Endocytosis: **EH domains lend a hand**

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A number of proteins that have been implicated in endocytosis feature a conserved protein-interaction module known as an EH domain. The three-dimensional structure of an EH domain has recently been solved, and is likely to presage significant advances in understanding molecular mechanisms of endocytosis.

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Budding protein biochemists are often admonished that they should not consider proteins as mere strings of amino acids, but rather as complex folded structures that are much more than the mere sum of their constituents. While this is certainly true, it is now also apparent that many proteins consist of independently folded domains that contribute modular functions. The design advantages of this 'beads on a string' organization are obvious: like a youngster with a set of blocks, evolution can mix and match domains to create new proteins with novel combinations of catalytic and binding activities. The idea that the function of a protein can be inferred from the modular domains that it possesses is now so ingrained that identification of a novel protein module by sequence similarity, establishment of the biochemical activity of the putative domain, and determination of its three-dimensional structure often precedes any detailed information about the biological role of the proteins in which they are found. Such is the case with the newly-minted EH domain, the structure of an example of which has recently been revealed by NMR spectroscopy [1]. By all indications this is an early salvo in an imminent barrage of information about the molecular details of the endocytosis machinery.

The EH domain was first noted as a repeated segment in Eps15, a substrate for the tyrosine kinase activity of the epidermal growth factor (EGF) receptor — hence the name 'Eps15 homology' or EH domain [2,3]. Eps15 is known to associate with components of the clathrin-mediated endocytic machinery, and other proteins containing the domain have also been implicated in endocytosis and vesicle transport in yeast and in vertebrates. The EH domain itself has approximately 100 residues, and is repeated three times in Eps15. Filter-binding studies using bacterially-produced EH domains first

suggested that they mediated protein–protein interactions [3], and a variety of approaches, including phage display and yeast two-hybrid analysis, identified the tripeptide asparagine-proline-phenylalanine (NPF in the single-letter code) as the core binding site for most EH domains [4–8]. Most known EH domain proteins contain multiple copies of the module, suggesting that binding to targets might be aided by multiple, cooperative interactions.

Overduin and colleagues [1] have now solved, by NMR spectroscopy, the three-dimensional structure of the central EH domain (EH_2) of human Eps15. The structure consists of two so-called 'EF hands' — an EF hand is a common helix–loop–helix structural motif that binds calcium via conserved negatively-charged side chains in the loop [9]. In the EH_2 domain, the two EF hands are closely apposed, with the helices packed into a bundle and the two loops close together on the same face (Figure 1). The overall structure of the EH_2 domain is in fact quite similar to that of calbindin, a small calcium-binding protein that consists of two functional EF hands [10]. Whether this is a case where a successful structural motif has been recruited for other uses, or rather reflects a functional role for calcium in EH domains, remains to be determined.

Figure 1
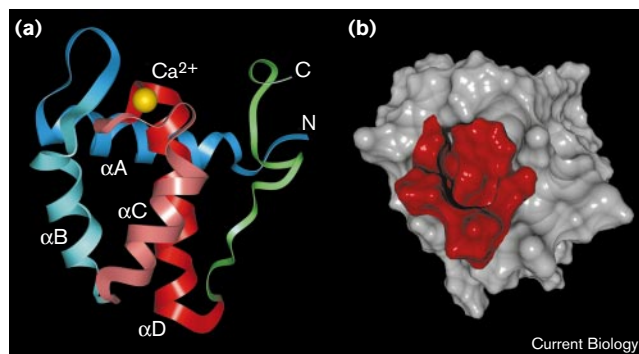
Structure of the second EH domain (EH_2) of human Eps15. **(a)** A ribbon diagram, in which the two EF-hand helix–loop–helix motifs are indicated in blue (αA and αB) and red (αC and αD). The carboxy-terminal region is indicated in green, and the calcium ion is indicated as a yellow sphere. **(b)** Surface representation of the asparagine-proline-phenylalanine binding pocket of EH_2. Residues whose chemical shifts change upon ligand binding — Gly148, Leu152, Leu155, Leu156, Val162, Leu165, Gly166 and Trp169 — are indicated in red. The images were created by Tonny de Beer, Jim Mamay and Michael Overduin using InsightII and VMD (Visual Molecular Dynamics Version 1.2). High resolution true color graphic rendering was accomplished on the 'Flamingo4' parallel processing cluster running Solaris X86.

Although it had been noted previously that the sequences of some EH domains have the hallmarks of the EF motif [3], to date calcium has not been shown to affect EH domain function. The carboxy-terminal EF hand of EH_2 binds calcium with very high affinity, whereas the other does not bind calcium detectably [1]. Calcium is therefore unlikely to play a regulatory role for this particular domain, as fluctuations of calcium concentration in the physiological range would not be expected to affect binding. Nevertheless, the confirmation that the EH domain is composed of EF hands, at least some of which are capable of binding calcium, certainly raises the possibility that some EH domains are regulated by the intracellular calcium levels. Those domains that are likely to bind calcium can be identified readily by the presence of conserved negatively charged residues involved in coordinating the calcium ion [9].

The binding site in the Eps15 EH domain for an NPF-containing peptide was determined by mapping changes in the NMR signals — the 'chemical shifts' — upon titration with the peptide. A pocket was identified on the face of the domain opposite the termini, formed by conserved hydrophobic residues on helices αB and αC (Figure 1). Because the affinity for the peptide was low — a K_D of 560 μM was determined by surface plasmon resonance [1] — it has not yet been possible to define the orientation of the peptide on the surface of the domain. However, the strong requirement for asparagine in the first position is suggestive: asparagine–proline dipeptides strongly favor a type I β-turn conformation [11], and asparagine–proline-containing peptide ligands for yet another protein binding module, the PTB domain, adopt a β-turn conformation which is apparently essential for binding [12,13]. Kay and colleagues [7] found that cyclized NPF peptides can have much higher affinity for EH domains than their linear counterparts, suggesting that cyclization locks the peptide into a conformation (such as a β-turn) that can interact productively with an EH domain.

It is clear that EH domain recognition involves more than the minimal NPF sequence. Residues surrounding the NPF core motif can influence binding [4,8], and in some cases carboxy-terminal NPFX or NPFXX sequences have been found to have higher affinity for EH domains than internal sites [7]. Thirteen mammalian and yeast EH domains were recently used to identify high-affinity ligands from a phage-displayed nonapeptide library. While most domains selected peptides with a NPF core, a subset could also bind to peptides containing FW or WW motifs, and one — that of End3, a yeast protein required for endocytosis — selected the sequences HTF and SWG [8]. Crystal structures of EH–ligand complexes or NMR studies with cyclized peptides will undoubtedly provide valuable insights into the determinants of peptide recognition. The specific contributions of the highly conserved
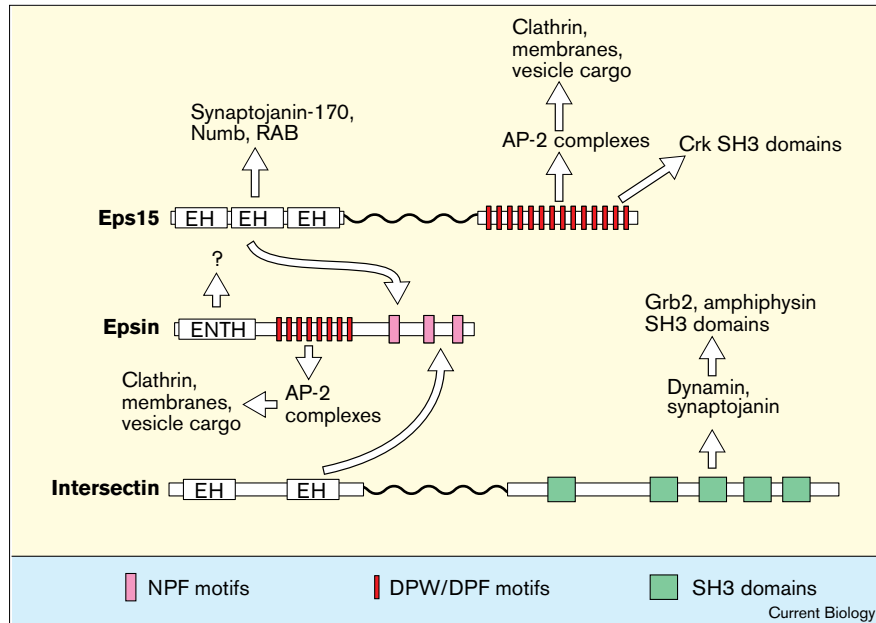
asparagine of the NPF motif may prove particularly enlightening for, as discussed below, the superficially similar DPF and DPW motifs are also commonly found in components of the vesicle transport machinery (including Eps15 itself). It is interesting to speculate that EH domains might have the potential to bind these sites under some circumstances.

Where does the EH domain fit into our emerging understanding of endocytosis? Circumstantial evidence from a variety of systems suggests that Eps15 and other EH-containing proteins play an important part in clathrin-mediated endocytosis. This process is initiated by binding of the adaptor protein complex AP-2 to cargo proteins on the membrane, and their subsequent concentration into localized patches (reviewed in [14]). The AP-2 complex recruits clathrin subunits which self-assemble into cage-like lattices in coated pits, which ultimately mature into coated vesicles that pinch off from the plasma membrane and are targeted to intracellular compartments [14]. Obviously the processes of cargo loading, coat assembly, vesicle budding, fission and targeting must be carefully controlled, and all evidence suggests that an elaborate set of protein–protein interactions drives these processes.

Eps15 associates with AP-2 complexes and thus indirectly with clathrin and coated pits [15–17]. Overall, the protein consists of the three amino-terminal EH domains, a central region with heptad repeats suggestive of a coiled-coil structure, and a carboxy-terminal domain containing approximately fifteen DPF tripeptide motifs and a number of PxxP binding sites for Src homology 3 (SH3) domains (Figure 2). Rotary shadowed electron microscope images and biophysical studies of Eps15 suggest that it exists as a tetramer in solution. The tetramer is a dimer of dimers: in each dimer, two Eps15 molecules interact side-by-side via their coiled-coil regions; in the tetramer, two dimers associate in anti-parallel fashion, with the EH-containing amino-terminal region of one apposed to the DPF-containing carboxy-terminal region of the other [18,19]. It is tempting to speculate that, in the absence of a competing high-affinity ligand, tetramerization might be facilitated by low-affinity interactions between the EH domains of one dimer and the numerous DPF motifs of a second dimer.

Each of these motifs, however, has other ligands. The carboxy-terminal DPF region of Eps15 binds to the amino-terminal 'appendage' region of the AP-2 component α-adaptin, suggesting a mechanism for recruitment of Eps15 to sites of coated pit assembly [15]. As for the EH domain, among its known binding partners are several proteins implicated in endocytosis. One is the 170 kDa form of synaptojanin [20,21], a phosphatidylinositol 5′-phosphatase that additionally contains multiple binding sites for SH3-domain-containing proteins, such as Grb2 and amphiphysin.

Figure 2

Examples of protein–protein interactions in proteins implicated in endocytosis. Eps15, epsin and intersectin are depicted diagramatically to scale. EH and ENTH domains are indicated by labeled boxes, and putative helical coiled-coil segments are indicated by wavy lines. Arrows indicate known protein–protein interactions. (See text for details.)

Another recently identified target of the Eps15 EH domains is epsin [5], a protein enriched at nerve terminals which itself consists of multiple domains. Epsin has a carboxy-terminal region containing three NPF motifs, which bind the three EH domains of Eps15; a central region containing eight DPW motifs, which competes with the DPF region of Eps15 for binding to the same site on α-adaptin; and an amino-terminal region containing a novel domain, termed the ENTH domain, which is found in a number of proteins that have been implicated in endocytosis [22] (Figure 2). One imagines that this will prove to be yet another modular protein-binding domain, whose ligands and structure will undoubtedly come to light soon.

The suggestion that both Eps15 and epsin play a part in endocytosis stems from their colocalization with sites of clathrin assembly and the observation that overexpression of the AP-2-binding domains of either protein has a dominant inhibitory effect on endocytosis of receptors such as the transferrin receptor [5,23]. Their precise role in assembly of the coated vesicle is currently unknown, but Eps15 and epsin are both more highly enriched in membrane fractions containing coated pits than in mature coated vesicles [5,16], suggesting a role in assembly and not in the finished vesicle. And for Eps15, it has been shown that clathrin coat assembly dissociates pre-formed AP-2–Eps15 complexes [24]. Immuno-electron microscope studies suggest that Eps15 is concentrated at the membrane-proximal rim of the assembling clathrin coat, again consistent with a role in the assembly process [16]. The size and tetrameric stoichiometry of Eps15 might allow it to serve as a dynamic bridge between adjacent AP-2 complexes, facilitating assembly of the clathrin coat by orienting the clathrin subunits to promote productive interaction [19].

Other evidence points to a more general role for EH-containing proteins in endocytosis and vesicle transport. An EH domain protein known as intersectin was isolated recently as a major dynamin-binding protein [7]. Dynamin is a GTP-binding protein that is localized to the neck of budding vesicles and is suspected of mediating the pinching off of the nascent vesicle from the plasma membrane [25,26]. Dynamin is rich in PxxP SH3-domain-binding motifs, and so binds to SH3-containing proteins such as the adaptor Grb2. Intersectin has a carboxy-terminal region with five SH3 domains, which mediate binding to dynamin, a putative coiled-coil central region and two amino-terminal EH domains (Figure 2). When the intersectin EH domains were used to fish out binding partners by the yeast two-hybrid method, several ENTH-domain-containing proteins were found, including epsin [7].

Studies on EH-containing proteins in other systems, especially yeast, underscore the role of these domains in endocytosis. For example, the yeast protein Pan1, which has a domain organization reminiscent of Eps15, is essential for endocytosis; its EH domains bind to proteins that contain ENTH domains and clathrin-binding sites [6]. One of the Pan1 EH-domain-binding proteins has an approximately 250 residue amino-terminal region with significant similarity to members of the vertebrate AP180/CALM family, which can directly bind clathrin and promote its polymerization [27]. It is not yet clear whether

this region is an ENTH domain, as its sequence similarity to other ENTH domains is modest at best [22]; structural and biochemical studies are clearly needed here. It has also recently been shown that an NPFxD motif, which is likely to bind directly to EH domains, can serve as an endocytic signal in yeast [28], suggesting that EH domains could play an active role in recruiting some cargo proteins into endocytic vesicles. The genetically tractable yeast system will undoubtedly complement biochemical studies in teasing out the complex interrelationships among these proteins.

Many questions remain, including the following. How is the binding of Eps15 to adaptor protein complexes and to other proteins regulated during the process of coat assembly? What is the specific role of proteins such as Eps15 in the assembly process? What is the significance of tyrosine phosphorylation of Eps15 and its binding to SH3-containing proteins such as the Crk SH2/SH3 adaptor [29]? What are the function and activities of the newly described ENTH domain? It will also be of interest to see if the EH domain's role is limited to the vesicle transport machinery, or if it has been put to other uses in the cell. In any event, it seems certain that our ability to recognize protein modules such as the EH domain and rapidly unravel their structure and biochemical activities will keep this field moving briskly.

Acknowledgements

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