Molecular Neurobiology

ISSN0893-7648/05/32(1): 73–87/$30.00

Peptide Motifs

Building the Clathrin Machinery

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Abstract

Clathrin-coated vesicles (CCVs) form at the plasma membrane, where they select cargo for endocytic entry into cells, and at the trans-Golgi network (TGN) and the endosomal system, where they generate carrier vesicles that transport proteins between these compartments. We have used subcellular fractionation and tandem mass spectrometry to identify proteins associated with brain CCVs. The resulting proteome contained a near complete inventory of the major functional proteins of synaptic vesicles (SVs), suggesting that clathrin-mediated endocytosis provides a major mechanism to recycle SV membrane proteins following neurotransmitter release. Additionally, we identified several new components of the machineries for clathrin-mediated membrane budding, including enthoprotin/epsinR and NECAP 1/2. These proteins bind with high specificity to the ear domains of the clathrin adaptor proteins (APs)-1 and -2, and, intriguingly, they each utilize novel peptide motifs based around the core sequence ØXXØ. Detailed mutational analysis of these motifs, coupled with structural studies of the ear domains, has revealed the basis of their specificity for clathrin adaptors. Moreover, the motifs have now been recognized in multiple proteins functioning in clathrin-mediated membrane trafficking, revealing new mechanisms in the formation and function of CCVs. Thus, proteomics analysis of isolated organelles can provide insights ranging from peptide motifs to global organelle function.

Index Entries: AP-1; AP-2; α-ear, γ-ear; clathrin-coated vesicles; endocytosis; enthoprotin/epsinR; GGA; mass spectrometry; NECAP; synaptic vesicles.

Introduction

Eukaryotic cells employ numerous portals of entry at the plasma membrane, including phagocytosis, macropinocytosis, and clathrin-mediated endocytosis (CME) or caveolin-mediated endocytosis *(1)*. Of these, CME represents the best-understood pathway, and it regulates many important physiological processes essential to all cells. For example, the receptors for vital nutrients such as iron and cholesterol are internalized via clathrin-coated pits (CCPs) and

Received 8/17/04; Accepted 9/1/04.
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vesicles (CCVs). CME regulates cell communication as it modulates the surface levels of signaling receptors by mediating receptor downregulation following activation *(2,3)*. Additionally, it is now widely recognized that endocytic uptake of certain G protein-coupled and tyrosine kinase receptors is, in fact, necessary for their ability to engage intracellular signaling pathways *(2,3)*. Moreover, pathogens (such as the influenza virus *[4]*) and bacterial toxins (such as Shiga toxin *[5]*) subvert the clathrin-mediated pathway to gain entry into cells.

Clathrin-mediated membrane budding also occurs at the membranes of the trans-Golgi network (TGN), leading to the generation of carrier vesicles that transport cargo from the TGN to the endosomal system *(6,7)*. For example, mannose-6-phosphate receptors (MPRs) bind to mannose-6-phosphate-tagged lysosomal hydrolases in the lumen of the TGN and package these enzymes into CCPs for transport to endosomes/lysosomes *(8)*. Deficiencies in MPR expression lead to the secretion of lysosomal hydrolases with resultant abnormalities in lysosomal function and the development of lysosomal storage disease *(9)*. More recently, clathrin-mediated membrane budding has been implicated in the formation of transport vesicles from endosomes *(6,7,10–12)*.

In the nervous system, additional and unique roles are observed for clathrin-mediated budding, particularly for CME. For example, the levels of amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors at the cell surface are partly controlled via CME, contributing to certain forms of synaptic plasticity *(13,14)*. Constitutive endocytosis of γ-aminobutyric acid A receptors plays an important role on inhibitory neurotransmission *(15)*. Moreover, CME is believed to be essential for the recycling of synaptic vesicle (SV) membrane proteins following the collapse of SVs into the plasma membrane during neurotransmitter release *(16,17)*. However, neurotransmitter release without membrane collapse and subsequent recycling through CCVs (referred to as kiss-and-run secretion) has also been proposed, although the relative prevalence of these two mechanisms remains unclear *(18)*. The need to develop a complete understanding of the molecular basis of clathrin-mediated budding is underscored by the many important physiological functions controlled by this process.

Subcellular Proteomics: Functional Conclusions on CME and SV Recycling

To better understand the clathrin-budding machinery, we used subcellular proteomics toward the characterization of brain CCVs *(19,20)*. Subcellular proteomics is a technique that uses recent advances in protein and peptide separation technologies combined with innovations in the application of tandem mass spectrometry (MS/MS) to analyze the protein components of organelles or other subcellular compartments that have been isolated by subcellular fractionation *(21,22)*. The ability to determine the complete protein complement of an organelle and to monitor dynamic changes in protein composition under various conditions can allow for global insights into organelle function that are not available from the study of individual protein components. Moreover, the subcellular proteomics approach provides important advantages over the proteomic analysis of whole cells or tissues. First, because of the extremely broad range of protein expression levels in a tissue lysate, less abundant proteins are below detection or masked by higher abundance proteins *(21)*. These problems are lessened by the isolation of an organelle, which generates a greatly reduced protein complexity with the enrichment of lower abundance proteins specific to the compartment *(22,23)*. Second, identified proteins are linked to a functional unit—that is, the organelle under study. In our studies, CCVs were isolated using modified versions of well-developed procedures involving differential centrifugation and velocity and density gradients in sucrose *(20,24)*. CCV proteins were separated by one-

dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the complete gel lanes (cut into even sized horizontal gel slices) were analyzed in an automated system by nanoscale reverse phase liquid chromatography quadrupole time-of-flight (LC Q-ToF) MS/MS. From three independent preparations, 209 proteins were reproducibly identified *(20)*.

We were intrigued by the observation that the amount of protein detected by Coomassie blue staining in any given gel slice of fractionated CCV proteins appeared to correlate to the total number of MS/MS determined peptides that had been assigned to proteins in that slice *(20)*. This suggested a correlation between peptide counts and protein levels. To further explore this, we examined peptide counts for components of known protein complexes on CCVs. Two major components of clathrin coats are clathrin (composed of clathrin heavy chain with associated clathrin light chains) and adaptor protein (AP)-1 and AP-2 *(25)*. As outlined in further detail later, AP-1 and AP-2 are heterotetrameric protein complexes that play central roles in the formation of CCVs at the TGN/endosome and the plasma membrane, respectively. When normalized for protein size (larger proteins generate more peptides per mole), we were detected the expected 1:1 ratio for clathrin heavy and light chains and the expected 1:1:1:1 ratios for the subunits of the AP-1 and AP-2 complexes *(20)*.

These results suggest that peptide accounting is a valuable approach toward determining relative levels of protein expression within a proteome. In fact, Yates and colleagues described an approach called spectral sampling, in which they demonstrated a linear relationship between the level of sampling for a protein (number of MS/MS spectra) and the relative abundance of that protein within complex mixtures *(26)*.The relationship remained linear for six different marker proteins over two orders of magnitude *(26)*. Stable isotope-labeling methods, such as the isotope coding affinity tag technique, are excellent tools for measuring small differences of protein expression between proteins from distinct proteomes *(27,28)*. However, the peptide accounting/spectral sampling approach appears to detect differences over a broader dynamic range *(20,26)*. Moreover, it provides an easy method to globally examine relative levels of protein expression within a single proteome.

For CCVs, approx 50% of all assigned peptides came from the 18 proteins that were identified as known components of the clathrin coat *(20)*. Interestingly, we noted an approx 3:1 molar ratio for AP-2 over AP-1 *(20)*. In a quantitative proteomic analysis of liver CCVs, we found this ratio was inversed, and the opposition of these ratios was confirmed by Western blot *(29)*. This suggests that in whole brain, AP-2-dependent endocytosis predominates over AP-1-mediated intracellular trafficking. One possible explanation for this abundance of clathrin-mediated trafficking from the plasma membrane in brain comes from the observation that the single largest category of proteins identified in the brain CCV proteome consisted of the 32 that cover most of the known components of SVs *(20)*. CCVs have long been implicated in the recycling of SVs following SV collapse into the plasma membrane concomitant with neurotransmitter release *(16,17)*. However, recent studies have suggested that kiss-and-run is a more predominant mode of SV retrieval in specific neuronal populations *(30,31)*. Our identification of a near complete inventory of known and well-characterized SV proteins suggests the recycling of SVs as main function of CCVs in brain. Although this does not address the relative levels of full fusion vs kiss-and-run, our results indicate full fusion of SVs as a prevalent mechanism of neurotransmitter release. Therefore, the global analysis of the protein components of CCVs has provided important insights into the functional activities of the organelle.

Machinery for Clathrin-Mediated Membrane Budding

Subcellular proteomics also provides a valuable approach toward the identification of

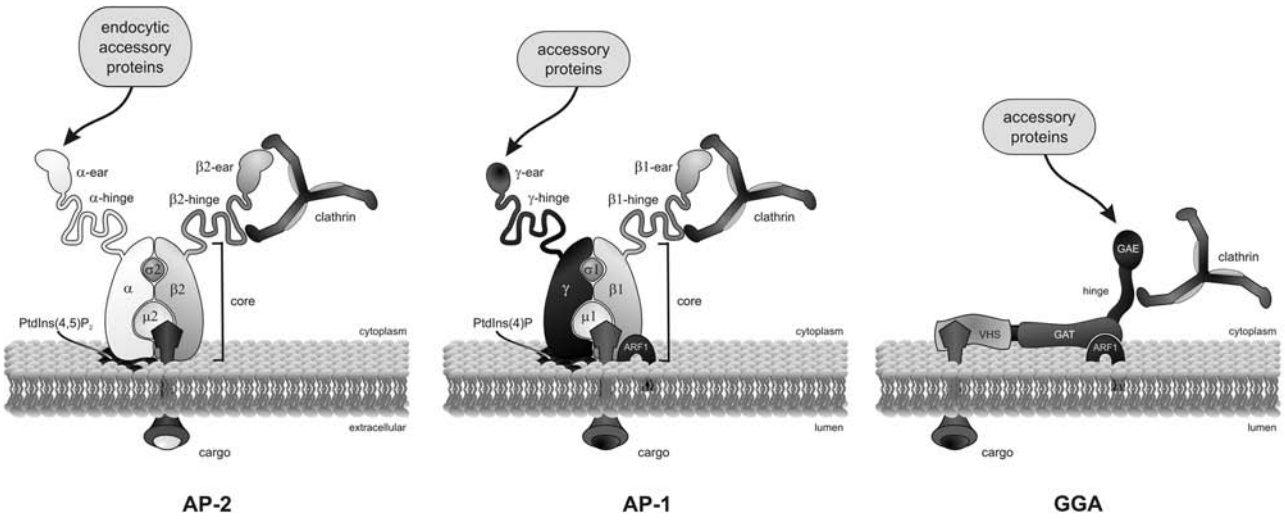

Fig. 1. Domain structures of the major clathrin adaptor proteins. AP-2 and AP-1 are heterotetramers composed of α-, β2-, μ2-, and σ2-adaptin subunits and γ-, β1-, μ1, and σ1-adaptin subunits, respectively. The α- and γ-subunits bind to PtdIns(4,5)P_2 and PtdIns(4)P, respectively, which helps anchor the adaptors at membranes. The β-subunits bind to clathrin triskelia and, along with the μ-subunits, help recruit cargo into nascent clathrin-coated buds. The α-ear and γ-ear bind to accessory proteins that regulate various aspects of clathrin-mediated membrane budding. The GGAs are monomeric and are composed of Vps27p, Hrs, STAM (VHS), GAT, and GAE domains. The VHS domain binds to cargo, whereas the GAT domain binds to ARF1, linking the GGAs to the membrane. Clathrin triskelia bind to the hinge region, and the GAE domain binds to accessory proteins for clathrin budding.

novel components of cellular compartments *(22,23)*. The machinery that regulates the formation and function of CCVs is very complex. Key components are clathrin, AP-1, and AP-2 *(25,32)*. AP-2 is composed of two large subunits (α- and β2-adaptin), a medium subunit (μ2-adaptin), and a small subunit (σ2-adaptin) (Fig. 1). The N-terminal regions of α and β2, together with the μ2- and σ2-subunits, form the core of the AP-2 structure, and the C-termini of α- and β2-adaptin are globular structures referred to as ear domains, which project from the core on long, unstructured flexible hinges *(7,33,34)*. AP-1 is composed of γ-, β1-, μ1-, and σ1-adaptin subunits with an analogous structural organization as the α-, β2-, μ2-, and σ2-subunits of AP-2 (Fig. 1).

Each of the AP subunits serves distinct functions (Fig. 1). The α- and γ-adaptin subunits bind to specific inositol phospholipids, facilitating their interactions with appropriate membranes *(35–37)*. The β-subunits bind to clathrin and promote its assembly into clathrin cages, and cargo proteins concentrate in nascent CCPs through the interaction of targeting sequences with the μ- and β-adaptin subunits in their cytoplasmic domains *(38–40)*. Finally, the ear domains of α- and γ-adaptin act as recruitment platforms for proteins that regulate various aspects of CCV formation and function *(34,39)*. These features allow the AP complexes to operate as master regulators in the formation of CCVs that contain appropriate cargo (Fig. 1). Intracellular clathrin-mediated trafficking also uses the monomeric clathrin adaptors Golgi-localized, γ-ear-containing, Arf-binding proteins (GGAs) 1, 2, and 3 *(41)*, in which various domains within the monomer fulfill similar functions as the subunits of the heterotetrameric AP complexes (Fig. 1). The VHS domain recruits cargo such as MPR, whereas the GAT domain contributes to membrane targeting through interaction with guanosine triphosphate-bound Arf *(41)*. A flexible linker

region that functions in clathrin recruitment connects to the C-terminal γ-adaptin ear-like (GAE) domain, which interacts with regulatory proteins, similar to the γ-adaptin ear (Fig. 1).

At the plasma membrane, a large assortment of endocytic regulatory proteins has been found to contribute to CCV formation (Table 1; for reviews, *see also* refs. *2,3,32,39,40,42–51*). Many of these proteins bind directly to membranes, clathrin and/or AP-2, and each other, forming a complex "interactome." They can function cooperatively with AP-2 or independent of the adaptor to recruit clathrin to the plasma membrane, to stimulate its assembly into clathrin lattices, and to recruit cargo into nascent CCPs. They also regulate the levels of inositol phospholipids and contribute to the biophysical curvature of the membrane, mediate the fission of deeply invaginated CCPs, control CCV uncoating following vesicle release, and link CCPs and CCVs to signaling molecules and components of the actin cytoskeleton. Many of these proteins are expressed in brain at higher levels than in non-neuronal cells, and they are often enriched in presynaptic terminals (Table 1), consistent with an important role for CCVs in the recycling of SVs. Although CCV formation has been studied most intently at the plasma membrane, complex regulatory protein networks appear to exist for clathrin-mediated membrane transport at the TGN and endosomes *(34)*.

The complex interactions among the components of the clathrin-budding machinery are largely mediated through specific modular domains and peptide motifs (Table 1). Some of these modules, such as src homology 3 (SH3) domains, are recognized in proteins that function in a wide variety of biological processes *(52)*. Within the endocytic machinery, amphiphysin, endophilin, PACSIN/syndapin, and intersectin use SH3 domains to interact with proline-rich motifs found in dynamin and synaptojanin, recruiting these enzymes to sites of endocytosis (Table 1; ref. *42*). Phosphotyrosine-binding (PTB) domains are broadly used by signaling proteins to interact with tyrosine-phosphorylated residues *(53)*. Within endocytic proteins, such as autosomal recessive hypercholesterolemia (ARH) protein and disabled2, PTB domains bind to phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) and to FXNPXY motifs found in the cytoplasmic tails of cargo receptors; therefore, they function in the recruitment of cargo to CCPs *(39)*. The Bin/amphiphysin/ Rvs (BAR) domain, which was originally studied in endocytic proteins (including amphiphysin and endophilin), binds to highly curved negatively charged membranes and may function as curvature-sensing module *(54,55)*. The function of the BAR domain has recently been extended to multiple protein families with variable vesicle trafficking functions *(55)*.

Other modules appear to be more restricted to components of the clathrin-budding machinery (Table 1). For example, the binding of endocytic proteins containing Eps15 homology (EH) domains to others bearing the tripeptide NPF supports the formation of endocytic protein networks *(43)*. Epsin N-terminal homology (ENTH) and AP180 N-terminal homology (ANTH) domains have been found almost exclusively in proteins of the clathrin-budding machinery *(48)*. By binding to specific inositol phospholipids, these modules help anchor their parent proteins to membranes and play a role in the regulation of membrane curvature by modulation of the biophysical properties of membranes *(48)*. Other endocytic regulatory proteins contain short peptide motifs (referred to as clathrin boxes) that allow direct interactions with the globular N-terminal domain of the clathrin heavy chain *(56–58)*. Together, these modules and peptide motifs allow for the organization of the endocytic machinery necessary for the proper formation of CCVs that bear appropriate cargo.

Additionally, many of the components of the endocytic machinery bind to the ear domain of the α-adaptin subunit of AP-2 (α-ear) (Table 1). The α-ear is a two-lobed structure consisting of an N-terminal β-sandwich domain and a C-terminal platform domain composed of mixed α-helices and β -sheets (refs. *59* and *60*; Fig. 2). The identification of peptide motifs that mediate α-ear binding have resulted in important

Table 1
Accessory Proteins Involved in Endocytosis

Protein	Function	Binding domains/motifs	Partners	Tissue
AAK1	Ser/thr kinase, phosphorylates μ2-adaptin, regulates recruitment of YXX∅-based sorting motifs to AP-2	DPF, WXXF-acidic NPF DLL	α-ear platform/sandwich ? ?	Brain/liver, brain expression presynaptic
Amphiphysin	Recruits dynamin/synaptojanin to membranes, possibly coupled to membrane curvature	BAR domain SH3 domain DPF (amphi. I), FXDXF PRD Clathrin box	Membranes (electrostatic) Dynamin, synaptojanin α-ear platform Endophilin Clathrin terminal domain	Amphiphysin 1, enriched in brain, presynaptic Amphiphysin 2, brain specific splice variants presynaptic
Aftiphilin	Unknown	WXXF-acidic ∅XX∅	α-ear sandwich γ-ear/GAE domain	Brain, presynaptic, TGN
AP180	Clathrin membrane recruitment and assembly	ANTH domain DLL DPF, FXDXF	PtdIns(4,5)P_2 Clathrin terminal domain α-ear platform	AP180, enriched in brain, presynaptic CALM, ubiquitous homolog
ARH	Endocytosis of lipoprotein receptors	PTB domain Clathrin box ?	LDLR, PtdIns(4,5)P_2 Clathrin terminal domain β2-ear	Hepatocytes
Auxilin 1	Recruits Hsc70 to CCVs, stimulates clathrin uncoating activity of Hsc70	DNAJ domain DPF Clathrin box	Hsc70 α-ear platform Clathrin terminal domain	Enriched in brain, presynaptic
Auxilin 2/GAK	Recruits Hsc70 to CCVs, stimulates clathrin uncoating activity of Hsc70, ser/thr kinase, phosphorylates μ2-adaptin	DNAJ domain DPF, WXXF-based Clathrin box	Hsc70 α-ear platform/sandwich Clathrin terminal domain	Ubiquitous
β-arrestin	Endocytosis of GPCRs Clathrin recruitment, not assembly	Arginine residues Clathrin box Argine/lysine residues	β2-ear Clathrin terminal domain PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3	Ubiquitous
Dab2	Endocytosis of lipoprotein receptors Modulates cystoskeleton organization	PTB domain PRD Clathrin box DPF, FXDXF WXXF-acidic NPF	LDLR, PtdIns(4,5)P_2 CIN85, Grb2 Clathrin terminal domain α-ear platform α-ear sandwich ?	Ubiquitous
Dynamin	GTPase, drives fission reaction, release of CCVs from membrane	PRD PH domain	Amphiphysin, endophilin, Intersectin, PACSIN Inositol phospholipids	Dynamin 1, enriched in brain, presynaptic Dynamin 2, ubiquitous Dynamin 3, enriched brain/testis
Endophilin	Regulates membrane recruitment and phosphatase activity of synaptojanin, possibly coupled to membrane curvature	BAR domain SH3 domain	Membranes (electrostatic) Dynamin, synaptojanin, Amphiphysin	Endophilin 1, enriched in brain, presynaptic Endophilin 2, ubiquitous Endophilin 3, enriched brain/testis

Epsin	Clathrin recruitment and assembly, membrane curvature, adaptor for ubiquitinated cargo	ENTH domain Multiple DPW NPF Clathrin box	PtdIns(4,5)P_2 α-ear platform EH domains of Eps15, intersectin Clathrin terminal domain	Epsin 1, enriched in brain, presynaptic Epsin 2, enriched in brain Epsin 3, restricted to epithelial wounds
Eps15	Regulation of clathrin assembly	Multiple DPF EH domain	α-ear platform NPF motifs in epsin, AP180 Synaptojanin170	Enriched in brain, presynaptic
HIP1	Regulation of clathrin assembly through clathrin-light chain interactions, cargo adaptor for AMPA receptors	ANTH domain DPF, FXDXF Clathrin box Helical domain Talin-like domain	Phospholipids α-ear platform Clathrin terminal domain Clathrin-light chain ?	Enriched in developing brain
HIP1R/HIP12	Regulation of clathrin assembly through clathrin-light chain interactions, productive linkage of CCPs to actin	ANTH domain Helical domain Talin-like domain	Phospholipids Clathrin-light chain Actin	Enriched in brain
Hsc70	Uncoating ATPase	ATPase domain	Auxilin, auxilin 2/GAK	Ubiquitous
Intersectins-s	Scaffold at endocytic sites, couples endocytic and signaling pathways	EH domains SH3 domains	NPF motifs in epsin Dynamin, synaptojanin, N-WASP, Sos, cdGAP	Intersectin 1-s, Ubiquitous Intersectin 2-s, ubiquitous
Intersectins-l	Scaffold at endocytic sites, couples endocytic and signaling pathways, GEF for Cdc42, regulates actin assembly	EH domains DH/PH SH3 domains	NPF motifs in epsin Cdc42 Dynamin, synaptojanin, N-WASP, Sos, cdGAP	Intersectin 1-l, enriched in brain Intersectin 2-l, ubiquitous
PACSIN/ Syndapin	Links endocytosis and actin cytoskeleton	Coiled-coil SH3 domains	Oligomerization Dynamin, N-WASP, synaptojanin, Sos, huntingtin (PACSIN 1)	PACSIN 1, enriched in brain PACSIN 2, ubiquitous PACSIN 3, heart, muscle, and lung
NECAPs	Unknown	WXXF-acidic ∅XX∅	α-ear sandwich γ-ear/GAE domains	NECAP 1, enriched in brain NECAP 2, ubiquitous
Stonin 2	Potential regulation of synaptic vesicle recycling	NPF WXXF-acidic μ2 homology domain	EH domains of Eps15 α-ear sandwich and μ2 synaptotagmin I	Enriched in brain, neuronal
Synaptojanin 145	Dephosphorylates inositol phospholipids, regulates coat stability	WXXF-acidic PRD	α-ear sandwich Amphiphysin, endophilin, intersectin, PACSIN	Enriched in brain, presynaptic
Synaptojanin 170	Dephosphorylates inositol phospholipids, regulates coat stability	DPF, FXDXF WXXF-acidic PRD	α-ear platform α-ear sandwich Amphiphysin, endophilin, intersectin, PACSIN	Ubiquitous, developing brain

For function, binding domains/motifs and binding partners, only major activities related to endocytosis are indicated. AAK1, adapter associated kinase; AP-2, adapter protein 2; BAR, Bin/amphiphysin/Rvs161/167; SH3, src-homology 3; PRD, proline-rich domain; GAE, γ-adaptin ear-like; TGN, trans-Golgi network; AP180, adapter protein 180; ANTH, AP180 N-terminal homology; PtdIns, phosphatidylinositol; CALM, cathrin assembly lymphoid myeloid leukemia; ARH, autosomal recessive hypercholesterolemia; PTB, phospho-tyrosine binding; LDLR, low-density lipoprotein receptor; CCVs, cathrin-coated vesicles; GAK, cyclin G-associated kinase; GPRC, G protein-coupled receptor; Dab2, disabled2; CIN85, c-cbl-interacting protien of 85 kDa; PH, pleckstrin homology; PACSIN, PKC and CK2 substrate in neurons; ENTH, epsin N-terminal homology; EH, Eps15 homology; HIP, huntingtin-interacting protein; cdGAP, cdc42 GTPase-activating protein; GEF, guanine-nucleotide exchange factor; cdc, cell division cycle; Sos, son-of-sevenless; NECAP, adaptin-ear-binding coat-associated protein.

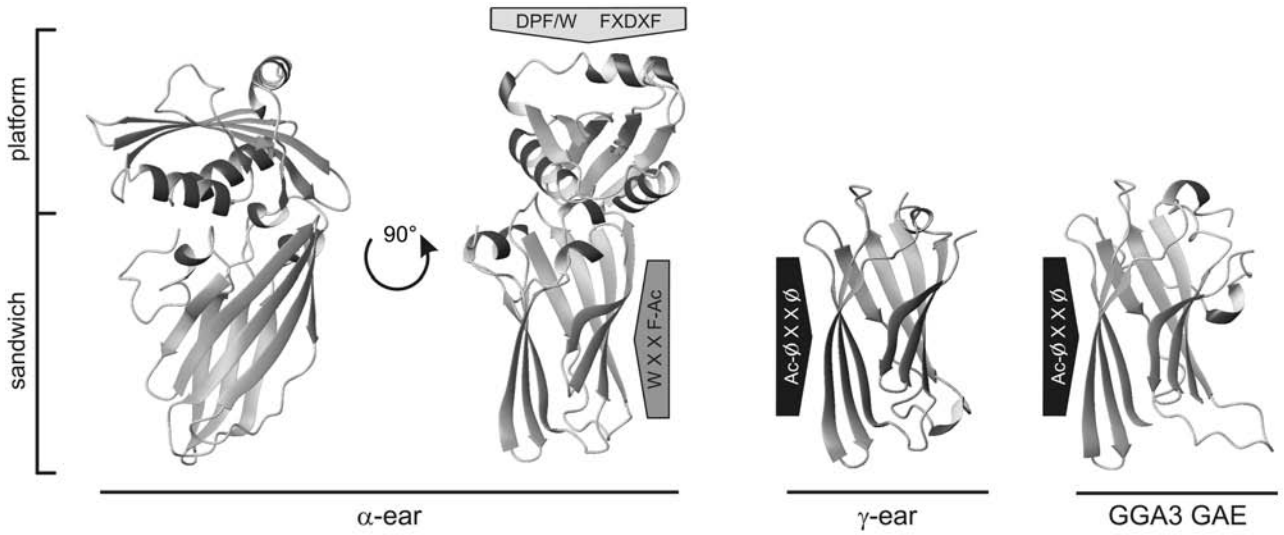

Fig. 2. Atomic structure of the ear domains of clathrin adaptors. The backbone trace of the α-ear (PDB entry 1KYU), γ-ear (PDB entry 1GYU), and GGA3 GAE domain (PDB entry 1P4U) are indicated. The α-ear, composed of a platform domain with mixed β-sheets and α-helices and a sandwich domain composed of all β-sheets, is shown in two orientations. Note the structural similarity of the sandwich domain of the α-ear with the γ-ear/GGA3 GAE. DPF/W and FXDXF motifs bind to a site on the platform domain of the α-ear, whereas WXXF-acidic motifs (WXXF-Ac), bind to a site in the sandwich domain. The binding site for WXXF-acidic motifs is on the opposite face of the α-ear compared to the binding site for acidic-ØXXØ (Ac-ØXXØ) on the structurally homologous γ-ear/GGA3 GAE.

breakthroughs in our understanding of CME. Two such motifs, DPF/W or sequences that match the consensus FXDXF, were originally described *(59–61)*. These motifs bind to overlapping sites on the platform domain (refs. *59–61*; Fig. 2). Their identification allowed for predictions of the AP-2-binding potential of proteins bearing these signatures and contributed to the identification of new components of the regulatory machinery for CME *(59–63)*. Analogously to the role of the α-ear, the γ-ear of AP-1 and the GAE domains of GGAs appear to recruit accessory proteins to clathrin bud sites on the TGN and endosomal membranes. In fact, these domains are structurally similar to the sandwich domain of the α-ear (refs. *64* and *65*; Fig. 2). Until recently, however, no consensus motifs for γ-ear/GAE domain binding had been identified. Moreover, little was known regarding potential ligands for the sandwich domain of the α-ear. Through CCV proteomics, we have identified new components of the clathrin-budding machineries at the TGN and plasma membrane *(19,20)*. Intriguingly, the characterization of several of these proteins has led to the identification of a consensus motif for γ-ear/GAE domain binding and has led to the identification of a new motif that binds to the sandwich domain of the α-ear.

Subcellular Proteomics: Expanding the Machinery for Clathrin-Mediated Budding on Internal Membranes and Identification of a γ-Ear/GAE Domain-Binding Motif

The CCV proteome revealed eight proteins that had only been described as potential open reading frames at the time of the study *(19,20)*. Of these, six have been linked to vesicle trafficking. We named one of these proteins entho-

protin *(19)*, which is also referred to as Clint and epsinR *(66–68)*. Enthoprotin encodes an N-terminal ENTH domain and is highly enriched on CCVs *(19)*. ENTH domain-containing proteins function predominantly in CME, with epsin as the most well-characterized example *(46)*. The epsin ENTH domain binds to PtdIns(4,5)P_2 at the plasma membrane *(69,70)*. After PtdIns(4,5)P_2 binding, the ENTH domain undergoes a structural change that generates a new α-helix (referred to as α0) from a previously unstructured region at the N-terminus *(71)*. This helix has a hydrophobic face that inserts into the inner leaflet of the membrane and contributes to the biophysical deformation of the membrane necessary to evoke membrane curvature *(72)*. The enthoprotin ENTH domain also contains an N-terminal sequence that has the potential to form an α0 with a hydrophobic face *(68)*, although its ability to undergo a conformational change on lipid binding has not been directly examined.

Unlike epsin, the enthoprotin ENTH domain binds weakly to phospholipids with a slight preference for PtdIns(4)P *(66–68)*, which is enriched at the TGN *(37)*. Interestingly, enthoprotin binds to the γ-ear and GAE domains of AP-1 and GGA2, respectively, as well as to clathrin itself; it also colocalizes with marker of the TGN and the endosomal system *(19,66–68)*. Therefore, enthoprotin is believed to function in the organization of clathrin bud sites and is the first example of an ENTH domain-containing protein that functions in clathrin-mediated trafficking between the TGN and the endosomal/lysosomal system. In fact, recent studies have suggested that enthoprotin functions in retrograde transport from early endosomes to the TGN, where it may operate to recruit specific cargo (e.g., soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptors to nascent clathrin-coated buds *(73,74)*.

A two-hybrid screen using the yeast GGA protein Gga2p and the γ-adaptin subunit of yeast AP-1 led to the identification of the protein Ent3p, which appears to be the yeast homolog of enthoprotin *(75)*. Detailed characterization of enthoprotin and Ent3p has led to the identification of short peptide sequences that mediate binding of the proteins to γ-ear/GAE domains *(68,75–77)*. Variations of the sequences are present in other AP-1- and GGA-binding partners, including γ-synergin, rabaptin-5, and p56 *(76, 78–80)*. In vitro binding assays, coupled with mutational analysis and screening of phage-displayed recombinatorial peptide libraries, resulted in the identification of a consensus sequence for the γ-ear–GAE domain interaction Ø-[GA]-[PDE]-[ØLM] (where Ø is an aromatic residue), more simplistically referred to as a ØXXØ motif (the N-terminal Ø is denoted as position 0 with the C-terminal Ø as position +3; refs. *77* and *80*).

The identification of this consensus motif has been invaluable in the identification of additional components of the machinery for intracellular clathrin trafficking. One example is the protein aftiphilin, which was identified in database searches for proteins containing sequences conforming to the ØXXØ GAE domain-binding motif *(80)*. Aftiphilin contains eight copies of the motif and its interaction with AP-1 and GGA has been confirmed *(80–82)*. Nuclear magnetic resonance (NMR) and X-ray crystallography studies have analyzed the GAE domains of GGA1, GGA3, and the γ-ear of AP-1 in combination with ØXXØ motifs from p56, rabaptin-5, and enthoprotin, respectively, revealing a conserved binding site for the motif mediated through amino acids in β-strands 4, 5, 7, and 8 (refs. *77,83,* and *84*; Fig. 2). Interestingly, hydrophobic portions of sidechains of basic amino acids within the ears provide the interaction interface for the hydrophobic amino acid sidechains of the motif *(83,84)*.

Subcellular Proteomics: Expanding the Machinery for CME and Identification of a New α-Ear-Binding Motif

Two additional proteins identified in the proteomics analysis of brain CCVs (now referred to as NECAP 1 and 2) are highly

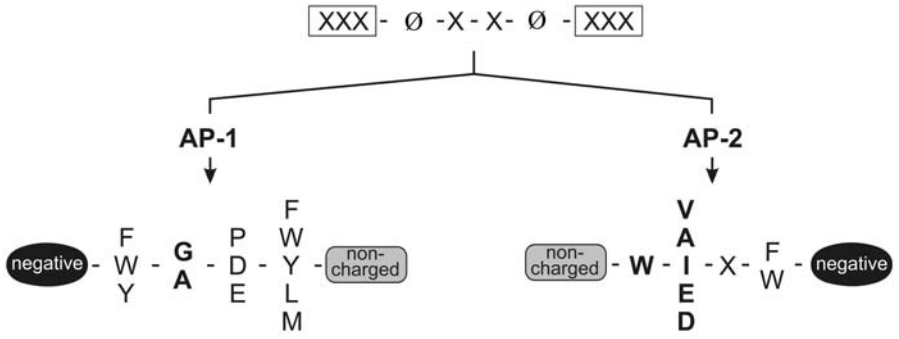

Fig. 3. Summary of the properties of α-ear- and γ-ear-binding motifs. Both the AP-1 γ-ear-binding motif and the AP-2 α-ear-binding motif are based around ØXXØ cores (where Ø is an aromatic residue). Allowable amino acids at each position within the motifs are indicated. In addition to the cores, negative charge upstream and downstream of the γ-ear-binding motif and α-ear-binding motif, respectively, contribute to interactions of the motifs with the ears.

homologous to each other but share no homology or common domains with any characterized protein *(85)*. The NECAPs are enriched in the coat protein fraction of CCVs and colocalize, in part, with markers of CCPs at the cell surface *(85)*. LC Q-ToF analysis of proteins (affinity selected from brain extracts using full-length GST–NECAP fusion proteins) allowed for the identification of AP-2 as a major NECAP-binding partner. The NECAPs were found to directly interact with the α-ear of AP-2, despite the lack of DPF/W or FXDXF consensus α-ear-binding motifs. Instead, the sequence WVQF, located at the very C-terminus, is necessary and sufficient for α-ear binding *(85)*. Extensive characterization of the sequence and its interaction with the α-ear has led to the realization of a new consensus α-ear-binding motif, referred to as a WXXF-acidic motif (the W is denoted as position 0 with the F as position +3). Intriguingly, this motif is reminiscent of the ØXXØ motif for γ-ear/GAE domain binding, but it demonstrates no binding to these domains *(86)*. However, the NECAPs also contain the sequence WGDF, which binds to the γ-ear/GAE domains of AP-1 and GGAs *(80)*.

The presence of the two motifs in the NECAPs has allowed us to define the parameters that establish the binding specificity of each motif for its specific clathrin adaptor (ref. *86*; Fig. 3). Unlike the ØXXØ motif, which tolerates different aromatic residues at positions 0 and +3, the WXXF-based motif uses W exclusively at position 0, and only F or W are tolerated at position +3 (refs. *77,80,86*; Fig. 3). The two motifs further require different subsets of amino acids at the +1 and +2 positions. The ØXXØ motif most often contains P, D, or E at position +2, which are usually paired with G and A at position +1, as these combinations allow the peptide to be shaped into the type I β-turn essential for the outer hydrophobic positions to contact the γ-ear/GAE domain simultaneously *(83)*. In contrast, the +1 position of the WXXF-acidic motif directly contributes to the interaction with the α-ear, leading to a restricted degree of flexibility for amino acids at this position *(86)*. Therefore, the +1 position appears to establish a second level of binding specificity. The residue at position +2 of the WXXF-acidic motif does not contact the α-ear and is freely interchangeable into any amino acid. Importantly, however, acidic charge outside of the core (positions 0 to +3) also contributes to specificity for ear binding (Fig. 3). In particular, WXXF-acidic motifs require acidic charge C-terminal of the core to bind to the α-ear *(86)*. This can be provided by acidic amino acids; alternatively, in the case of

the NECAPs (where the motif is located at the extreme C-terminus of the protein), the charge is provided by the free main chain carboxyl group. In contrast, all ØXXØ γ-ear/GAE domain-binding motifs reported to date are preceded by at least one acidic amino acid (Fig. 3). Elimination of the negative charge interferes with γ-ear/GAE domain binding.

NMR studies and mutational analysis have led to the identification of the binding site for WXXF-acidic motif on the α-ear (ref. *86*; Fig. 2). Interestingly, this site is located in the N-terminal sandwich domain of the α-ear, whereas the overlapping binding sites for DPF/W and FXDXF are located in the C-terminal platform domain *(59–61,86,87)*. Amino acids within β-strands 2, 3, 4, and 7 contribute to the binding site centered around Q782 and K727 *(86,87)*. Although the α-ear sandwich domain and the γ-ear/GAE domains represent homologous structures and the different motifs share a common basic structure, the binding sites are on opposite faces of the ears (Fig. 2). Therefore, the differences between the motifs and their binding sites likely are a product of independent co-evolution. WXXF-acidic motifs have also been demonstrated to contribute to α-ear binding in synaptojanin 170, AAK1, GAK, and stonin 2, confirming WXXF-acidic motifs as an autonomous α-ear-binding signature *(87–89)*. Importantly, overexpression of NECAP deletion constructs that contain the α-ear-binding motif inhibit the clathrin-mediated uptake of transferrin receptor in a dominant-negative manner, emphasizing the importance of proteins harboring WXXF-acidic α-ear-binding motifs for normal AP-2 function *(85)*.

Therefore, it appears that at least three distinct peptide motifs (DPF/W, FXDXF, and WXXF-acidic motifs) mediate interactions with the α-ear. Individually, the motifs have affinities in the approx 10 to 120-μ*M* range, with WXXF-acidic motifs having the highest relative affinity. Thus, the platform domain binds proteins containing DPF/W and FXDXF motifs and proteins bearing these motifs may compete for AP-2 interactions. Although WXXF-acidic motifs do not compete with DPF/W- and FXDXF-bearing proteins, they can compete with each other for the sandwich-binding site. Therefore, the various endocytic accessory proteins may need to function at different endocytic steps of the endocytic cycle or be otherwise regulated in their interactions with AP-2. However, it is worth noting that AP-2 is very abundant and likely is present at levels that exceed the total of the entire complement of accessory proteins. Thus, it is conceivable that large networks of endocytic accessory proteins form around AP-2. Because each of these proteins has a distinct function during endocytosis, AP-2 could function as master scaffold that brings together the various activities in a coordinated manner to regulate CCV formation and function.

Interestingly, many of the endocytic accessory proteins contain multiple copies of the platform motifs. For example, epsin and Eps15 contain tandem repeats of DPW and DPF motifs, respectively, and amphiphysin I contains both a DPF and an FXDXF motif. Therefore, these proteins could crossbridge multiple AP-2 molecules, leading to their higher apparent affinity for AP-2 interactions. Additionally, several WXXF-acidic motif-containing proteins (including synaptojanin170, stonin2, and the kinase AAK1) also harbor one or multiple copies of the platform domain motifs *(86–89)*. Therefore, these proteins could synchronously engage both domains of the α-ear, leading to strong avidity effects. Combinations of motifs for platform and sandwich domains, together with differences in the number of copies of either motif, could provide a means for hierarchial protein recruitment to the α-ear and provide a mechanism to control CCV formation and function.

Conclusions

Large-scale sequencing of expressed genes, along with the completion of the human genome and the genomes of various model animals, has provided invaluable insights into gene expression and function. However, a complete understanding of cellular processes

depends on decoding this genomic information into functional proteins and protein assemblies. Any given cell may express 10,000 genes, which can give rise to much larger numbers of functional proteins because of splice variants and posttranslational modifications *(21)*. Subcellular proteomics provides a mechanism to begin to decode genomic information using samples of manageable complexity *(21)*. In fact, numerous organelles and suborganellar compartments have now been analyzed by subcellular proteomics, and each study has revealed new concepts regarding the functional roles of the organelles *(22,23)*. The analysis of CCVs has further revealed the power of subcellular proteomics in this regard and, similarly to the other studies, has been invaluable as a tool for protein discovery. In particular, the association of novel proteins with an organelle provides an immediate framework on which to build models of protein function. This was especially vital for the NECAPs, for which bioinformatics analysis failed to reveal any protein domains or modules to guide functional studies. The identification of novel CCV proteins allowed the surprising identification of new motifs for intracellular and endocytic clathrin-mediated membrane trafficking, which then sparked the identification of additional potential binding proteins for AP-2 and AP-1/GGAs. Therefore, subcellular proteomics is a valuable approach in the study of nervous system function.

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research and from a grant from Genome Quebec to the project: Réseau Protéomique de Montréal, Montreal Proteomics Network (RPMPN).

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