FEBS 25686 FEBS Letters 513 (2002) 11–18

Minireview

The ENTH domain

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Received 20 November 2001; revised 11 December 2001; accepted 17 December 2001

First published online 4 January 2002

Edited by Gianni Cesareni and Mario Gimona

Abstract The epsin NH2-terminal homology (ENTH) domain is a membrane interacting module composed by a superhelix of α-helices. It is present at the NH2-terminus of proteins that often contain consensus sequences for binding to clathrin coat components and their accessory factors, and therefore function as endocytic adaptors. ENTH domain containing proteins have additional roles in signaling and actin regulation and may have yet other actions in the nucleus. The ENTH domain is structurally similar to the VHS domain. These domains define two families of adaptor proteins which function in membrane traffic and whose interaction with membranes is regulated, in part, by phosphoinositides.

Key words: VHS domain; Ubiquitin; Clathrin; AP-2; Endocytosis; EH domain

1. Introduction

Following the discovery of the clathrin coat in the mid-sixties [1] major progress has been made towards its molecular characterization. Endocytic clathrin coats, in particular, have been thoroughly investigated [2–5]. Their main components are the heavy and light chains of clathrin [4], and the hetero-tetrameric clathrin adaptor AP-2 [6]. In addition, a large number of accessory factors that cooperate with clathrin and AP-2 in the endocytic reaction, and may also assist the newly formed vesicle during the early stages of the endocytic pathway, have been identified [3,7]. Studies of these factors, which typically have a modular structure, led to the identification of the EH domain, and subsequently, of the ENTH domain. EH domains, which are discussed elsewhere in this volume [8], are small protein modules first recognized in the NH2-terminal region of Eps15 (Eps15 homology). Eps15 is a main substrate for the tyrosine kinase activity of the EGF receptor [9] and is also a major AP-2 interactor [10]. Thus, while it has a special role in EGF receptor signaling and/or internalization, it also participates in all forms of endocytosis, including synaptic vesicle recycling at the synapse [11]. Studies of the EH domain revealed that it functions as a protein–protein interaction module and defined the sequence NPF as a critical consensus for EH domain binding [12].

2. Epsin: the founding member of ENTH domain containing proteins

A search for interactors of the EH domains of Eps15 in brain led to the identification of a major 90 kDa synaptically enriched protein band (based on SDS–PAGE motility) referred to as epsin (epsin 1) (for Eps15 interacting protein) [13]. A molecular characterization of epsin demonstrated its identity to a 90 kDa protein band previously shown to bind the ear domain of the clathrin adaptor AP-2 [14], thus corroborating a link of this protein to the endocytic machinery. Independently, epsin was isolated as an interactor of the EH domains of intersectin, another accessory factor in endocytosis [15,16] and of POB1, a component of the Ral GTPase network [17]. A function of epsin in the endocytic reaction was further confirmed by its property to bind clathrin [18–20] and by evidence that disruption of epsin function in living cells inhibits receptor-mediated endocytosis of the EGF and transferrin receptors [13].

3. Domain structure of epsin and definition of the ENTH domain

Inspection of the epsin 1 sequence and alignment with related sequences allowed definition of several major domains within this protein [13] (Fig. 1). The NH2-terminal approximately 150 amino acids are highly conserved in evolution. Hence, they were proposed to represent a separately folded protein module, the epsin NH2-terminal homology (ENTH) domain [13,21]. The remaining portion of the epsin contains several stretches of low complexity amino acid composition and signature motifs for binding to other endocytic proteins. Its central region contains multiple DPW repeats [13], which fit the consensus DP[W/F] of the core of binding sites for the ear domains of the α- and β-adaptin subunits of AP-2 [22]. It also includes two consensus sequences for binding to the heavy chain of clathrin (clathrin boxes) [4,18,20,23]. Accordingly, the central region of epsin binds both AP-2 and clathrin [18,20]. The COOH-terminal part of the protein comprises

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0014-5793/02/$22.00
PII: S0014-5793(01)03306-3

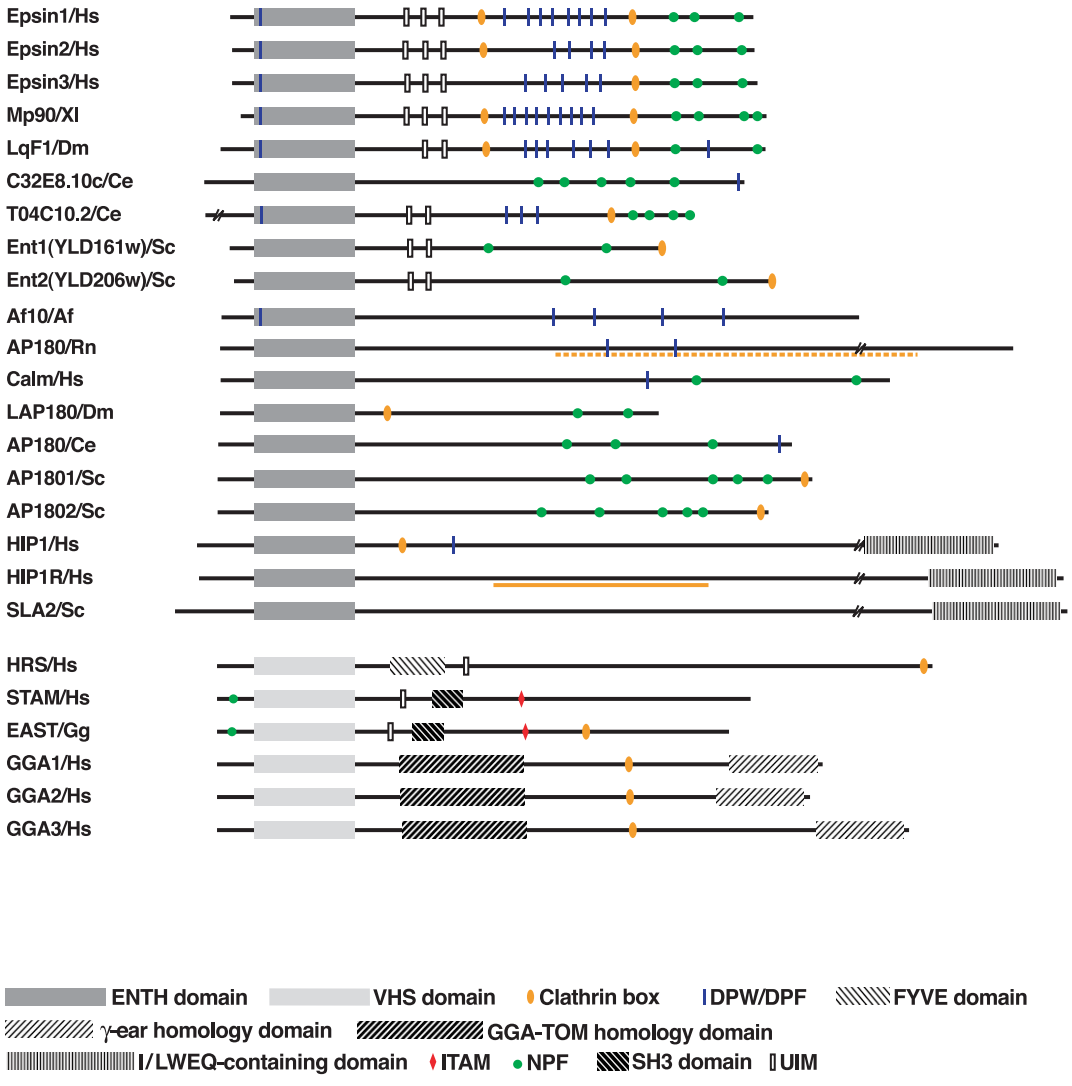

Fig. 1. Domain cartoon of a subset of proteins containing ENTH or VHS domains. The drawing illustrates the presence in many of them of motifs or domains which mediate an interaction with clathrin coat proteins, actin and other components of the cell cortex. See text for the significance of these domains. While only DPF/W motifs (AP-2 interaction motifs) are shown, additional DXF motifs, which may also mediate interaction with AP-2, are present in some of these proteins. Orange ovals represent either sequences which fits the clathrin binding consensus (clathrin box) as defined in [4,23], or closely related sequences experimentally shown to bind clathrin. Other clathrin box-like sequences, possibly representing additional clathrin binding sites, are not indicated. Mammalian AP180 was shown to bind clathrin through a series of short motifs more distantly related to typical clathrin boxes (region indicated by an orange dotted line) [37]. HIP1R binds clathrin via coiled-coil interactions (region indicated by an orange continuous line) (modified from [21]).

three NPF repeats required for binding to Eps15, intersectin and other EH domain containing proteins [13,15,17]. Recently, epsin was shown to be mono-ubiquitinated (Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, R.M., Bossi, G., Chen, H., De Camilli, P. and Di Fiore, P.P., unpublished observation) and the sequence between the ENTH domain and the central DPW domain was found to contain three ubiquitin interacting motifs (UIMs) [24]. This region does bind ubiquitin, and, surprisingly, is also needed for ubiquitination (Polo et al., see above).

At least some of the interactions of epsin are regulated by phosphorylation. At synapses, epsin undergoes constitutive phosphorylation and its stimulation-dependent dephosphorylation correlates with its assembly into endocytic complexes [25]. Mitotic phosphorylation of epsin at serine 382 (rat epsin 1) inhibits its interaction with clathrin coat components and possibly reflects one of the mechanisms leading to inhibition of clathrin-mediated endocytosis in mitotic cells [25,26]. Studies on yeast epsin homologs [27] predict that epsin may be a substrate for GAK kinases [28], the mammalian homologs of yeast Prk/Ark kinases implicated in actin function and endocytosis [29].

4. The epsin family

Proteins that have the same overall domain structure of epsin 1 and contain most of its signature motifs are present in all eukaryotic organisms (Fig. 1). The human genome con-

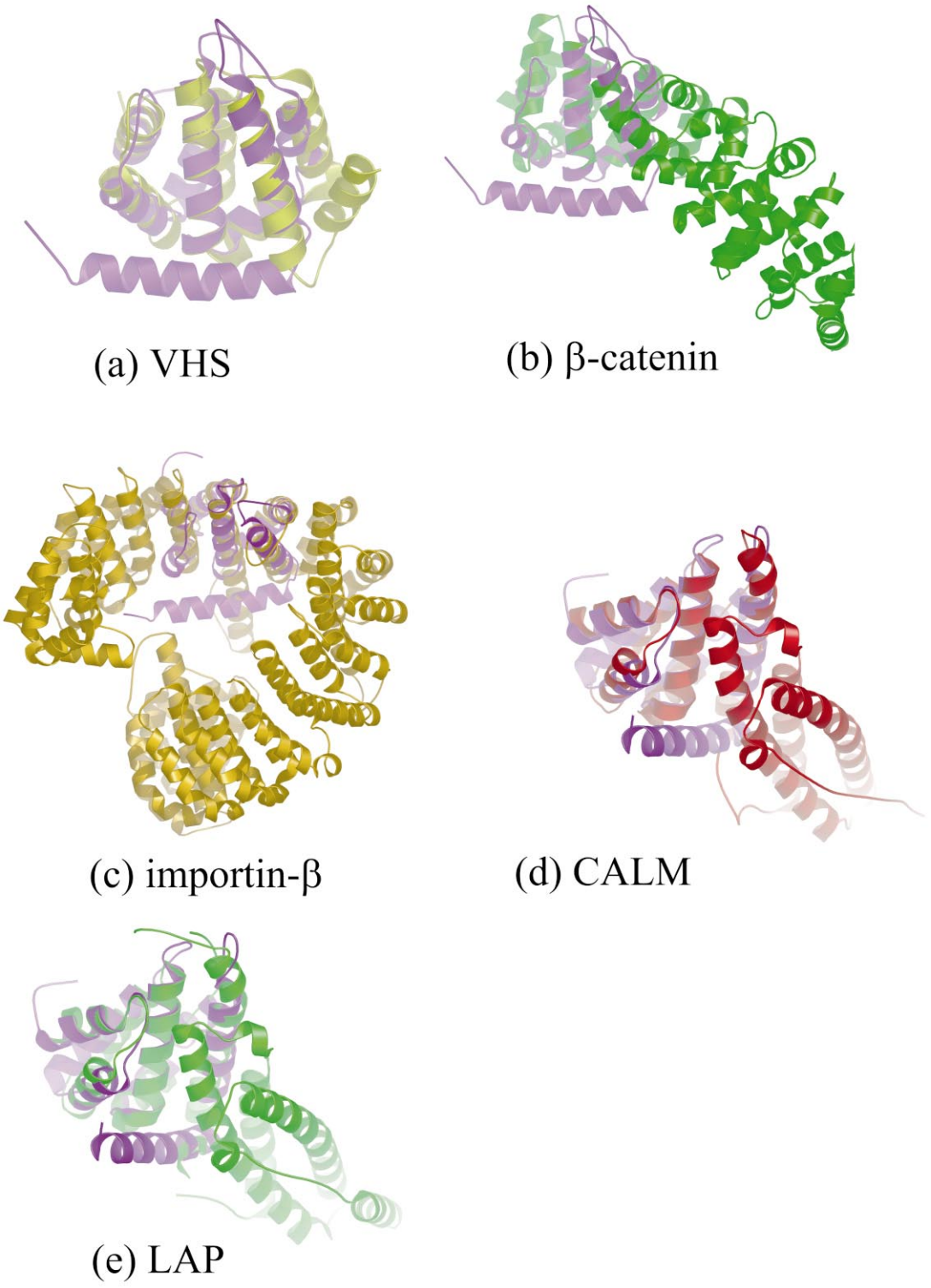

Fig. 2. Comparison of the ENTH domain of epsin 1 (magenta) with similar structures. Superposition of the ENTH domain with the VHS domain (green) (a), the armadillo repeat region of β-catenin (green) (b), the HEAT repeat region of importin-β (gold) (c), the ENTH domain of CALM domain (red) (d), the ENTH domain of the LAP protein (green) (e).

tains at least three epsin genes. Epsin 1 is expressed at high concentration in brain [13], epsin 2 has a more widespread tissue distribution [20] while epsin 3 is keratinocyte specific [30]. A *Xenopus* epsin was identified as a mitotic phosphoprotein in oocytes (MP90) [26]. One epsin orthologue, liquid facets (LqF), is present in *Drosophila*. Developmental defects due to mutations in LqF are enhanced by mutations in the genes encoding clathrin heavy chain (*CHC*) or the GTPase dynamin (*shibire*), thus providing genetic evidence for a link between epsin family members and endocytosis [31]. Two characterized epsin homologs, Ent1 and Ent1, are present in *Saccharomyces cerevisiae*. In these two proteins, the clathrin binding consen-

sus is localized at the COOH-terminus, downstream of the NPF motifs, which bind yeast EH domain proteins [32]. Both Ent1 and Ent2 participate in endocytosis and in the regulation of actin function, in agreement with strong evidence linking actin to endocytosis in yeast. While neither the ENT1 or the ENT2 gene is essential, an *ent1*Δ,*ent2*Δ double mutant is not viable. This mutant can be rescued by a truncated form of Ent2 that comprises the ENTH domain and the COOH-terminal clathrin binding domain, but not by Ent2 constructs missing the ENTH domain [32]. Thus, the ENTH domain represents a critically important portion of epsin.

5. Other ENTH domain containing proteins

In addition to proteins likely to be bona fide epsin homologs, several other protein families contain an ENTH domain (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01417) (Fig. 1). While some of these proteins are substantially divergent from epsin outside this domain, which is always localized near the NH2-terminus, they often contain some additional elements of similarity. Thus, many such proteins contain UIMs, DX[W,F] motifs [3], clathrin boxes or closely related sequences, and NPF motifs [21] (Fig. 1). For two of these protein families, the presence of an ENTH domain, predicted by distant amino acid sequence similarity, has been confirmed by crystallographic studies. One of these two families is the AP180/CALM family of accessory clathrin adaptors. AP180 is a third major component of neuronal endocytic clathrin coats besides clathrin and AP-2 [33–35]. CALM is its non-neuronal homolog [36]. Both AP180 and CALM bind AP-2 and clathrin. Accordingly, they contain AP-2 binding motifs [3] as well as multiple repeats of a small amino acid stretch that contains a core consensus similar to clathrin boxes [37]. CALM also contains NPF motifs and bind EH domain containing proteins [36]. Based on results from cell-free studies [34] and on ultrastructural observations of AP180 mutants of *Drosophila* (*lap* mutants) and *Caenorhabditis elegans* (*unc11* mutants) [38,39], AP180/CALM proteins are thought to play a role in the generation of homogeneously sized high curvature vesicles. Furthermore, *unc11* mutants accumulate abnormal levels of synaptobrevin/VAMP in the plasma membrane [39], raising the possibility that AP180 may participate in the recapture of synaptobrevin into endocytic vesicles after endocytosis. Two ENTH containing AP180 homologs are present in yeast: yAP180A and yAP180B. They bind clathrin and EH domain containing proteins and have a putative role in endocytosis and actin function [40].

The other protein family with an NH2-terminal region that is structurally similar to the ENTH domain is the one comprising HIP1 and HIP1R, so called because of their property to interact with huntingtin (huntingtin interacting protein and huntingtin interacting protein related, respectively), the Huntington disease protein. HIP1 binds AP-2 and clathrin via typical consensus sequences (DPF or DXF and a clathrin box respectively) [41–43], and both HIP1 and HIP1R interact with clathrin via coiled-coil domains [42,44]. A unique feature of HIP1 and HIP1R among ENTH domain containing proteins is the presence of a talin homology domain in the COOH-terminal region [45]. This comprises the I/LWEQ motif through which they bind actin. Thus, these two proteins, which are partially localized on clathrin-coated pits and vesicles, may act as linkers between clathrin coats and the actin cytoskeleton [44,45]. The yeast homolog of HIP1/HIP1R is the Sla2 protein, which also contains an ENTH domain and a talin homology domain, and which is implicated in actin function and endocytosis [46,47]

6. Atomic structure of the ENTH domain

The structure of the ENTH domain of epsin 1 was solved by X-ray crystallography (Fig. 2). The ENTH domain forms a compact globular structure, composed of eight α-helices connected by loops of varying length ([48]; see also RCSB/PDB accession number 1EDU). The general topology of the domain is determined by three helical hairpins that are stacked consecutively with a right-handed twist. This stacking gives the ENTH domain a rectangular appearance when viewed face on [48]. The most highly conserved amino acids fall roughly into two classes: (i) internal residues that are involved in packing and therefore are necessary for structural integrity, and (ii) solvent accessible residues that may be involved in protein–protein interactions [48].

More recently, other ENTH domain structures have been solved: the ENTH domain of rat CALM [49] (Figs. 2d and 3), of its *Drosophila* homolog Lap (Fig. 2e) [50] and of Hip1R (McMahon, H., personal communication). These ENTH domains are very similar to each other in spite of low amino acid sequence similarity (Fig. 2d,e). Only the COOH-terminal helix of epsin's ENTH domain does not superimpose well on the corresponding region of CALM, but this difference may reflect an abnormal orientation of this helix when disconnected from downstream regions. In the CALM construct used for crystallographic analysis, this helix is followed by an additional α-helical stretch [49].

7. Structural neighbors of the ENTH domain

The ENTH domain is most similar in atomic structure to the VHS domain [51–53] (Fig. 2a). The VHS and ENTH domains align with a root mean square deviation (RMSD) of approximately 1.8 Å, indicating an extremely similar fold despite low primary sequence similarity. The VHS domain, which is discussed in another paper of this volume [54] was first identified as an NH2-terminal homology region found in Vps27, Hrs and STAM [51] (Fig. 1). Hrs and its yeast homolog Vps27 have been implicated in endocytic traffic. STAM is a signal transducing adaptor molecule involved in cytokine-mediated signaling [55]. The VHS domain is also present in other mammalian proteins that participate in the regulation of membrane traffic to the endosome-lysosomal system either from the plasma membrane or from the Golgi complex. These include EAST, which, like Hrs, is a substrate for EGFR and also binds to Eps15 [56–58], and the GGA proteins, which function in transport from the Golgi to the lysosomes [59–61]. As in the case of ENTH domain proteins, functional links of VHS domain proteins to actin and signaling have been reported [51,54]. Like the ENTH domain, the VHS domain is always found at the NH2-terminus of proteins and can be followed by UIM motifs [24]. In addition, some VHS and ENTH domain proteins share some of their binding partners (for example Eps15 and clathrin) [58,62,63] (Fig. 1).

The ENTH structure is also similar to an *armadillo* repeat segment of β-catenin (Fig. 2b) [64], to the HEAT repeat unit

of karyopherin-β (importin-β) (Fig. 2c) [65,66], and to the scaffolding subunit of protein phosphatase 2A [67]. Both *armadillo* and HEAT repeats are protein–protein interaction modules composed exclusively of α-helices, and form structures with two primary faces, one concave and one convex. *Armadillo* and HEAT repeats bind to target proteins with their concave face [64–67]. Structural superimposition of the ENTH domain with either type of repeats aligns their concave faces, which contain the largest patch of conserved surface residues.

8. Interactions of the ENTH domain

The ENTH domain of AP180/CALM contains a basic amino acid cluster that was shown biochemically to bind inositolpolyphosphates as well as PtdIns(4,5)P_2 [49,68]. The ligation of three lysines and a histidine within this cluster by phosphate groups of the inositol ring has been confirmed by crystallographic studies [49] (Fig. 3A). Surprisingly, the ENTH domain of epsin, which lacks this basic region [49], also binds PIP2 and PIP3 [69]. The residues of this domain that exhibit the largest chemical shift upon phospholipid binding are not in register with the PtdIns(4,5)P_2 binding amino acids of AP180/CALM, but are on the same side of the domain (Fig. 3B). Although some discrepancies between the two studies which addressed these lipid interactions must be further addressed [49,69], it would appear that the ENTH domains are phospholipid binding modules, whose affinity for phosphoinositides is further enriched in some family members by the presence of clusters of basic amino acids. The interaction with membrane phospholipids of ENTH domains must be reversible and regulated because a major pool of these proteins has a cytosolic localization [13,20]. We note that an interaction with phosphoinositides was also shown for the NH2-terminal region of the α-adaptin subunit of AP-2 [70], which was proposed to align structurally with the NH2-terminal portion of the ENTH domain of AP180/CALM (see Fig. 2G in [49]). Thus, recruitment to the lipid bilayer of the clathrin adaptor AP-2 and of ENTH domain proteins may be mediated by similar mechanisms. Interestingly, the structurally related VHS domain is also a phospholipid binding module, whose interaction with membrane is further enhanced, at least in some proteins, by flanking FYVE domains which bind PtdIns(3)P (Fig. 1) [51–53].

Interactions of ENTH domains with proteins have been described. The interaction of the ENTH domain of epsin 1 with the transcription factor PLZF may suggest a role of epsin as a transcriptional regulator (see below) [48]. The same domain also strongly interacts with tubulin in vitro (Kay, B., McPherson, P. and Traub, L., personal communications; our unpublished results) and possibly with the coatomer [48], but the significance of these findings is unknown. No interactions with transmembrane proteins have been reported so far. However, the reported binding of the VHS domain to cytoplasmic tails of membrane proteins [60,61] raises the possibility that a similar property may also apply to the ENTH domain.

9. ENTH domain proteins and ubiquitin

An unexpected twist in the understanding of epsin function came with the isolation of *Drosophila* epsin (LqF, see above)

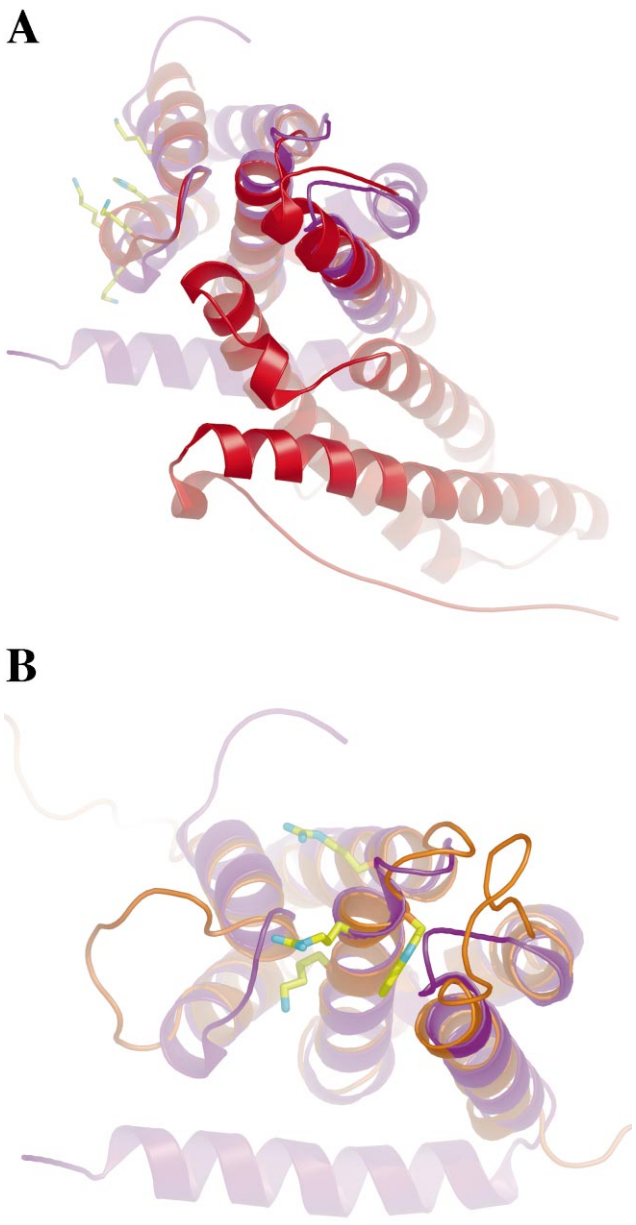

Fig. 3. Proposed PtdIns(4,5)P_2 binding sites in the ENTH domain. A: Superposition of the ENTH crystal structure (magenta) [48] and the crystal structure of the CALM protein (red) [49]. Residues that are involved in PtdIns(4,5)P_2 binding to CALM [49] are explicitly shown as sticks. B: Superposition of epsin's ENTH crystal structure (magenta) [48] and its solution NMR structure (red) [69]. Residues that are implied in PtdIns(4,5)P_2 binding (Fig. 2g of [69]) are explicitly shown as sticks.

as an enhancer of mutations in Fat facet (Faf) [31]. Faf is a de-ubiquitinating enzyme whose activity is necessary in a signaling pathway underlying the proper development of photoreceptor cell clusters in the eye. Genetic data suggest that epsin may be a substrate for Faf [31]. Accordingly, epsin has now been shown to be mono-ubiquitinated and its state of ubiquitination is stimulated by growth factor receptor activation (Polo et al., see above). It remains unclear whether ubiquitination–de-ubiquitination of epsin is a mechanism to control its half-life, or to regulate its interactions and localization. Since ubiquitination of membrane associated proteins mediate their targeting to lysosomes, ubiquitination may

underlie a post-internalization function of epsin in the endocytic pathway [71,72]. A further link between epsin and ubiquitin metabolism has been revealed by the identification of UIMs in epsin and other ENTH and VHS domain containing proteins [24] and by the demonstration that the UIMs of epsin bind ubiquitin (Polo et al., see above).

10. Nuclear cytoplasmic shuttling of ENTH domain proteins

Epsin and other well-characterized ENTH domain containing proteins are localized at the cell periphery, in proximity of endocytic sites, actin and signaling scaffolds [13,44,45,49]. Yet, incubation of cells with leptomycin, a fungal metabolite that inhibits the Crm1-dependent nuclear export pathway, leads to the rapid accumulation of both epsin and CALM in the nucleus, suggesting that these ENTH domain containing proteins continuously shuttle in and out of the nuclear envelope [48,73]. A similar shuttling, involving a reversible binding to Ran GTPases, has been demonstrated for Eps15 [73]. Epsin does not contain a conventional nuclear localization sequence, but it is interesting to note that its ENTH domain shares some similarity to the karyopherin/importins (see above) (Fig. 2c). Furthermore, β-catenin, another structural neighbor of the ENTH domain (Fig. 2b) and of karyopherin, also shuttles in and out of the nucleus but lacks a classical nuclear localization sequence. The list of proteins with a main function in the cortical region of the cell, but which also have a second life in the nucleus as transcriptional regulators, is rapidly increasing (for example [74–77]). They include actin [78] and proteins that, like epsin, can bind both plasma membrane phospholipids and transcription factors. One well-characterized such example is tubby [79]. Perhaps, this dual localization reflects an origin of the nuclear matrix from a prokaryotic cortical cytoskeleton that acts as a scaffold for DNA. In eukaryotic cells, where the DNA has been segregated from the cytoplasm by the nuclear envelope, nuclear cytoplasmic shuttling of proteins of the cell cortex may play an important role in mediating a signaling feed-back between the transcriptional machinery and changes in the extracellular environment.

11. Putative adaptor function of ENTH domain containing proteins

Based on their structural properties and interaction, as well as on the phenotype resulting from their functional disruption, it appears that a main function of the majority of ENTH domain containing proteins is to act as accessory clathrin adaptors in endocytosis (Fig. 4). Thus, they bind to the phospholipid bilayer, and help recruiting clathrin coat components and EH domain containing clathrin accessory factors, such as Eps15 and intersectin. Consistent with this hypothesis, AP180 and CALM are enriched in clathrin-coated vesicles [13,33] and disruption of AP180 at the synapse leads to defects in the clathrin-dependent endocytosis of synaptic vesicles. An accumulation at clathrin-coated pits was also observed for epsin [13,20], HIP1 [41] and HIP1R [44], although the recovery if these proteins in purified clathrin-coated vesicle fractions is variable [13,80], possibly depending, in part, upon the variable preservation of phosphoinositides on the vesicle membrane. A similar clathrin adaptor function has been demonstrated for one class of VHS domain proteins, the GGA proteins, which comprise an adaptin homology region and clathrin binding sites. The VHS domain of the GGA proteins not only binds phospholipids but also cytoplasmic tails of vesicle cargo proteins, thus participating in cargo selection [60,61]. An interesting possibility, to be explored by future studies, is that ENTH domains as well may interact with the cytosolic tail of membrane proteins and thus contribute to the sorting of cargo transmembrane proteins into budding vesicles. Via interactions with clathrin accessory factors such as Eps15, intersectin and POB1, ENTH domain proteins may help to coordinate the endocytic reaction with a local reorganization of the actin cytoskeleton and of signaling scaffolds.

Mono-ubiquitination of the cytoplasmic tails of several membrane proteins has been shown to be important for their internalization [81]. UIMs may interact with their ubiquitinated tails and thus contribute both to the selection of these proteins as cargo for the nascent vesicle and to the recruitment of ENTH domain containing proteins to the membrane (Fig. 4). An additional function of UIMs may be to bind ubiquitin-loaded E3 enzymes, thus facilitating the ubiquitination of ENTH domain proteins (Polo et al., see above). Support to the second possibility comes from essential requirement for the UIMs in the mono-ubiquitination of epsin and of other UIM domain containing proteins tested (Eps15 and Hrs) (Polo et al., see above). In either case, presence of ubiquitinated ENTH domain proteins on internalized vesicles may help to direct them to the endosome-lysosomal system [71,72].

UIMs are also present in VHS domain containing proteins, which may additionally contain the FYVE domain, a PtdIns(3)P binding module (see Stenmark et al. [63]). Thus, phosphoinositides and membrane-bound ubiquitin may func-

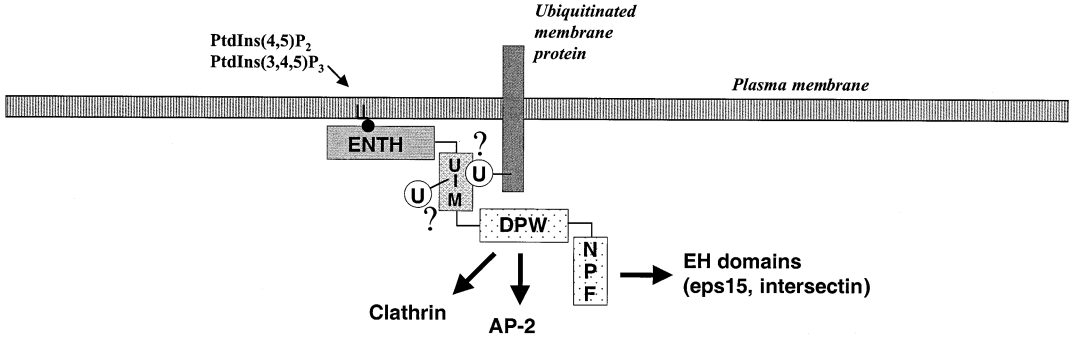

Fig. 4. Interactions of epsin illustrating its putative adaptor function. The 'so called' DPW domain include both DPW repeats and two clathrin boxes [20]. While the interaction of epsin's UIM domain with ubiquitin has been documented (Polo et al., see text), it remains unclear whether epsin does bind ubiquitinated protein cargo of clathrin-coated vesicles. It is also unclear whether the ubiquitination of epsin, which requires an intact UIM, interferes with the ubiquitin binding properties of the UIM. U = ubiquitin.

tion as membrane coreceptors for both VHS and ENTH domain containing proteins. While the predominant phosphoinositide on the plasma membrane and nascent endocytic vesicles is PtdIns(4,5)P2, an important phosphoinositide on endosomes is PtdIns(3)P. It is therefore of interest that ENTH domain containing proteins appear to be involved in the endocytic reaction, while Hrs, which contains a VHS domain followed by the FYVE domain is implicated in downstream stations of the endocytic pathway. A progressive shift from an ENTH-based membrane associated scaffold to a VHS-FYVE domain-based scaffold may occur along the endocytic pathway.

12. Conclusions and future perspectives

ENTH domain containing proteins have come to center stage in cell biology as important cross-roads of many different areas of cell biology. The ENTH domain is a membrane interacting module which is found in a variety of proteins whose shared characteristic is to act at early stages of the endocytic pathway as coat components and coat adaptors. These proteins have additional roles in signaling and actin regulation. Their further characterization may help shed light on the elusive link between actin and endocytosis. Priorities for future research include a precise elucidation of the link of ENTH domain containing proteins to ubiquitin metabolism and an understanding of how the multiple interactions of these proteins are regulated. A well-coordinated sequence of conformational changes and posttranslational modification is likely to be essential for a proper function of these proteins in the context of the cell cytoplasm.

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