The Yeast Epsin Ent1 Is Recruited to Membranes through Multiple Independent Interactions

Received for publication, November 14, 2002, and in revised form, January 6, 2003
Published, JBC Papers in Press, January 14, 2003, DOI 10.1074/jbc.M211622200

Rubén Claudio Aguilar, Hadiya A. Watson‡, and Beverly Wendland§
From the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

In addition to its well known role in targeting proteins for proteasomal degradation, ubiquitin (Ub) is also involved in promoting internalization of cell surface proteins into the endocytic pathway. Moreover, putative Ub interaction motifs (UIMs) as well as Ub-associated (UBA) domains have been identified in key yeast endocytic proteins (the epsins Ent1 and Ent2, and the Eps15 homolog Ede1). In this study, we characterized the interaction of Ub with the Ede1 UBA domain and with the UIMs of Ent1. Our data suggest that the UIMs and the UBA are involved in binding these proteins to biological membranes. We also show that the Ent1 ENTH domain binds to phosphoinositides in vitro and that Ent1 NPF motifs interact with the EH domain-containing proteins Ede1 and Pan1. Our findings indicate that the ENTH domain interaction with membrane lipids cooperates with the binding of membrane-associated Ub moieties. These events may in turn favor the occurrence of other interactions, for instance EH-NPF recognition, thus stabilizing networks of low affinity binding partners at endocytic sites.

Endocytosis is a multistep process in which cells selectively internalize plasma membrane proteins and lipids, as well as extracellular macromolecules such as nutrients and peptide hormones. It is an effective way to control the composition of the plasma membrane, and thus the physiological responses of the cell, so it is a tightly regulated pathway. Many cytosolic proteins are required for the early stages of clathrin-dependent endocytosis, including the adaptor protein complex AP2, the coat protein clathrin, and more recently, a class of proteins called accessory factors (1, 2). Because accessory factors interact with AP2 and are required for endocytosis, but are not enriched in purified clathrin-coated vesicles, they have been suggested to play a regulatory rather than a structural role in clathrin-coated vesicle formation (3). However, the precise functions, interactions, and order of operation of most accessory factors have not yet been determined.

One accessory factor of interest to many investigators is the protein epsin, which is conserved from yeast to humans. Rat epsin 1 (Eps15 interactor 1) was first identified in a yeast two-hybrid screen as a protein that binds to another endocytic accessory factor, Eps15 (4). Epsin and eps15 are localized to endocytic sites at the plasma membrane and interact directly through the Asn-Pro-Phe (NPF) tripeptide motifs of epsin binding to the Eps15 homology (EH) domains of Eps15 (4). The yeast epsins Ent1 and Ent2 were similarly identified as binding partners of the EH domain-containing endocytic protein Pan1 (5, 6). Several independent lines of evidence have indicated an important role for epsin in the internalization step of endocytosis. First, overexpressing fragments of epsin in cultured cells inhibits internalization of ligands such as transferrin and epidermal growth factor (4, 7, 8). Second, mutation of the yeast epsins results in growth defects and reduced internalization of plasma membrane lipids and proteins (6, 9, 10). Third, Drosophila melanogaster epsin (liquid facets) exhibits genetic interactions with genes encoding other endocytic machinery components (11). Fourth, in vitro studies show that epsin can stimulate clathrin polymerization into the spherical basket that encases endocytic vesicles (12). Finally, recent work suggests that epsin binding to phospholipids initiates the membrane curvature necessary to form an endocytic vesicle (13).

Epsins from all species share several features (Fig. 1A), including a conserved epsin amino-terminal homology (ENTH) domain that binds the phospholipid phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2), two or three copies of the ubiquitin interaction motif (UIM, Fig. 1A) that binds ubiquitin (Ub), and a carboxyl-terminal region containing numerous short linear peptide sequences that serve as ligands for binding to components of the endocytic machinery such as AP2 (mammalian epsins), EH domains, and clathrin (Fig. 1A; Refs. 4, 6, 7, 10, and 14). To understand the function of such multivalent proteins, it is necessary to characterize the nature of each interaction in isolation as well as in the context of the entire protein, where cooperativity may influence some of its properties. In this study, we have defined the binding partners for each of the characterized domains of the yeast epsin Ent1, using in vitro recombinant protein binding, yeast two hybrid, and immobilized phospholipid binding experiments.

The yeast epsins are almost exclusively localized in cortical patches at the plasma membrane (6, 9), but the cis- and trans-acting components necessary for their membrane association have not been determined. Here we show that Ub covalently attached to transmembrane proteins (often a requirement for internalization; reviewed in Ref. 15) or to peripheral membrane

§ Burroughs Wellcome New Investigator in the Pharmacological Sciences. To whom correspondence should be addressed: Dept. of Biology, The Johns Hopkins University, Mudd Hall, Rm. 35, 3400 N. Charles St., Baltimore, MD 21218. Tel.: 410-516-0460; Fax: 410-516-5213; E-mail: bwendland@jhu.edu.

‡ Supported by a Ford Foundation Dissertation Fellowship.

§ Supported by a Ford Foundation Dissertation Fellowship.

1 The abbreviations used are: EH, epsin homology; Ub, ubiquitin; WT, wild type; GAL4bd, GAL4 DNA-binding domain; GAL4ad, GAL4 transcription activation domain; ENTH, epsin amino-terminal homology domain; UIM, ubiquitin interaction motif; UBA, ubiquitin-associated domain; NSF, asparagine-proline-phenylalanine tripeptide; PI(4,5)P2, phosphatidylinositol (4,5) bisphosphate; GST, glutathione S-transferase; CBD, clathrin-binding domain.
proteins mediates the recruitment of Ent1 to membranes. By examining the requirements for recruitment of Ent1 to biological membranes, we have uncovered evidence for cooperativity between the ENTH domain and the UIMs for efficient membrane binding. Other data indicate that the NPF-containing regions of Ent1 also contribute to additional layers of cooperative interactions. Together, our data are consistent with a model in which epsin might function as an endocytic adaptor protein. We suggest that epsin binds PI(4,5)P$_2$-enriched membranes and mediates the recruitment of Ent1 to biologically active membranes.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Constructs**—The Gal4AD and BD fusion constructs were prepared by ligating the appropriate PCR fragments (digested with BamHI-SalI) into the BamHI-XhoI and BamHI-SalI sites of the pGADT7 (LEU2, Clontech) and pGBT9 (TRP1, Clontech) vectors, respectively. To prepare His$_6$-tagged and GST fusion proteins, cDNAs were subcloned into the pET28a (Novagen) or pGEX-4T-1 (Amersham Biosciences) vectors, respectively. Amino acid substitutions were made using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Murine p63 in pS2—1 and SV40 large T-antigen in pACT2 were obtained from Clontech.

**Two-hybrid Assays**—The yeast strain AH109 (MATa, trp1-901, leu2–3, 112, ura3–52, his3–200, gal4Δ, gal80Δ, LYS2::GAL1,αs– GAL1::HIS3, GAL2::PARK2, ade2, URA3::MEL11–15, MEL1::GAL1–lacZ, Clontech) was maintained on YPD agar plates. Transformations were performed according to the Clontech's Yeast Protocols Handbook.

**Recombinant His$_6$-tagged and GST Fusion Protein Expression and Purification**—Protein expression was induced in Rosetta cells (Novagene) transformed with pET28a or pGEX-derived constructs by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM final concentration) followed by a 5-h incubation at 30°C. The cells were harvested, resuspended in TBST (10 mM Tris, 140 mM NaCl, 0.1% Tween 20, pH 8.0), and incubated with 1 mM ml lysozyme (Sigma) for 30 min at 4°C, and sonicated. The lysates were centrifuged for 30 min at 15,000 rpm at 4°C in a Sorvall centrifuge, and the supernatants were collected.

For His$_6$-tagged protein purification, the lysates were incubated in batch with nickel-nitrilotriacetic acid Superflow resin (Qiagen, Valencia, CA) in the presence of 5 mM imidazole (Sigma) for 1 h at 4°C. The unbound fraction was separated by centrifugation, and the resin containing the bound His$_6$-tagged protein was packed into a disposable polypropylene column (Pierce). The column was washed with TBST/15 mM imidazole, and the protein was then eluted with TBST/150 mM imidazole, and fractions containing the His$_6$-tagged protein were pooled. The imidazole from the His$_6$-tagged protein and the glutathione from the GST fusion protein fractions were eliminated by using a PD-10 desalting column (Amersham Biosciences).

For GST fusion protein purification, the lysates were incubated in batch with agarose beads coupled to glutathione for 2 h at 4°C and then washed 4 times with TBST. The beads were then incubated with 20 mM glutathione (Sigma) in TBST for 30 min at room temperature, and the supernatant containing the purified fusion protein was collected.

**Yeast Membrane Preparation**—C.120 cells (MATa lys2–801 leu2–3 112 ura3–52 his3–Δ200 trp1–1 ubi1::TRP1 ubi2::URA3 ubi3::Δubi4 ubi4::Δ2::LEU2) expressing copper promoter-driven His-Ub (p6209) (described in Ref. 16) or pep4A cells (MATa leu2–3 ura3–52 his3–Δ200 trp1–Δ901 (p2–ho1 supC2Δ9 pep4A::LEU2) expressing copper promoter-driven myc-tagged ubiquitin were grown overnight in selective medium to mid-log phase and induced with 0.1 mM CuSO$_4$ for 3 h. Twenty to fifty A$_{600}$ (1 A$_{600}$ corresponds to ~1.2 × 10$^9$ cells) were harvested by centrifugation (13,000 g) and incubated for 10 min at room temperature in softening solution (Triton X-100, 8.0), and incubated with 1 mg/ml lysozyme (Sigma) for 30 min at 30°C. Spheroplasts were washed once, and pellets were Dounce-homogenized in HEPES/KOAc lysis buffer containing 50 mM N-ethylmaleimide (Sigma) and protease inhibitor mixture (Boehr, Indianapolis, IN). Homogenates were spun at 300 × g for 5 min, and the resulting pellets were stored at –80°C before use in membrane-binding experiments.

**GST and Ub-Agarose Pull-down Assays**—All binding assays were incubated for 1 h at 4°C in the presence or absence of 10 mM free ubiquitin (Sigma). For GST pull-downs, glutathione-agarose beads (Sigma) loaded with either GST or GST fusion proteins were incubated with purified His$_6$-tagged recombinant protein. For Ub binding, Ub coupled to agarose beads (Sigma) was incubated with either purified His$_6$-tagged recombinant protein or $^{35}$S-labeled in vitro transcription/translation products. After washing with TBST, bound radiolabeled samples were quantified in a scintillation counter, whereas all other samples were boiled in Laemmli's protein sample buffer and resolved by SDS-PAGE followed by immunoblotting using either a rabbit polyclonal antibody raised against the Ent1 protein (previously described in Ref. 9) or an anti-His$_6$ tag mouse monoclonal antibody (Clontech).

**In Vitro Transcription/Translation and Binding of the in vitro-transcribed/translated Products to Yeast Membranes**—$^{35}$S-labeled proteins were obtained by in vitro transcription/translation of pET28a constructs using the TNT T7 Quick coupled transcription/translation system (Promega, Madison, WI) and Easytag expression protein labeling mixture (PerkinElmer Life Sciences) according to the manufacturer's instructions. The transcription/translation products were diluted 1:100 in TBS and centrifuged (150,000 g, 1 h, 4°C), and the resulting pellet was resuspended in TBS. The integrity of the protein was determined by a conventional trichloroacetic acid precipitation assay (only tracers showing at least 10% incorporation were used for further experiments). The predominance of a single radiolabeled species was verified by SDS-PAGE followed by autoradiography. The concentration of radioactive material for different mutants was normalized to $^{35}$S incorporation and the number of methionines present in the corresponding Ent1 truncations.

3–10 × 10$^5$ cpm of $^{35}$S-labeled proteins were incubated with membrane suspensions prepared from A$_{600}$ of cells in a total volume of 300 μl, for 1 h at either room temperature or 4°C, and in the presence or absence of 10 mM free ubiquitin. Following incubation, the membrane pellets were washed in TBS, centrifuged (136,000 g, 1 h, 4°C), and their specific activity was determined by a conventional trichloroacetic acid precipitation assay (only tracers showing at least 10% incorporation were used for further experiments). The predominance of a single radiolabeled species was verified by SDS-PAGE followed by autoradiography. The concentration of radioactive material for different mutants was normalized to $^{35}$S incorporation and the number of methionines present in the corresponding Ent1 truncations.
Ent1 Recruitment to Membranes

Ent1 Binds Ub via Its UIMs in a Cooperative Fashion—It has been shown previously that Ent1 binds Ub via its UIMs (10). We confirmed and further characterized the specificity of this interaction. In an in vitro pull-down assay, we found that recombinant His$_6$-Ent1 protein bound Ub-agarose beads as assessed by immunodetection of starting and bound material with an anti-His$_6$ antibody (Fig. 1A, lanes 1 and 2). Importantly, this interaction was specifically inhibited by the presence of an excess (10 mM) of free Ub (Fig. 1A, lane 3).

We next investigated whether both UIMs are necessary for the recognition of Ub. Both conserved serines within the Ent1 putative UIMs (Ser$_{177}$ and Ser$_{201}$) were mutated to aspartate, the recognition of Ub. Both conserved serines within the Ent1 PAM250 matrix ("selected based on random permutation probabilities according to Dayhoff’s PAM250 matrix (i.e. the residue substitutions least likely to cause structural perturbation in the protein)." $^{35}$S-labeled His$_6$-Ent1 wild type, single, and double mutants were produced by in vitro transcription/translation, incubated with Ub-conjugated agarose beads, and bound radioactivity was quantified. Compared with Ent1 WT, all five of the Ent1 UIM double mutants exhibited significantly impaired ubiquitin binding (data not shown), and as demonstrated by the 34% reduction for the S → D double mutant (Fig. 1C). Also, mutation of a single conserved serine within either UIM decreased the binding of the mutant proteins for Ub-agarose beads by at least 50% (Fig. 1C). These results suggest that the two UIMs cooperate for the interaction of Ent1 with Ub.

Ent1 Binds Yeast Membranes in a Ub- and UIM-dependent and Cooperative Fashion—It has been demonstrated that certain cell-surface transmembrane proteins, such as Ste2 and Ste6, are ubiquitinated as a prerequisite to their internalization (17, 18). Thus, it is possible that these and/or other Ub-modified proteins such as peripheral membrane proteins may act as docking sites for Ub-binding proteins such as Ent1. To test this possibility, we prepared a crude membrane fraction from yeast that contains plasma membrane, which is the major localization site of many endocytic proteins in vivo (Fig. 2A), including the yeast epsins (6). We confirmed by Western blotting that these membranes contained an abundance of Ub-conjugated proteins (Fig. 2A), and tested whether these membranes could recruit recombinant His$_6$-Ent1 in a Ub-dependent manner.

Purified His$_6$-Ent1 was incubated with a suspension of membranes for 1 h at 4°C in the presence or absence of 10 mM free Ub. After washing, the presence of the recombinant protein associated with the membrane pellet was assessed by SDS-PAGE, followed by Western blotting with an anti-His$_6$ antibody. Our results showed that His$_6$-Ent1 interacts with membranes prepared from either MATa (Fig. 2B, lane 1) or MATa cells (Fig. 2B, lane 3). 10 mM free Ub greatly reduced this membrane recruitment (Fig. 2B, lanes 2 and 4). We also observed that even in cases where our His$_6$-Ent1 preparations contained some protein degradation products (some as abundant as the intact protein), only full-length Ent1 was recruited to the yeast membranes. Additionally, an excess of yeast cytosolic proteins did not compete the interaction (data not shown).

To characterize further this membrane association process, we studied the recruitment of Ent1 UIM mutants. For these experiments, $^{35}$S-labeled in vivo transcription/translation products were incubated with membrane suspensions for 1 h at 4°C, and after washing, the radioactivity associated with the membrane pellet was quantified. As shown in Fig. 2C, and
consistent with their reduced binding to Ub-agarose, the S → D single and double mutants exhibited at least 50% reduced recruitment to membranes as compared with WT Ent1. We observed reduced membrane binding with all five UIM double mutants (S → D, S → E, S → K, S → R, and S → C), and the reduction was comparable with the binding of WT Ent1 to membranes in the presence of free Ub (ranging from 40% to 60% inhibition, depending on the experiment). The remaining bound material may be nonspecific binding or, alternatively, could represent a Ub-independent component of membrane recruitment (see below). The finding that a single mutation introduced into either the first or second UIM of Ent1 was sufficient to drastically decrease the membrane binding capability of the protein is again consistent with cooperative binding of the two UIMs to Ub.

We also carried out saturation experiments by incubating increasing concentrations of 35S-labeled Ent1 with a constant amount of membrane in the presence or absence of free Ub (data not shown). Although we were not able to confirm that binding equilibrium was achieved after 1 h at 4 °C, we roughly estimated an avidity dissociation constant of ~20 μM for each UIM.

The ENTH Domain Cooperates with the UIMs for Membrane Recruitment—The ENTH domains of mammalian epsins bind phosphoinositides (7); therefore, we tested whether the Ent1 ENTH domain also interacts with lipids, thus possibly contributing to Ent1 membrane association. Purified His6-tagged proteins plus 3% fatty acid-free bovine serum albumin were incubated with nitrocellulose strips containing a variety of immobilized phospholipids. After washing, the bound proteins were detected using an anti-His6 antibody and visualized by chemiluminescence. Both His6-Ent11–454 (full-length) and His6-Ent11–149 (ENTH domain) bound phosphoinositides immobilized onto nitrocellulose (Fig. 3A). As expected, an Ent1 amino-terminal truncation lacking the ENTH domain (Ent1149–454) did not exhibit phospholipid binding activity (Fig. 3A). It should be noted that, because of the qualitative nature of this binding assay, slight differences observed between full-length and the ENTH domain binding were considered insignificant.

Given that the Ent1 ENTH domain binds phosphoinositides and its UIMs bind ubiquitin, we next explored a possible interplay between the UIMs and the ENTH domain for membrane recruitment. To this end, we prepared two Ent1 truncations (see Fig. 1A), one lacking the first 148 amino acids that comprise the ENTH domain (Ent1149–454, containing both UIMs plus the carboxyl-terminal part of the protein) and another consisting of amino-acids 1–240 (Ent11–240, containing the ENTH domain plus both UIMs and a putative coiled coil region).

We assayed the binding of the Ent11–240 and Ent1149–454 truncations at room temperature rather than 4 °C to preserve physiological membrane characteristics such as membrane lipid fluidity. As before, 35S-labeled in vitro transcription/translation products were incubated with membranes, and the membrane-associated radioactivity was quantified. Each Ent1 truncation bound the membranes, consistent with Ub-dependent recruitment. However, we found that Ent11–240 bound membranes to a greater extent than Ent1149–454 (Fig. 3B). This suggested that, in addition to the UIMs, the ENTH domain also contributed to the membrane recruitment of Ent1. Moreover, we also found that the highly phosphorylated soluble inositol phosphate IP6 (phytic acid) that binds ENTH domains (13, 19) was able to reduce significantly (by 20–40%) some of the membrane recruitment of Ent11–240 (Fig. 3B). In contrast, efficient membrane recruitment of Ent11–240 was still observed on incubation with ISP (myoinositol hexasulfate) (Fig. 3B). Membrane recruitment of the Ent1149–454 truncation was the same in the presence of either IP6 or ISP, washed, and the bound radioactivity (in cpm) was measured in a scintillation counter. Values are the means ± S.D. of triplicates, from one of two independent experiments. Statistical significance in comparison with binding showed by Ent11–240 incubated with ISP is indicated by ** (p < 0.005).

The UBA Domain of Ede1 Binds Yeast Membranes in a Ub-dependent Manner—Ede1 is an endocytic protein (the yeast homolog of eps15) with a multimodular domain architecture, including 3 EH domains and a UBA domain (14, 20). Because the Ede1 UBA domain binds Ub (10, 2) and given that this

---

R. C. Aguilar, H. A. Watson, and B. Wendland, unpublished results.
The UBA domain of Ede1 binds yeast membranes in a Ub-dependent fashion. A schematic representation of Ede1 domain organization. The endocytic protein Ede1 comprises a multimodular domain structure, from amino to carboxyl-terminus: two EH domains, a Pro-rich region (PPP), coiled coils (CC), and a Ub-associated domain (UBA). B, the Ede1 UBA domain recruitment to biological membranes is inhibited by free Ub. Membrane suspensions were incubated with Ub, Pro-rich region (PPP), coiled coils (CC), and a Ub-associated domain (UBA). As shown in Fig. 4B, the Ede1 UBA domain recruitment to biological membranes is inhibited by free Ub. Membrane suspensions were incubated with 35S-labeled Ede1 UBA domain (see text for details) for 1 h at 4°C in the presence or absence of 10 mM Ub and washed, and the bound radioactivity (in cpm) was measured in a scintillation counter. Values are the means ± S.D. of triplicates obtained from two independent experiments. Statistical significance in comparison with binding showed by Ede1 UBA domain in absence of free Ub is indicated by ** (p < 0.005).

Ent1 Is Involved in a Network of Interactions with Other Endocytic Proteins That Localize to Membranes—Ent1 exhibits a cortical patch membrane localization pattern similar to that of yeast epsins (6, 9, 20, 21). It is possible that Ede1 and Ent1 colocalize through recognition of ubiquitous membrane proteins. Alternatively, they could colocalize through an EH/NPF interaction, because the yeast epsins were originally identified in a yeast two-hybrid screen with the EH domains of Pan1 (5, 6). We found that Ent1 is indeed able to interact in a two-hybrid system with Ede1 EH1 domains, specifically with the third EH domain (EH3), and not the first or second EH domains (Fig. 5A). The carboxyl-terminal Ent1149−454 protein was sufficient to bind the Ede1 EH3 domain, consistent with the NPF motifs being the binding determinants involved in this interaction (Fig. 5A). In fact, we established that this interaction is indeed mediated by Ent1 NPF motifs, as mutation of both NPF motifs to NPM abolished the binding (Fig. 5B). Mutation of a single NPF motif decreased but did not abrogate the EH domain-dependent recognition, suggesting that each Ent1 NPF motif participated in the interaction with Ede1 (Fig. 5B). Interestingly, we also found that the Ent1 interaction with Pan1 is much weaker than with Ede1 (Fig. 5A), suggesting a preference for Ede1 over Pan1.

To confirm the Ent1/Ede1 interaction, we performed GST pull-down experiments using a GST-EH3 fusion protein. As shown in Fig. 5C, His6-Ent1 bound Ede1 EH3 and clathrin. GST pull-down experiments were conducted by incubating (1 h at 4°C) His6-Ent1 bound Ede1 EH3 (H3) with GST, clathrin, Ede1 EH3, or clathrin-binding domain (CBD) with glutathione-agarose beads loaded with GST alone (−) or with GST fused to Ede1 EH3, the terminal domain (TD) of clathrin or Ub. After washing with ice-cold buffer, the presence of the Histagged proteins was detected by Western blot with an anti-His antibody. A 10% sample of the total His6-tagged proteins added is shown.

The mechanisms mediating the recruitment of the endocytic molecular machinery at sites where internalization takes place are not completely understood. In this study, we present evidence suggesting that the yeast epsin Ent1, an integral part of the endocytic machinery, is recruited to biological membranes by multiple, cooperative interactions with lipids and proteins through its numerous binding domains. Importantly, we show...
that biological membranes containing plasma membrane proteins, endocytic machinery, and ubiquitinated proteins can recruit epsins and Ede1. It has not yet been clearly determined which specific ubiquitinated proteins are the targets in these membranes; however, an attractive possibility would be membrane receptors and/or ubiquitinated endocytic machinery. In fact, preliminary evidence from cross-linking His6-Ent1 to MATA' membranes indicated that ubiquitinated Ste6 is one of the interacting partners for Ent1.2

Many studies have uncovered roles for ubiquitination in the endocytosis and lysosomal/vacuolar degradation of membrane receptors (reviewed in Ref. 15), which may be facilitated byUb binding to some endocytic proteins (10, 14, 23). We have demonstrated that Ent1 binds Ub in a UIM-dependent manner, and that, similarly to Vps27 (10), the two Ent1 UIMs cooperate in this interaction. Whether each UIM binds to different Ub moieties to generate a stable multivalent-complex or instead both UIMs together bind a single Ub unit requires further investigation. A UIM is predicted to form an α-helix (14), and other Ub-binding domains such as UBA and CUE domains are composed of three α-helices. In fact, the CUE domain binds Ub via an interface of two α-helices,3 so it is possible that two UIMs might similarly join to form a binding site for a single Ub.

We propose that at 4 °C, Ent1 recruitment to membranes is primarily UIM-dependent, as binding is decreased (from 40 to 60%) either by addition of free ubiquitin or by mutation of the UIMs. Similarly to the cooperative Ub/UIM interaction we observed with recombinant protein, our studies showed that the individual UIMs cooperate for membrane association. Analysis of the membrane binding data, considering the individual contributions of each UIM bound to Ub ([UIM1-Ub], [UIM2-Ub], and [UIM1-Ub-UIM2] complexes), gave an estimated apparent dissociation constant of ~20 μM, which is similar to the binding affinity of Ub to the single UIM present in the mammalian protein Hrs (24). However, a remarkable consequence of the UIM-bivalency of Ent1 is that the effective overall avidity of the protein for membrane-linked Ubs falls in the 10−8 m range (data not shown). Additionally, the apparently high affinity of Ent1 for membranes could represent Ent1 cooperativity, as a Hill coefficient significantly greater than 1 (nH = 1.78 ± 0.04) was calculated from membrane binding experiments and Ent1/Ent1 interactions were observed in a yeast two-hybrid study.2

Because a wild-type ENTH domain of yeast epsin is required for endocytosis and viability in yeast (6), ENTH domain-binding activity may be most central to the endocytic function of epsin. Previous studies have shown that ENTH domains bind phosphoinositides (7), and we have confirmed this for the Ent1 ENTH domain. Thus, it was surprising to find that a major contribution to membrane binding is provided by the recognition of Ub by the Ent1 UIMs (at 4 °C). These results suggest that alteration of membrane biophysical properties due to lipid phase transitions (likely to occur at temperatures below 20 °C) and/or the weakening temperature-dependent entropic interactions (the primary driving force for hydrophobic contacts) would result in unfavorable conditions for ENTH domain binding to membranes at 4 °C. Supporting this interpretation, our experiments conducted at room temperature indicated that the ENTH domain not only binds lipids, but also cooperates with the UIMs for binding to membranes. The order of events for ENTH domain- and UIM-mediated recruitment of Ent1 to sites of endocytosis remains to be determined. However, one possibility is that the ENTH domain localizes the epsins to phosphoinositide-rich plasma membrane domains, whereas the UIMs provide specific targeting to ubiquitinated transmembrane and/or peripherally associated proteins.

It should be noted that our recruitment to membranes assay using radiolabeled protein resulted in a degree of background variability for each experiment, although all experiments showed similar statistically significant trends. We are currently working on optimizing reconstitution assays using liposomes and/or noninvasive in vivo techniques. However, these membrane binding experiments do have the advantage of using biological materials that provide an indication of in vivo interactions.

We have shown Ub-dependent membrane recruitment of the Ede1 UBA domain, suggesting that Ede1 may also bind ubiquitinated membrane-associated proteins. We also found that Ede1 and Ent1 bind one another through a synergistic EH/NPF interaction, which illustrates a recurring theme of cooperativity for Ent1 interactions. Because an Ent1 amino-terminal truncation (Ent1149–454) still bound membranes (Fig. 3B), it is possible that EH domain-containing proteins like Ede1 may also recruit Ent1 to membranes via EH/NPF interactions, independently of Ent1 binding to lipids.

Additionally, and as expected, the Ent1 carboxyl-terminal CBD was found to be sufficient for binding to the terminal domain of clathrin. Because a yeast strain lacking the five protein complexes previously recognized as homologues of clathrin assembly factors and/or adaptors (deleted for genes encoding yAP180A, yAP180B, and the β-subunits of AP1, AP2, and AP3; Ref. 25) is viable, can perform endocytosis, and form clathrin-coated vesicles, it is clear that other factors remain to support these activities. Epsin is an obvious candidate, particularly in light of the recent demonstration that rat epsin can stimulate clathrin cage assembly (12).

Based on our results, we propose a model (Fig. 6) in which Ent1 is recruited to nascent endocytic sites as a combined result of its lipid-membrane binding property (through its ENTH domain) and its UIM-mediated specificity for ubiquitinated proteins, depicted here as cargo. This could be followed by the recruitment of Ede1 that may also interact with cargo and/or covalently linked Ub moieties via its UBA domain and cooperatively bind Ent1 NPFs through its EH domain. In turn, other accessory proteins could bind to both Ent1 and Ede1 and the endocytic coat would be assembled by interaction with the Ent1 CBD, among other clathrin-binding determinants. Furthermore, we suggest that the epsins may play a key role as adaptors...
linking ubiquitinated cargoes to the endocytic machinery, recruiting proteins with specific activities and/or exerting undiscovered functions by themselves that make endocytosis possible.

One interesting prediction of our model is that multiple mutations would be necessary to affect significantly the function/localization of the epsins. In agreement with this hypothesis, neither mutation nor deletion of the Ent1 UIMs is sufficient to render a phenotype in yeast cells expressing the mutant protein as the exclusive source of epsin (10). Our model predicts that such UIM mutants would still be able to properly localize at endocytic sites via their ENTH and NPF interactions, consistent with the finding by Shih et al. (10) that both deletion of Ede1 and Ent1 UIM inactivation are required for a significant endocytic defect in vivo. In fact, mutating the ENTH domain is the only known case in which single-domain mutations produce a phenotype (6). This is consistent with the findings of Ford et al. (13) and supports our model in which epsin binds to membranes via its ENTH domain as an early and required step for membrane localization.

We also expect Ent1 recruitment to membranes to be highly regulated by reversible post-translational modifications such as phosphorylation (9, 23, 26) that may influence the stability of the endocytic network. For example, phosphorylation of Ent1 Thr394 and Thr416, flanking the second NPF motif, has been shown to affect the endocytic function of Ent1 (9). Consistent with studies of phosphoregulation of endocytic complex function in nerve terminals (25), our preliminary data indicate that this modification impairs the Ent1/Ede1 interaction. On the other hand, it was recently demonstrated that UIM-containing proteins can be ubiquitinated in an UIM-dependent manner (23). Although the functional consequences of covalent Ub modification of epsins are unknown, it is possible that an intramolecular Ub/UIMs interaction may compete for the recognition of Ub linked to cargo by the UIMs (27).

In summary, we provide evidence suggesting that the epsins, as well as other proteins such as Ede1, are recruited to membranes through a network stabilized by cooperative interactions throughout their multiple modular domain architecture. Future in vitro reconstitution studies will be necessary to determine the order of action of these and other proteins in initiating and assembling the endocytic machinery.

Acknowledgments—We thank Catharine Sciambi for excellent technical assistance. We also thank Pietro De Camilli, David Katzen, Douglas Fambrough, and Jon Shaw for critical reading of the manuscript and members of the Wendland Laboratory for helpful comments and suggestions throughout the course of the work.

REFERENCES

1. Brodsky, F. M., Chen, C. Y., Knecht, C., Towler, M. C., and Wakeham, D. E. (2001) Annu. Rev. Cell Dev. Biol. 17, 517–568
2. Kirchhausen, T. (2000) Annu. Rev. Biochem. 69, 699–727
3. Slepnev, V. I., and De Camilli, P. (2000) Nat. Rev. Neurosci. 1, 161–172
4. Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P., and De Camilli, P. (1998) Nature 394, 793–797
5. Wendland, B., and Emr, S. D. (1998) J. Cell Biol. 141, 71–84
6. Wendland, B., Steele, K. E., and Emr, S. D. (1999) EMBO J. 18, 4383–4393
7. Roh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S., and Takenawa, T. (2001) Science 291, 1047–1051
8. Morinaka, K., Koyama, S., Nakashima, S., Hinoi, T., Okawa, K., Iwamatsu, A., and Kikuchi, A. (1999) Oncogene 18, 5915–5922
9. Watson, H. A., Cope, M. J., Groen, A. C., Druhan, D. G., and Wendland, B. (2001) Mol. Biol. Cell 12, 3668–3679
10. Shih, S. C., Katzen, D. J., Schnell, J. D., Sutanto, M., Emr, S. D., and Hicke, L. (2002) Nat. Cell Biol. 4, 389–393
11. Cadavid, A. L., Ginzel, A., and Fischer, J. A. (2000) Development 127, 1727–1736
12. Kalthoff, C., Alves, J., Urbanke, C., Knorr, R., and Ungewickell, E. J. (2002) J. Biol. Chem. 277, 8209–8216
13. Ford, M. G., Mills, I. G., Peter, B. J., Vailis, Y., Praefcke, G. J., Evans, P. R., and McMahon, J. T. (2002) Nature 419, 361–366
14. Hofmann, K., and Falquet, L. (2001) Trends Biochem. Sci 26, 347–350
15. Hicke, L. (2001) Nat. Rev. Mol. Cell. Biol. 2, 195–201
16. Ling, R., Colon, E., Dahmus, M. E., and Callis, J. (2001) Anal. Biochem. 282, 54–64
17. Hicke, L., Zanolari, B., and Riezman, H. (1998) J. Cell Biol. 141, 349–358
18. Loayza, D., and Michaelis, S. (1998) Mol. Cell. Biol. 18, 779–789
19. Saiardi, A., Sciambi, C., McCaffery, J. M., Wendland, B., and Snyder, S. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14206–14211
20. Gagny, B., Wiederkehr, A., Dunouin, P., Winsor, B., Riezman, H., and Haguenauer-Tsapis, R. (2000) J. Cell Sci. 113, 3309–3319
21. Ni, L., and Snyder, M. (2001) Mol. Biol. Cell 12, 2147–2170
22. ter Haar, E., Musacchio, A., Harrison, S. C., and Kirchhausen, T. (1998) Cell 95, 563–573
23. Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P., and Di Fiore, P. P. (2002) Nature 416, 451–455
24. Raiborg, C., Roche, K. G., Gilleoity, D. J., Madhus, I. H., Stang, E., and Stenmark, H. (2002) Nat. Cell Biol. 4, 394–398
25. Huang, K. M., D’Hempt, K., Riezman, H., and Lemmon, S. K. (1999) EMBO J. 18, 3997–3908
26. Chen, H., Slepnev, V. I., Di Fiore, P. P., and De Camilli, P. (1999) J. Biol. Chem. 274, 3257–3260
27. Wendland, B. (2002) Nat. Rev. Mol. Cell. Biol. 3, 971–977
The Yeast Epsin Ent1 Is Recruited to Membranes through Multiple Independent Interactions
Rubén Claudio Aguilar, Hadiya A. Watson and Beverly Wendland

J. Biol. Chem. 2003, 278:10737-10743.
doi: 10.1074/jbc.M211622200 originally published online January 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211622200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 13 of which can be accessed free at http://www.jbc.org/content/278/12/10737.full.html#ref-list-1