observed at the gel/liquid crystalline phase boundary in PG/PC mixtures with varied acyl chain composition [44]. α-Synuclein also binds very strongly to lipid mixtures with phase separations, presumably binding at the phase boundaries [20,68]. This feature likely reflects the stabilization of the membrane upon insertion of the amphipathic helix into surface defects. The preference of MinD for lipid vesicles containing polyunsaturated acyl chains provides another example of membrane binding enhancement by lipid disordering [55].

Response to Negative Curvature Strain

CCTα also binds to membranes enriched in type II lipids such as DAG and unsaturated PE [69,70]. Type II lipids induce negative curvature in monolayers due to their inverted cone geometry, and create strain when present in a bilayer, which frustrates the curling tendency of each monolayer [71]. This strain is relieved upon insertion of an amphipathic helix into the interfacial region. The relative potency of a series of unsaturated PEs as CCT activators correlated very well with the calculated curvature strain energy [70]. The activation of CCT by dioleoylglycerol was also correlated with the curvature strain, and this activation was antagonized by lyso-lipids that induce positive curvature [70]. The activation of CCT by translocation to cell membranes by phospholipase C treatment to generate DAG *in situ* was also antagonized by lyso PC [72]. These data suggest that CCT might respond to negative curvature strain in cell membranes, but this remains only conjecture due to the inability to estimate this physical parameter in cell membranes. Another possibility – that the physical property generated by DAG and PE is surface hydrophobicity – was suggested by changes in the positioning of a polarity-sensitive fluorescent probe [70], but has also not been evaluated in cell membranes.

Response to Positive Curvature

Early work suggested that CCT binds >1000 times more effectively to SUVs than MLVs [44]. Careful analyses of the relationship between CCT binding and the geometric curvature are not yet available. ARF-GAP, like CCT, responds to DAG, and it was suggested that ARF-GAP senses packing voids at the surface created by the small head-group of DAG and other lipids [57]. A response to negative curvature strain energy could provide an alternative explanation for these findings. However, recent work has demonstrated that mammalian and yeast Arf-GAPs bind more avidly to vesicles as the radius of curvature increases [18,73]; thus Arf-GAPs respond to positive rather than negative curvature strain. This process regulates the uncoating of budding vesicles from the Golgi as discussed below under lesson #2. Membrane binding and the GTPase-promoting activity of Arf-GAP was greatest with vesicles of a diameter approximating that of Golgi –derived vesicles (R= 35 nm) [18,73]. The membrane curvature responsive motif, identified by mutagenesis, CD analysis, proteolytic protection, and membrane floatation, is the amphipathic helix illustrated in Fig. (**1B**) [18]. Alanine substitution of three bulky hydrophobic residues in this motif reduced the vesicle binding affinity, emphasizing the importance of the hydrophobic face for membrane insertion [18].

The amphipathic helices of α-synuclein bind to highly curved synaptic vesicles [74], and binding shows a strong dependence on the radius of curvature of model lipid vesicles [19,20,74]. Binding of α-synuclein to SUVs of DPPC led to a very large heat release detected by isothermal titration calorimetry, and a stabilization of the gel phase and melting cooperativity of the lipids, demonstrated by differential scanning calorimetry [19] or fluorescence anisotropy [75]. It was suggested that α-synuclein binds to surface defect sites in gel state SUVs, resulting in acyl chain ordering and annealing of surface defects [20]. Binding to surface defects is also compatible with curvature recognition for α-synuclein.

LESSON #2. AMPHIPATHIC HELICES BIND MEMBRANE SURFACES WEAKLY, AND THEIR BINDING IS THEREFORE SUBJECT TO REGULATION

Components of the Binding Reaction

Most amphitropic proteins distribute between soluble and membrane fractions in disrupted cell lysates where the cellular volume is diluted typically by a factor of 100. This leads to an underestimation of the percent bound in the intact cell. Factors that increase the membrane partition coefficient will result in redistribution towards the membrane. Changes in membrane affinity can also be seen via fluorescence microscopy as a transition from a diffuse fluorescence to an image outlining an organelle or the PM. For example, CCTα, a nuclear protein, translocates from the nucleoplasm to the inner membrane of the nuclear envelope upon stimulation of CHO cells with phospholipase C or oleic acid [76,77].

How strongly do the AHA proteins bind? Partition coefficients can be determined *in vitro* with lipid vesicles and pure proteins or the isolated AH peptide, but may only vaguely resemble the partition coefficient *in situ*, due to uncertainties in the lipid content of the membrane target site and contribution of other proteins to the membrane association. Measurement of binding affinities for AHA proteins is useful for analysis of the effect of mutations within the motif on binding strength and to determine lipid selectivity. In fact few binding energies are available for this class of proteins. The components of the membrane binding reaction of AH motifs in amphitropic proteins are likely the same as described for amphiphilic peptides in general, but with a strong hydrophobic component. These factors have been discussed elsewhere [1,78,79].

Partition coefficients for CCTs domain M and its subregions into phospholipid vesicles of various compositions have been measured and binding free energies calculated [31,32]. Side-by side analyses of the binding of CCT and domain M peptide have not been done using the same vesicles and analytical method. However independent analysis of a 62mer domain M peptide and full length CCT to PC/PG vesicles of the same composition give similar binding affinities, with ΔG_{bind} of -8.6 kcal/mol for the peptide and -8.8 kcal/mol for CCT [31,46]. Electrostatic attraction was estimated to contribute ~2 kcal/mol toward the total binding energy of 8.6 kcal/mol for a domain M peptide. This was derived from the difference in binding free energy of a 33mer peptide corresponding to the N-terminal basic region of domain M to PG/PC (1/1) vesicles and the same peptide with glutamine substitutions at the 8 interfacial lysines [32]. A similar ΔG_{el} was obtained from the loss of binding of this peptide in high ionic strength. The remaining 6.6 kcal is presumably due to the favorable enthalpy of helix formation [79-81] and/or favorable entropy associated with dehydration of ordered water around the non-polar side chains [82,83].

The importance of α-helix formation as a driving force for binding is supported by the common observation that the AH motifs are largely unstructured in an aqueous environment, and that phospholipid vesicle binding is associated with an induction of α-helical character [15,18,31, 35,54,84,85]. Exceptions to this rule include the N-terminal AH-motifs of ARF1 and Sar1, which are helical in both soluble and membrane-bound forms [86,87]. In these instances the hydrophobic residues of the AH motif pack into the interior of the soluble protein fold, and in response to GTP binding this orientation is disfavored in favor of exposure of the hydrophobic face for membrane insertion. Thus the nucleotide-induced conformational change is a key contributor to the binding equilibrium (see below). The amphipathic helices of vinculin are also present in the soluble form of this protein, where they self assemble as part of a five-helix bundle, resembling the fold of apolipoproteins [53,88]. The driving force for membrane binding of these AH-motifs appears to be a conformational change induced by the electrostatic attraction towards acidic lipids of a basic tail adjacent to the 5-helix bundle [88].

Features that Maintain Weak Affinity

In general these AH motifs on their own have dissociation constants in the micromolar range to millimolar range. The low affinities are maintained by relatively low-specificity Van der Waals interactions, although molecular dynamics simulations of amphipathic helices including ARF reveal that hydrogen bonding between peptide and lipid headgroups is prevalent [38,89]. The importance of the character of the non-polar face has not been explored for the most part. The non-polar face of CCT stretches some 7.5 nm in length and contains at least 18 hydrophobic side chains. Yet there is a curious interruption of the non-polar face with 3 serines (see Fig. **1**). Substitution of the three serines to alanine creates a domain with higher affinity, due to the hydrophobic driving force, and thus lower reliance on electrostatics [31]. This translates into a reduced selectivity for anionic lipids. Binding of the 3Ser → Ala mutant to PC vesicles was detected with a ΔG_{bind} = -5.6 kcal/mol, whereas this was not the case with the wild-type peptide. If these positions were occupied by very non-polar side chains such as isoleucine or phenylalanine, the affinity would possibly be too great to maintain selectivity for anionic lipids. A similar strategy may be employed in α-synuclein to augment its selectivity for anionic lipids. Five threonines are positioned within the hydrophobic face [37,39] see Fig. (**1B**). However, the effect of their substitution to a more hydrophobic residue has not been tested.

Regulation of Membrane Partitioning

Membrane Lipid Composition

One of the most common regulatory mechanisms is change in membrane lipid composition. As discussed above, most AH domains sense negative surface potential, implying regulation by anionic lipid content. Mechanisms for controlled variation in PIP_2 content, temporally and spatially, are well known [90]. Spatial control of the cardiolipin content in bacterial plasma membranes has also been described [91]. The short AH motif associated with the ENTH domain responds to PIP_2, but only as one portion of the PIP_2 binding pocket formed principally by other elements of the ENTH domain. Similarly, the N-terminal AH motifs of N-BAR domains are relatively short (<27 amino acids). Pairing with the BAR domain, which itself has strong selectivity for anionic lipids [15], would stabilize membrane association. Thus to provide longer-lived membrane adhesion short AH motifs may require coupling with another motif, which is the principle sensor of the changing membrane lipid content. For long AH segments, this coupling is not required, as in CCT, α-synuclein, and Arf-GAP1.

With respect to CCT, PIPs are the least effective activators of the enzyme *in vitro* [48], thus an electrostatic attraction induced by PIP domains does not seem plausible as a recruiting ploy for CCT. PA and fatty acids are minor anionic lipid species, and signal activated phospholipase A2 and D could exert controlled changes in the levels of these potential regulators. Moreover, these lipids are generated from PC hydrolysis, and if they were to serve as direct activators of CCT to accelerate resynthesis of PC, this would provide a mechanism for a PC metabolic cycle and PC homeostasis. However, there is no compelling evidence for direct CCT regulation by these lipids in response to physiological stimuli [92,93]. CCT membrane partitioning in cells can be promoted by exogenous oleic acid which results in oleic acid enrichment of membranes [32,94] but this is not a physiological situation. PC synthesis rates are linked to fatty acid synthesis rates [95], but this does not necessarily imply that fatty acids directly regulate CCT translocation.

Since the most abundant anionic phospholipids, PI and PS, should be dominant in maintaining the membrane surface potential, the question arises, are local fluctuations of PS and PI a factor in controlling the membrane partitioning of AHA proteins? Unfortunately, mechanisms for controlled variation of PS and PI content of cell membranes are poorly understood. Recent analysis of PS domain induction by annexin A2 suggest its potential role in the spatial organization of cell membrane PS [96].

The response to low mol% DAG *in vitro* by CCT and ARF-GAP raise the possibility of regulation in cells by varying the membrane DAG content. The domain responsive to DAG has not been identified for certain in either of these proteins, but is presumed to be the AH motif. Although full-length CCT binds to PC vesicles containing 15% DAG with an affinity much higher than that for 100% PC, the binding is still 30 times lower affinity than to 50% PG vesicles [46]. The binding of CCT domain M peptides to DAG-containing vesicles is too weak to measure (using a method in which the lower limit of K_a for detection is 10^3 M^{-1}) [31]. The response of the full-length enzyme to DAG-vesicles may rely on its dimeric nature. The binding affinity of the CCT dimer should be the square of the binding affinity of domain M for these vesicles.

Phosphorylation State of Protein

CCT is a phosphoprotein, with 16 potential serines in the C-terminal domain P, adjacent to the amphipathic domain M. This domain is not required for efficient catalysis nor for membrane binding [23,25,97] . The degree of phosphorylation of membrane associated CCT is less than that of soluble CCT in cells. Inhibitors of phosphatases lead to elevated phosphorylation status and an increase in soluble CCT [98]. Mutation of all 16 sites to alanine increased the membrane partitioning in cells, and mutation to aspartate to mimic the negatively charged phosphates decreased the membrane partitioning [99]. Dephosphorylation of the purified enzyme *in vitro* also enhanced its affinity for anionic phospholipid vesicles [45]. The mol% anionic lipid required for binding was lowered upon enzyme dephosphorylation. All these findings support the notion that CCT phosphorylation antagonizes membrane binding. As domain P is adjacent to the AH domain M a competition between the phospholipid phosphates and the phosphates on domain P for the lysines in domain M was proposed [45], but this idea remains experimentally untested. In support of a physiological role for control by phosphorylation changes, the phosphorylation status of CCT exhibits a periodicity throughout the cell cycle that correlates inversely with the rate of PC synthesis [100]. Although there is only scant supporting data, phosphorylation status may also regulate the reversible membrane association of FtsA, a putative AHA-protein functioning in tethering of the Z ring to the mid-cell region in bacteria [91,101].

Ligand-Dependent Conformation Switches

There are several AHA proteins whose membrane binding is regulated by the binding of nucleotide ligands. ARF and Sar1 are GTPases responsible for linking coat proteins and other proteins to COPI and COPII vesicles that traffic between the ER and Golgi. MinD is an ATPase that recruits MinC to the membrane and thereby inhibits cell division at the poles of bacterial cells so that division will occur at the midplane. Another bacterial ATPase, DnaA, contains a *putative* AH motif, and controls the unwinding of DNA to initiate its replication at OriC [102]. The GTP/ATP forms of these proteins are the active forms. The binding of GTP or ATP stabilizes the exposed helical conformation of the AH motif to insert into the bilayer, whereas GDP or ADP-binding stabilizes a buried conformation of the AH motif.

ARF1 contains a short AH motif at its N-terminus, which is also myristoylated. This motif is required for membrane binding and in ARF-GTP the AH inserts into the bilayer [38,57]. In ARF-GDP the non-polar face of this helix is buried [57,86] and interacts with the L2/L3 loop that stabilizes the nucleotide binding pocket in its GDP-binding form [103,104]. In Arf-GTP the L2/L3 loop is extruded along with a displacement of the Switch 1 motif, which participates in nucleotide binding.

MinD has a C-terminal AH motif, that is required for membrane binding [84]. Although this element is unstructured and not observed in crystals of MinD [105], peptides derived from this sequence undergo coil-to helix transitions assessed by CD in the presence of acidic lipid vesicles [106]. ATP binding stabilizes the membrane-bound form of MinD [55], and the membrane bound ATP-ligated form polymerizes on the membrane surface [17]. Szeto et al [84] proposed that the oligomerization of Min-ATP on the membrane binding would generate a multimer of helical binding motifs to greatly enhance the binding affinity for regions of high anionic lipid density.

Curvature

The clearest example of curvature sensing is Arf-GAP, a GTPase activating regulatory protein for the G protein, ARF. This curvature recognition (see also Lesson #1, response to positive curvature) controls the uncoating of COPI vesicles as follows [18,73]: Insertion of Arf1's N-terminal AH into the membrane induces positive membrane curvature (by analogy to Sar1 [61]), while cooperating with the exchange factor to release GDP and bind GTP. ARF-GTP then engages COPI and the bud grows as the coat bends the membrane surface. Arf-GAP is recruited to the membrane via its AH domain, which is thought to insert into surface packing voids present at the highly curved center of the bud. Membrane-bound Arf-GAP now acts on membrane-bound Arf-GTP, catalyzing its conversion to Arf-GDP, which dissociates from the coatomer and the lipid vesicle. This in turn leads to uncoating of the vesicle, presumably after fission takes place. Thus membrane curvature generation and sensing by AH motifs underlies the coating and uncoating (respectively) of these vesicles. The insertion of the AH of Arf-GAP, is triggered by positive curvature, and furthermore, its binding will stabilize the curvature of the COPI vesicle, as discussed under lesson #4.

LESSON #3: THE AH MOTIF CAN SERVE AS AN AUTOINHIBITORY DOMAIN

Domain M of CCT

Wang and Kent [23] were the first to show that the region now known as domain M inhibits catalytic function, and that this inhibition is relieved upon membrane binding. CCTΔ236, missing domain M plus the C-terminal domain P, is constitutively active (lipid- independent catalysis); whereas a truncation mutant missing only domain P requires lipid for activity [23,25]. Using CD and three deconvolution programs Taneva *et al* [35] showed that domain M takes on a mixture of conformations in the soluble form of CCT, and transforms into an α-helix in the presence of anionic lipids. Where domain M docks onto the N-terminal region of CCT

is not known. A proteolytic accessibility/mass spectrometry study suggested that the C-terminal region of domain M (residues 274 to 303) interacts with the C-terminal section of domain N (residues 36-69) [28]. These regions are inaccessible to both chymotrypsin and Arg C proteases in the soluble form. The domain N segment becomes more accessible in the membrane-bound state, in keeping with dissociation of domain M; domain M becomes membrane buried, and remains protease insensitive. Further support for an interdomain interaction between domain M and N is emerging from work with chimeras of CCTα and CCTβ2, which have divergent N-terminal domains, but are otherwise very similar. CCTβ2 binds more weakly than CCTα to membranes and thus may have stronger domain M/N interactions. A chimera comprised of domain N of CCTβ fused onto the remainder of CCTα generated a protein with weaker affinity for membranes than WT CCTα, while a chimera with domain N of CCTα fused to the rest of CCTβ bound with stronger affinity than WT CCTβ (M. Dennis, S. Taneva, R. Cornell, unpublished). These data show that domain N interactions dictate the affinity of domain M for the membrane.

Other Examples

ARF

The AH motif can be considered as part of an autoinhibitory domain [104]. Deletion of this AH generates an ARF that can be fully activated by a Sec7-domain nucleotide exchange factor [107]. The AH interaction with the L2/L3 loop prevents the rearrangement required to adopt the open, intermediate form between the inactive GDP-bound and the active GTP-bound form. How does membrane binding of the N-terminal myristate and amphipathic helix of ARF1 trigger GDP dissociation and GTP binding? The helix - membrane interaction dissociates the AH from the rest of ARF and thereby allows relocation of Switch 1, Switch 2, and the intervening L2/L3 segment. Exchange of GDP for GTP on ARF1 requires an exchange factor, ARNO, but is also dependent on displacement of the N-terminal AH by anionic lipid. There are, in effect, two cooperating exchange factors, ARNO and acidic lipid. The model emerging is that lipid binding of the AH motif assists ARNO 's action in moving the Switch 1 and 2 segments of ARF to pry open the GDP binding pocket and allow its release [103,104,108].

Vinculin

This cytoskeletal linker protein binds F-actin, talin, α-actinin, VASP, and acidic membrane domains in its fully functional form. In its non-functional form it remains in the cytosol with its AH motifs sequestered as a 5-helix bundle in an inter-domain interaction with the large globular "head" region. This interaction masks the binding sites for talin, actin and VASP [88,109]. In response to increased membrane acidic lipid content the basic C-terminal arm adjacent to the AH bundle draws the protein to the membrane, initiating an unfurling of the 5-helix bundle and membrane insertion of at least two of the AH-motifs. This model for the membrane activation of vinculin is supported by the requirement of the C-terminal arm for membrane binding [88], lipid photolabeling of the segments shown in Fig. (**1B**) [53], and by increased proteolytic sensitivity of membrane-bound vinculin [88].

LESSON #4. INSERTION OF AH MODULATES BILAYER PHYSICAL PROPERTIES

What effect do the amphipathic helices of AHA proteins have on bilayer properties when they bind and insert? Binding could impact on the electrostatics, packing strain, and curvature at the site of interaction.

Neutralization of Surface Negative Charge

The attraction of positively charged helices to a negatively charged membrane surface results in charge neutralization. This feature is by no means unique to AHA proteins, and since most AHA proteins are minor species, their binding will not induce large scale changes in membrane surface potential. α-Synuclein, on the other hand, is an abundant protein in neuronal cells, associating specifically with presynaptic vesicles. Since the AH motif in α-synuclein has a net charge of +3, its binding might have a substantial impact on the surface potential. Very recently analysis of phenotypes in transgenic mice has revealed that membrane-bound α-synapsin cooperates with the chaperone protein CSPα, to stabilize the correct folding of SNARE proteins, which are implicit in the synaptic vesicle – PM fusion mechanism [110,111]. One speculative idea is that α-synuclein *also* neutralizes the negatively charged phospholipid surface to promote the fusion event. MARCKS and GAP43 are proteins that bind tightly to and sequester PIP_2 into lateral domains [90], and although not yet experimentally supported, it is possible that some of the strongly basic AH motifs could sequester acidic lipids.

Elimination of Packing Stress

The two best-studied examples, α-synuclein and ARF-GAP, bind selectively to highly curved membrane vesicles, and may use surface defect-sensing to carry this out. By inserting into surface defect sites AH motifs can alleviate the curvature strain in vesicles of small radius, such as synaptic vesicles and vesicles involved in inter-organelle traffic, and in this way stabilize the curvature. Using isothermal titration calorimetry (ITC), Nuscher *et al*. [20] showed that the binding of the synaptic vesicle protein, α-synuclein, to PC SUVs in the gel phase was highly exothermic compared to SUVs of the same composition above T_m. The magnitude of the heat release could not be accounted for by helix formation alone. The phase transition temperature of DPPC vesicles increased and the transition narrowed upon addition of α-synuclein, as detected by DSC [20] and fluorescence anisotropy [75]. This suggested that α-synuclein binds to surface defect sites in gel state SUVs, ordering lipid acyl chains and annealing the defects at the sites of insertion of the amphipathic helix. This could explain the large negative enthalpy [20].

The binding of Arf-GAP to membranes varied inversely with the size of the lipid headgroup, and a surface-voids sensing mechanism for insertion of non-polar side chains of its AH motif was suggested [57]. When presented with very small, highly curved vesicles, the head-group size was less important, and it was argued that this is because the geometrical strain generates surface voids [18]. When the amphipathic helix of Arf-GAP inserts into these voids this strain will be relieved, leading to a more stably-packed surface *and* stabilization of the vesicle's high curvature.

Induction of Curvature

Nature has come up with several ways to bend a membrane [13]. One way, curvature induction by amphipathic helices, is a simple consequence of the asymmetric perturbation of lipid packing on only one leaflet of a vesicle. The promotion of curvature by AHA proteins is often assayed by tubulation; the transformation of flat lipid multilayers, LUVs or even monolayers into elongated tubes of low radius of curvature (i.e. 10-50 nm) [62]. Several proteins functioning in vesicular traffic promote the tubulation of large vesicles. These include proteins containing N-BAR domains (e.g. amphiphysin, nadrin [15] and endophilin [62]), ENTH domains such as epsin [16], as well as the GTPase, Sar1 [61]. Tubulation assays typically use ~1/1 protein/lipid weight ratios, corresponding to <100 lipid/protein molar ratio, and monitor the conversion of vesicle morphology by electron microscopic (EM) visualization. The protein concentrations are so high as to basically coat the vesicle surface. While this may be a ratio that inaccurately reflects the density of the protein on cell membranes, the assays intend to report on the potential of the peptide/protein to induce curvature by monitoring an exaggerated endpoint.

What is the role of the AH motif in these proteins in bending the membrane? The BAR domain is composed of three long helices interacting as coiled-coils, which self associate to form a banana-shaped dimer [15]. The concave face of the dimer has a radius of curvature of 12 nm, which would be compatible with the curvature of narrow neck of clathrin coated vesicles [112]. N-terminal to the BAR domain on amphyphysins, endophilin, and nadrins is a segment *predicted* to be an amphipathic helix. The helical content of amphiphysin N-BAR, but not the BAR domain missing the N-terminal segment, increased in the presence of lipid vesicles, as detected by CD, suggesting that lipids promote the formation of the amphipathic helical structure [15]. Endophilin generates narrow tubules *in vitro* with radii ranging from 10-50 nm [62]. Although deletion of the N-terminal AH motif reduced the efficiency, tubulation did not strictly require the AH motif; the intrinsically curved BAR domain on its own generated tubules [15]. Further analysis is needed to assess the quantitative contribution of the N-terminal AH and the BAR domain to curvature induction in these proteins.

The ENTH domain is a bundle of 8 helices which form a binding pocket for a $PI(4,5)P_2$ headgroup. The first helix of the bundle (the AH motif) is visible in the crystal diffraction pattern only in the complex with IP_3. This implies that membrane binding stabilizes the AH motif. Mutation of bulky hydrophobic residues on the membrane inserting face of epsin's AH drastically reduced its curvature activity [16]. The GTPases Arf1 and Sar1, which function in golgi ↔ ER traffic also have N-terminal amphipathic helices. Sar1 can induce curvature [61]. The membrane binding and curvature promotion of these proteins requires a GTP-induced protein conformational switch. As with epsin, mutagenesis to reduce the hydrophobicity of the AH motif in Sar1 crippled tubulation activity [61].

The curvature induction by these AHA proteins can easily be rationalized with their function in vesicle budding. MinD, a bacterial AHA-protein functioning in cell division site selection, also can induce tubule formation *in vitro* in its ATP-bound form [17]. This behavior may be a manifestation of a role *in vivo* to bind tubules associated with the invaginating midplane membrane during cell division [113]. A C-terminal AH motif has been identified as the membrane targeting domain of MinD based on the perturbation of membrane binding with mutants in this motif [114], phospholipid dependent α-helix induction of MinD-derived peptides [84], and membrane-triggered Trp fluorescence changes of C-terminal MinD mutants [59]. MinD-ATP binds to negatively charged lipid sites [55,84,106] which triggers polymerization [17,55], likely by enhancement of the surface concentration of MinD [91]. Although it is not clear whether the polymerization or the membrane insertion of the C-terminal AH membrane drives curvature, it is possible that both participate. The amphipathic helix action on the membrane would initiate curvature changes, and formation of the MinD polymer would stabilize tubes.

Less intuitive is the finding that CCT, an enzyme of PC synthesis, promotes tubulation *in vitro* and in cells. Addition of CCT to LUVs composed of brain lipids or a synthetic mixture (lipid/protein 100) converted the 400 nm vesicles to 20-50 nm diameter extended tubules [94]. CCT resides in the nucleus of CHO cells. Upon stimulation of CCT translocation to the nuclear envelope by oleic acid treatment, the frequency and extent of nuclear tubules increased. In mutant CHO cells lacking CCT, oleic acid did not produce this effect. Nuclear tubules, also referred to as the nucleoplasmic reticulum (NR), are invaginations of the nuclear envelope of uncertain function [115]. Immunogold labeled CCT was visualized by EM on the nuclear face of the invaginated structures. Over-expression of CCT also stimulated the proliferation of the NR [94]. The extent of NR proliferation was reduced or enhanced by mutations in domain M that alternately reduce or enhance CCT's membrane affinity [Ridgway, N., personal communication]. Why CCT should function in promoting the NR is of course unknown, since the function of the NR is unsolved, although there is some evidence to link it to nuclear→ cytoplasmic transport [115], or Ca^{+2} homeostasis [116].

What are the general requirements for curvature induction? Will any amphipathic helix do? A substitution of Leu-6 with the bulkier Trp residue in epsin's ENTH helix 0 did not affect membrane binding, but enhanced the tubulation ability [16]. This suggests that the character of the non-polar face, in particular the degree of lipid packing disruption, might be a determinant of curvature promotion. Work with acylated amphiphiles showed that the concentration of the acyl anchor which inserted into the membrane dictated the curvature efficiency [117]. Lee *et al.* [118] and Furuya *et al.* [119] explored some of the features required for tubule induction using a set of model basic amphipathic peptides. The most effective peptide, Hel 13-5, was comprised of 12 leucines + 1 tryptophan as the non-polar face and 5 lysines as the polar face. It transformed large vesicles of PC, PC/PG, as well as Golgi-like lipid mixture into nanotubes with radii of 20-25 nm [118,119]. The optimal features of the α-helical peptides for nanotube formation were (i) a high concentration of peptide/lipid; (ii) a length ≥ 18 amino acids, (iii) deep penetration of the hydrophobic residues. The charge of the polar face was not critical, nor was an α-helical conformation over

the full length of the peptide critical [119]. The width of the polar face was not varied in this study and all the peptides had a hydrophobic sector of ~260° [119], which is larger than naturally occurring AH motifs that promote budding. For example, the hydrophobic sector of epsin, amphyphysin or endophilin is only ~110°. Studies of this sort are needed that are designed to examine the features of natural curvature-inducing amphipathic helices.

SUMMARY AND FUTURE DIRECTIONS

AH motifs are being discovered in an ever-growing set of amphitopic proteins. A high proportion of the AHA-proteins described in this review function as nucleotide-binding molecular switches or regulators thereof. This includes Arf and Sar1, MinD and DnaA, Arf-GAP, and RGS proteins.

We have suggested here that the AH motif is used in amphitropic proteins to respond to changes in physical properties such as membrane negative charge density, surface voids, or negative curvature strain. Unfortunately, the data in support of this general conclusion arise mainly from *in vitro* work. Future work should strive to elucidate the mechanisms controlling membrane partitioning *in cells.* There have been a few strides forward on this front. For example, the recent work on the curvature-sensing properties of ARF-GAP provides an explanation for the regulation of membrane binding of this protein that explains how it operates spatially and temporally to control the uncoating of the budded vesicle [18,73]. MinD clearly responds to high negative charge density, as shown by defective localization in E. coli mutants with increased cardiolipin and PG content [55]. Proteins containing ENTH domains clearly respond to PIP_2 domains [120]. The binding of PIP_2 stabilizes the formation of the amphipathic helix 0 which participates along with other helices in the ENTH motif in forming a tight complex with PIP_2 [16,121]. For Arf1 and Sar1 it is GTP binding that serves as the switch to stabilize exposure of the non-polar face of the helix for membrane insertion. In this case there is no requirement for recognition of an altered membrane physical property, other than perhaps to target them to regions enriched in anionic lipids, since they do feature net positive charge. For other proteins, such as α-synuclein, RGS proteins, and CCT, the key modulators of membrane partitioning in cells remain elusive.

The AH motifs in CCT, Arf and vinculin are inhibitory domains whose displacement upon membrane binding serves to restructure the active site (CCT and ARF) or expose sites for key protein binding partners (vinculin). Do AH motifs in other AHA proteins have functions other than simple membrane partitioning? This will be known clearly only after a study of the conformation and interactions of the AH motif in the soluble form of these proteins. It is interesting that most of the AH motifs are located at the N- or C-termini of the AHA proteins. This may facilitate their disengagement from the folded protein to engage the membrane, with minimal restructuring of the rest of the protein.

Another important avenue for future work is to elucidate the features of AH motifs controlling curvature induction. Although most if not all amphipathic helices at sufficiently high protein/lipid ratios may generate positive curvature upon insertion into bilayers *in vitro*, is membrane bending an important function for them in cells? Future work would benefit from a side-by-side comparison of the curvature inducing potency of a comprehensive set of AH motifs. This functional parameter could be rationalized with structural features in these motifs (such as charge distribution, hydrophobic moment, non-polar sector, and length), and with binding parameters (including partition coefficient, the depth of bilayer penetration, and the contribution of electrostatic *vs.* hydrophobic forces). There is little empirical data on the binding properties of the AHA proteins. Many of them have only recently been discovered, but the time is ripe for in-depth biochemical and biophysical analyses. This knowledge might enable prediction of the factors to promote/disrupt curvature induction, and could be used to probe the functional importance of curvature inducing properties of AHA proteins in cells.

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ABBREVIATIONS

AH	=	Amphipathic helix
AHA protein	=	Amphipathic helix–containing amphitropic protein
CCT	=	CTP: phosphocholine cytidylyltransferase
PC	=	Phosphatidylcholine
PIP_2	=	Phosphatidylinositol (4,5) bis phosphate
IP_3	=	Inositol triphosphate
PG	=	Phosphatidylglycerol
DAG	=	Diacylglycerol
PA	=	Phosphatidic acid
PS	=	Phosphatidylserine
CD	=	Circular dichroism
ITC	=	Isothermal titration calorimetry
SUV	=	Small unilamellar vesicle
MLV	=	Multilamellar vesicle
LUV	=	Large unilamellar vesicle.

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