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EpsinR: an AP1/clathrin interacting protein involved in vesicle trafficking

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EpsinR is a clathrin-coated vesicle (CCV) enriched 70-kD protein that binds to phosphatidylinositol-4-phosphate, clathrin, and the gamma appendage domain of the adaptor protein complex 1 (AP1). In cells, its distribution overlaps with the perinuclear pool of clathrin and AP1 adaptors. Overexpression disrupts the CCV-dependent trafficking of cathepsin D from the trans-Golgi network to lysosomes and the incorporation of mannose-6-phosphate receptors into CCVs. These biochemical and cell biological data point to a role for epsinR in AP1/clathrin budding events in the cell, just as epsin1 is involved in the budding of AP2 CCVs. Furthermore, we show that two gamma appendage domains can simultaneously bind to epsinR with affinities of 0.7 and 45 μM, respectively. Thus, potentially, two AP1 complexes can bind to one epsinR. This high affinity binding allowed us to identify a consensus binding motif of the form DFxDF, which we also find in γ-synergin and use to predict that an uncharacterized EF-hand–containing protein will be a new gamma binding partner.

Introduction

Clathrin-coated buds have been observed on the plasma membrane, endosomes, the TGN, and on various other intermediate trafficking compartments (Stoorvogel et al., 1996; Zhu et al., 2001). They serve to package cargo from a donor compartment into transport vesicles.

To recruit cargo, a number of adaptor proteins are needed. These can be classified into four subunit polymeric adaptor protein complexes (APs;* AP1, AP2, AP3, and AP4; Hirst and Robinson, 1998; Boehm and Bonifacino, 2002) and monomeric adaptors, e.g., Golgi-localized, gamma-ear–containing ADP-ribosylation factor–binding proteins (GGAs; Dell'Angelica et al., 2000; Hirst et al., 2000). Each polymeric AP contains a specific μ-subunit that recognizes variations of a sequence motif (YxxΦ) on the cytoplasmic tails of cargo molecules (Kirchhausen et al., 1997; Owen and Evans, 1998). Each AP also contains two large homologous subunits (γ + β1 in the AP1 complex, α + β2 in the AP2 complex, and δ + β3 in the AP3 complex) whose appendage domains bind to core and accessory proteins necessary for clathrin-coated vesicle (CCV) formation. The α-appendage has been shown to bind proteins that contain DxF motifs, normally in multiple copies (Owen et al., 1999, 2000; Brett et al., 2002), and as part of this paper, we investigate the motif for binding the γ-appendage. AP2 adaptor complexes are found predominantly at the plasma membrane, whereas AP1 complexes are found concentrated at the TGN. The monomeric GGAs have recently been shown to bind cargo via a VHS domain (Doray et al., 2002; Misra et al., 2002; Shiba et al., 2002), and they also have a domain with very strong homology to the γ-adaptin appendage.

The AP of 180 kD (AP180) is a brain-enriched clathrin/adaptor-binding protein that promotes clathrin assembly on membranes (Ford et al., 2001). It is not a conventional adaptor protein in that it does not link cargo into the clathrin cage. The protein is essential for CCV formation in *Drosophila* (Zhang et al., 1998) and in *Caenorhabditis elegans* (Nonet et al., 1999), and overexpression of the COOH-terminal domain blocks clathrin-coated budding events (Ford et al., 2001). The AP180 NH_2-terminal homology (ANTH) domain of AP180 and its ubiquitous homologue, CALM, directs binding to PtdIns(4,5)P_2 in the membrane.

By domain analysis, the epsin family of proteins are close relatives of the AP180 family. They both have lipid-binding domains at the NH_2 terminus and clathrin/adaptor-binding

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*Abbreviations used in this paper: AP, adaptor protein complex; AP180, AP of 180 kD; CCV, clathrin-coated vesicle; EEA1, early endosome antigen 1; ENTH, epsin-NH_2-terminal homology; GGA, Golgi-localized, gamma-ear–containing ADP-ribosylation factor–binding protein; ITC, isothermal titration calorimetry; OSBP, oxysterol binding protein; PtdInsP, phosphatidylinositol phosphate.

Key words: epsin; GGA; TGN; endocytosis; ENTH

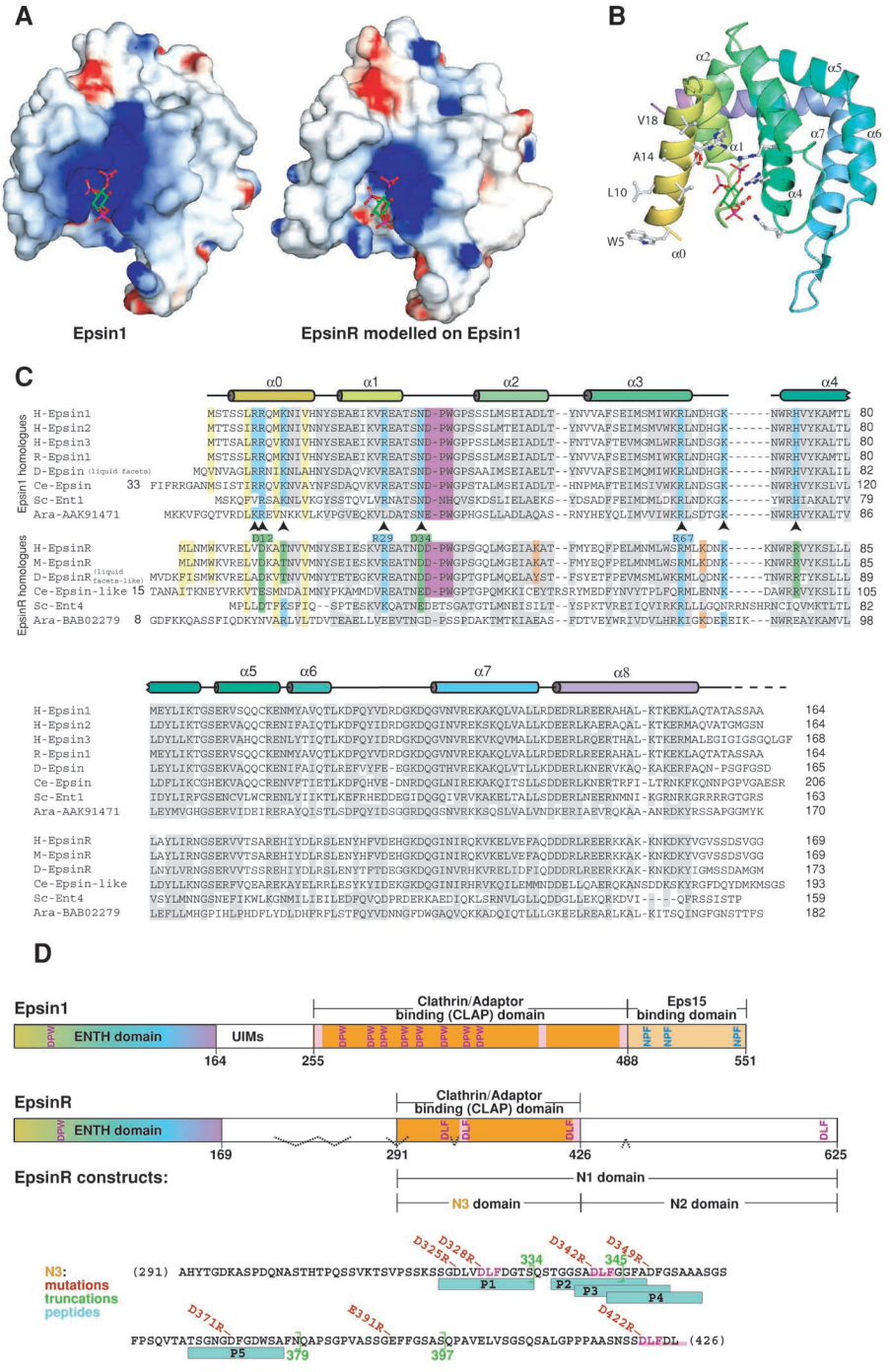

Figure 1. **The ENTH domain of epsinR.** (A) The ENTH domain of epsinR was modeled on epsin1 ENTH with Ins(1,4,5)P_3 bound. The major difference in surface electrostatic potential (red − 10 kT e^{-1}; blue + 10 kT e^{-1}) is in the PtdInsP binding pocket where several positively charged residues are missing in epsinR ENTH domain. (B) Ribbon diagram of the modeled epsinR ENTH domain showing the residues in the binding pocket and the succession of hydrophobic residues on the outer surface of helix zero (α0), just like in epsin1 ENTH domain. (C) Sequence homologies between epsin1 homologues and epsinR homologues. Both *Drosophila* (D) *and C. elegans* (Ce) have one homologue of each, but humans have three homologues of epsin1 (1–3), and yeast (Sc, *Saccharomyces cerevisiae*) also has multiple homologues. Hydrophobic residues on the outer surface of helix zero are marked in yellow. Conserved (blue) and nonconserved (green) Ptd(4,5)P_2 binding residues and other major differences (orange) are marked. Some of the key residues in the human epsinR sequence referred to in the paper are numbered. (D) Domain structure of epsin1 compared with epsinR. Clathrin binding motifs (pink), DPW/DLF adaptor binding motifs (purple) and regions of alternative splicing (dotted lines) are shown. The splice site at amino acid 460 is an insert of the residues QPLQNVSTVLQKPNPLYN. Below are shown all the constructs, mutations, truncations and peptides used in the paper. The ubiquitin-interacting motifs (UIMs) and the Eps15 binding motifs (NPFs) found in epsin1 are not present in epsinR. We also know that there are additional clathrin binding motifs in epsinR (at least one in the N2 domain) that we have not mapped.

domains centrally located. However, they are distinct in that the AP180 NH_2-terminal homology (ANTH) domain binds lipids on its surface, coordinating the headgroup via a KxxKxH motif, whereas the epsin-NH_2-terminal homology (ENTH) domain binds lipids in a pocket using residues spread over the first three helices. On binding, the first helix of the epsin ENTH domain folds around the lipid headgroup and exposes a hydrophobic surface on the outer surface of this new helix. We have proposed that on binding to membranes, this new helix buries itself between the lipid headgroups, pushing the lipids apart and thus promoting membrane curvature in the clathrin-coated bud (Ford et al., 2002). As this will need to be a coordinated event, the COOH terminus of epsin1 binds to both clathrin and the AP2 complex, thus inducing the curvature in newly forming coated pits.

Epsins were originally identified as Eps15-interacting proteins, but the family is now better described as being ENTH domain (phosphatidylinositol phosphate [PtdInsP] binding) proteins with clathrin/adaptor binding sequences. Epsins 1 and 2 are brain-enriched (Rosenthal et al., 1999), whereas epsin3 is expressed in wounded epithelia (Spradling et al., 2001). Previously, we identified another epsin in the database (Ford et al., 2002) that is more distantly related to epsins 1–3 in that it does not have any Eps15-binding motifs (NPFs), and so is not a classical "**eps**in," and thus, we named it epsinR (for epsin-related protein). The ENTH domain of epsinR is

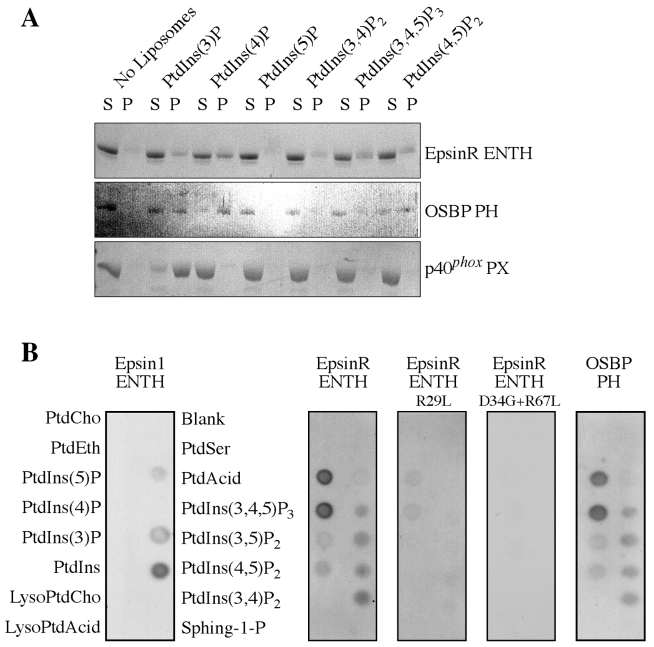

Figure 2. **EpsinR binds to PtdIns(4)P and PtdIns(5)P in vitro.** (A) Coomassie-stained gel of liposome-binding assays with epsinR ENTH domain, the PtdIns(4)P targeted GFP tagged PH domain of OSBP, and the PtdIns(3)P targeted PX domain of $p40^{phox}$. Pellet (P) and supernatant (S). (B) PtdInsP strips confirm the lipid specificity of epsin1 ENTH domain for PtdIns(4,5)P_2 and show that epsinR prefers PtdInsPs with a lower charge density. The R29L mutation weakens the interaction, and with the double mutation D34G + R67L, we lose the 4P and 5P interactions. OSBP also shows a very similar specificity to epsinR ENTH domain. The labeling on the first strip applies to the other panels.

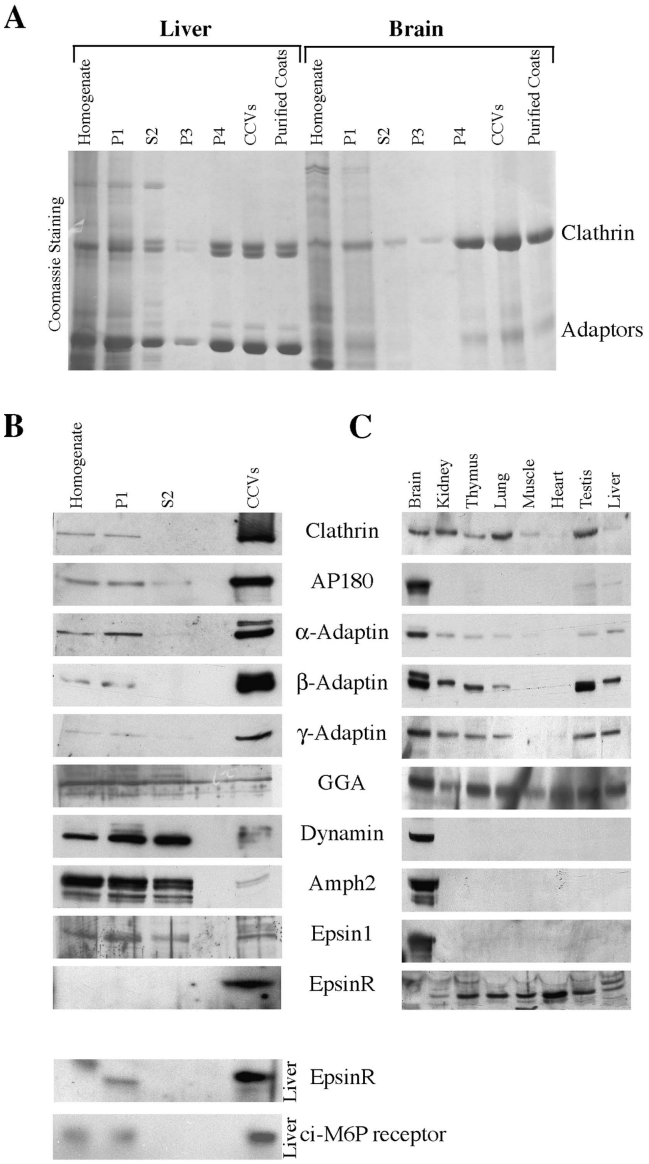

Figure 3. **EpsinR, a ubiquitous protein enriched in CCVs.** (A) Purification of rat liver and brain CCVs. A stronger adaptor band is seen by Coomassie staining in the liver CCV preparation, although by electron microscopy, the liver CCVs are not as pure as the brain CCVs (not depicted). (B) Brain fractions were blotted for various CCV-enriched and -nonenriched proteins, and these were compared with epsin1 and epsinR in the same preparation. Blots for epsinR and the cation-independent M6P receptor are also shown for liver fractions. (C) Brain-specific distribution of epsin1 compared with the ubiquitous distribution of epsinR. Equal concentrations of rat tissue were loaded.

conserved, although the lipid specificity is predicted to be different (Ford et al., 2002). The clathrin/adaptor binding domain is also conserved, although the motifs present are distinctly different (Fig. 1 D). EpsinR does not have the ubiquitin interacting motifs present in epsins 1–3 that bind to ubiquitin and are essential for the monoubiquitination of the protein (Oldham et al., 2002; Polo et al., 2002). EpsinR homologues are also present in other species, including *Drosophila* and *C. elegans* (Fig. 1 C), and have no NPF motifs, but have multiple DxF motifs in place of the multiple DPWs in the epsin1 homologues. In this paper, we investigate the function of mammalian epsinR in vesicle budding events.

Results

EpsinR ENTH domain binds PtdIns(4)P

The ENTH domain of epsinR is predicted to have essentially the same structure as epsin1 (48% sequence identity in this domain), but some of the key PtdIns(4,5)P_2 binding residues (R7, R8, and K11) are not conserved. R8 from epsin1 is replaced by D12, and N30 is replaced by D34 (Fig. 1). We predicted that epsinR should bind a monophosphate PtdInsP rather than PtdIns(4,5)P_2 (Ford et al., 2002). The lower overall positive charge density in the binding pocket can readily be seen from a model based on epsin1 ENTH domain (Fig. 1 A).

Epsin1 ENTH domain binds to PtdIns(4,5)P_2 in liposome-binding assays and by isothermal titration calorimetry (ITC) with a low micromolar affinity (Itoh et al., 2001; Ford et al., 2002). In liposome-binding experiments and in overlay assays, epsinR showed a very weak preference for liposomes containing PtdIns(4)P while also binding to PtdIns(5)P (Fig. 2). This was similar to the specificity of the PH domain of oxysterol binding protein (OSBP; Levine and Munro, 2002) that is targeted in vivo to Golgi membranes (also for epsinR; see Fig. 6 and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200208023/DC1). Mutants of epsinR showed no binding in the overlay assay when

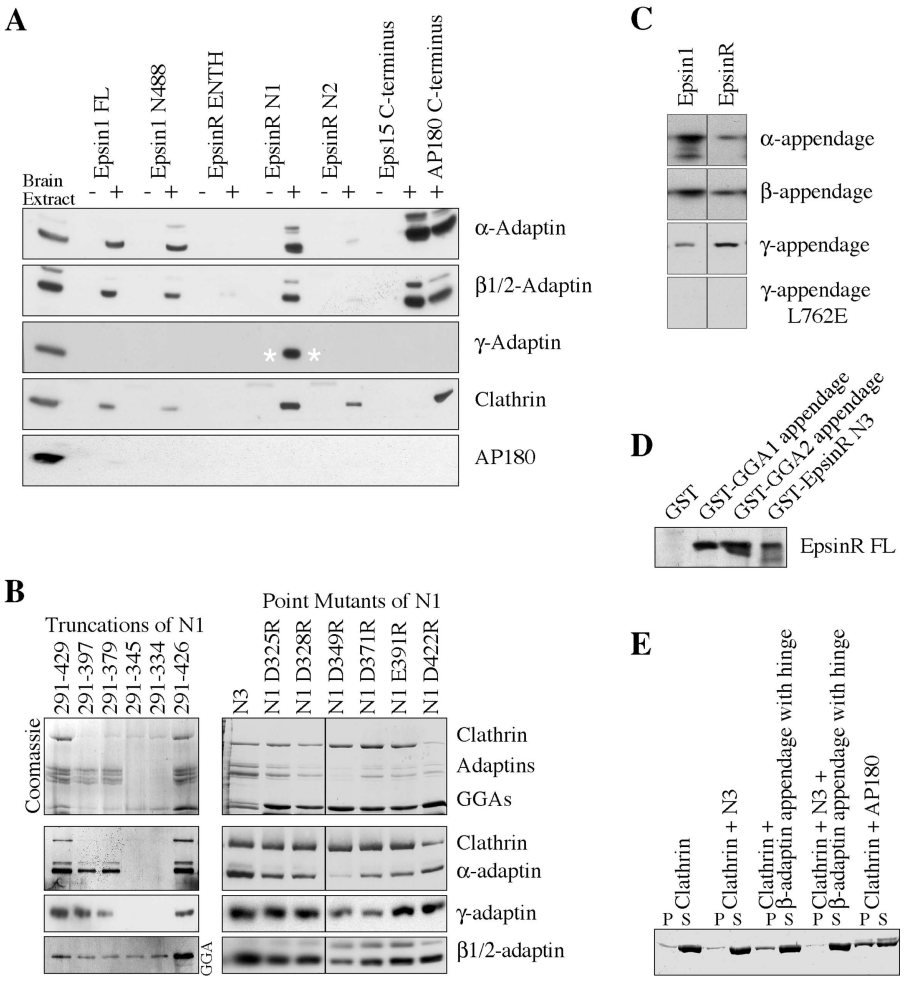

Figure 4. **Clathrin and AP1 adaptors bind to epsinR.** (A) Epsin1 and epsinR constructs were tested for binding to adaptors, clathrin, and AP180 in rat brain extract. Epsin1, Eps15 and AP180 all bind to α- and β-adaptins but only epsinR binds to γ-adaptin (asterisk). All constructs were GST-tagged, and +/− indicates the presence/absence of brain extract. (B) Truncations of N1 narrowed the major clathrin/adaptor-binding domain to 291–426 (N3), and further truncations abolish clathrin and then adaptor binding (Coomassie-stained gel). GGAs also bind to the N3 construct, and the first deletion affects the interaction (blot). Point mutagenesis shows that D422R primarily affects clathrin binding over adaptor binding while AP2 adaptors are affected by the D349 mutation. AP1 adaptors are affected by D349 and D371 mutations. GGA binding does not follow the same pattern as clathrin or the multimeric adaptors. (C) Full-length Myc-epsin1 and Myc-epsinR expressed in COS cells bind to the GST-appendage domains of α-, β-, and γ-adaptin, but not the L762E γ-adaptin appendage. (D) GST constructs of GGA1 and GGA2 appendage domains and the N3 construct of epsinR bind to full-length epsinR in brain extract. (E) Clathrin assembly is not promoted by the N3 domain of epsinR at neutral pH. Controls with β-adaptin appendage + hinge domain and full-length AP180 both show different extents of assembly. Clathrin in pellet (P) and supernatant (S) fractions after spinning are shown.

tested using equivalent protein concentrations and incubation times (Fig. 2). In vivo, PtdIns(4)P is thought to be more TGN-enriched, and PtdIns 4-kinase activity is found on the Golgi (Godi et al., 1999), whereas PtdIns(5)P has not been localized in cells. The weak binding to lipids in vitro may well mean that multimerization and/or the presence of other proteins may play a role in membrane recruitment (see Discussion). We have limited evidence for self-association of epsinR from pulldown experiments (see Fig. 4 D and unpublished data), and it is therefore possible that endogenous epsinR may have a higher avidity for membranes than observed for the monomeric ENTH domain.

EpsinR enrichment in CCVs

EpsinR has a ubiquitous tissue distribution both on Northern blots and by Western blots (Fig. 3 C; refer to Materials and methods for antibody used). Epsin1 is brain-enriched and colocalizes with plasma membrane clathrin and AP2 adaptors in cells, but is not enriched in CCVs (Chen et al., 1998; Ford et al., 2002). In contrast, epsinR showed a good enrichment in both brain and liver CCVs (Fig. 3, A and B). There are numerous possible explanations for different enrichments of epsins in brain CCVs, one being the activity of the major brain lipid phosphatase, synaptojanin (McPherson et al., 1996). Synaptojanin activity in CCVs would result in the loss of the 5′ phosphate from PtdIns(4,5)P_2, which would only lead to the release of epsin1 and the AP2 complex, while not having as profound an effect on epsinR and AP1. We also found endogenous and overexpressed epsinR enrichment in Golgi fractions using the Balch purification procedure (Balch et al., 1984) and a de-enrichment of the lipid-binding mutant epsinR-D34G (Fig. S1). On the basis of these combined results, we can conclude that epsinR is a widely distributed protein enriched in both CCVs and Golgi fractions.

EpsinR binds clathrin, APs, and GGAs

EpsinR contains a clathrin/adaptor-binding domain. Constructs encompassing this domain bound to clathrin and to AP1 and AP2 complexes (Fig. 4 A; blots for α-, β-, and γ-adaptins), but not to AP3 complexes (δ-adaptin blot, not depicted). The AP1 specificity of the epsinR N1 construct was notable in contrast to the other control proteins. The major clathrin/adaptin binding was further localized, with truncations and point mutagenesis (Fig. 4 B; see Fig. 1 D for constructs used), to the N3 construct of epsinR, with some further clathrin binding found in the N2 construct (Fig. 4 A and unpublished data). The N3 construct contains three DLF motifs that are potentially α- and β-appendage binding sequences (Owen et al., 1999, 2000; Brett et al., 2002). Point

A

proteins	K_D (μM) Site1	Site2
■ **EpsinR N3** + γ-adaptin appendage	0.7	45
● **EpsinR N3 D342R** + γ-adaptin appendage	0.9	160
▲ **EpsinR N3 D349R** + γ-adaptin appendage	3.2	54
EpsinR N3 D371R + γ-adaptin appendage	4.9	68
EpsinR N3 + α-adaptin appendage	58	
EpsinR N3 D342R + α-adaptin appendage	54	
β-adaptin appendage + epsinR-N3	0.8	22
GGA1 appendage + epsinR-N3	95	

B

peptides+ appendages:	sequence	K_D (μM) γ	β	α
EpsinR-P1	SGDLVDLFDGTS	none	none	>500
❑ EpsinR-P2	TGGSADLFGGFA	230	>500	>500
○ EpsinR-P3	SADLFGGFADFG	110	79	74
△ EpsinR-P□	FGGFADFGSAAA	>220	88	>500
◇ EpsinR-P□	TSGNGDFGDWSA	48	310	>500
◆ γ-s□ne□gin-P1	PEEDDFQDFQDA	13	>500	>500

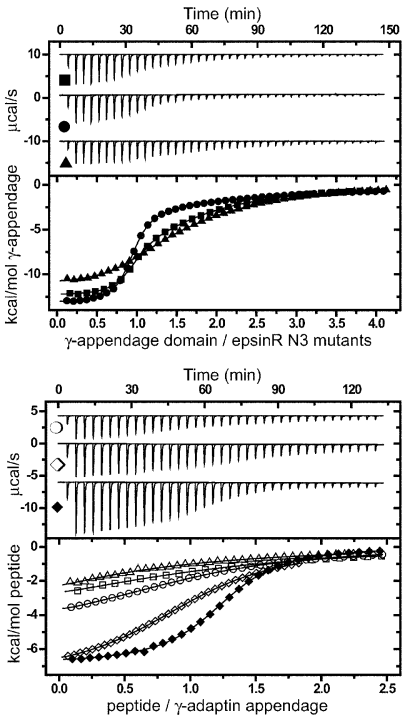

Figure 5. **Two γ-adaptin appendage domains can bind simultaneously with high affinity to epsinR.** (A) Affinity measurement by calorimetric titrations for α-, β-, and γ-appendage domains and GGA appendage domain with epsinR N3 constructs. Where the stoichiometry of the interaction was 2:1, the data showed a robust fit to a two-site model, and the K_Ds for both sites are shown. The bold lettering indicates the protein in the syringe. (B) Peptides from epsinR and γ-synergin binding to the γ-adaptin appendage domain. Profiles of typical calorimetric titrations and the integrated normalized data are shown on the right. A comprehensive table of calorimetric data is available in Table SI.

mutagenesis of these motifs had no major effect on adaptor interactions, but the D349R mutation did affect AP2 binding, and the shortening of the domain to G345 also affected AP binding (Fig. 4 B). There are at least two possible clathrin interaction motifs in the N3 construct. Mutagenesis of the first motif (D325R and D328R in *D*LV*D*LF) had a weak effect on clathrin binding, but mutagenesis of the second (D422R in *D*LFDLM) had a major effect on clathrin binding (Fig. 4 B). Truncations also pointed to the same motif as being important for clathrin binding.

Given the binding of epsinR to adaptor complexes in brain and COS cell extracts, we next tested which appendage domains were responsible for the interactions (Fig. 4 C). In this experiment, Myc-tagged epsins were overexpressed in COS cells and GST-appendage domains were used to fish the proteins out. All appendage domains tested bound to both epsin1 and epsinR; however, γ-appendage has a distinct preference for epsinR. The L762E mutant of the γ-appendage (Kent et al., 2002) abolished the interaction (Fig. 4 C). This preference for the γ-appendage was confirmed by ITC where the affinity for the α-appendage was much weaker than for the β- and γ-appendages (Fig. 5). For both the β- and γ-appendages, the binding stoichiometry was 2:1, and we were thus able to fit a curve describing two binding sites to the data. These were very robust fits, giving a site with a submicromolar affinity and a further lower affinity site in each case. These experiments also show that multiple appendage domains can interact at the same time. GGAs, which have a γ-adaptin appendage homology domain would also be predicted to bind epsinR. GST-GGA appendage domains interacted with epsinR (Fig. 4 D), and the reverse experiment also showed that GST-epsinR N1 construct bound to GGAs 1 and 2 (Fig. 4 B and unpublished data). However, despite the very high conservation of critical residues between the predicted binding sites in GGAs and γ-adaptin appendages, deletion constructs point to clear differences in the binding specificities (Fig. 4 B). The affinity of GGA1 for the epsinR N3 construct was also much lower than the affinity of the γ-appendage (Fig. 5). Of all the appendage domains tested the much higher affinities of γ- and β-appendages for epsinR imply that the AP1 complex is the major interacting adaptor.

Identification of the γ-appendage binding site

EpsinR shows a clear preference over epsin 1 for AP1 adaptors in brain cytosol (Fig. 4 A), and thus, there must be some additional recognition sequences for γ-adaptins (an AP1-specific subunit) in the epsinR protein. An unresolved issue in the recent crystallization studies on γ-adaptin appendage domain (Kent et al., 2002; Nogi et al., 2002) is the identity of this sequence. From comparisons of the appendage domain structures, the γ-appendage is missing the platform domains to which ligands bind in both α- and β-appendages. Thus, the binding motif for the γ-appendage is not likely to follow the DxF pattern (Owen et al., 1999, 2000).

Based on the truncation of N1, we saw a slight reduction in AP1 binding (as assessed by blotting for γ-adaptin) with a stop mutation after N379 and a major reduction in binding with a stop mutation after G345. We noted the presence of two DFG sequences sequentially deleted by these truncations. Point mutants of the sequences D349R and D371R both reduced AP1 binding. The presence of two binding sites is consistent with the affinity measurements for the N3 construct with the γ-appendage domain (Fig. 5). Mutagenesis of both D349R and D371R in N3 both greatly weakened the high affinity interaction, implying that this may be due to secondary folding. However, secondary structure analysis of the N3 construct by CD spectroscopy showed that it is largely unstructured, though there is a mild change in the presence of γ-appendage (Fig. S2).

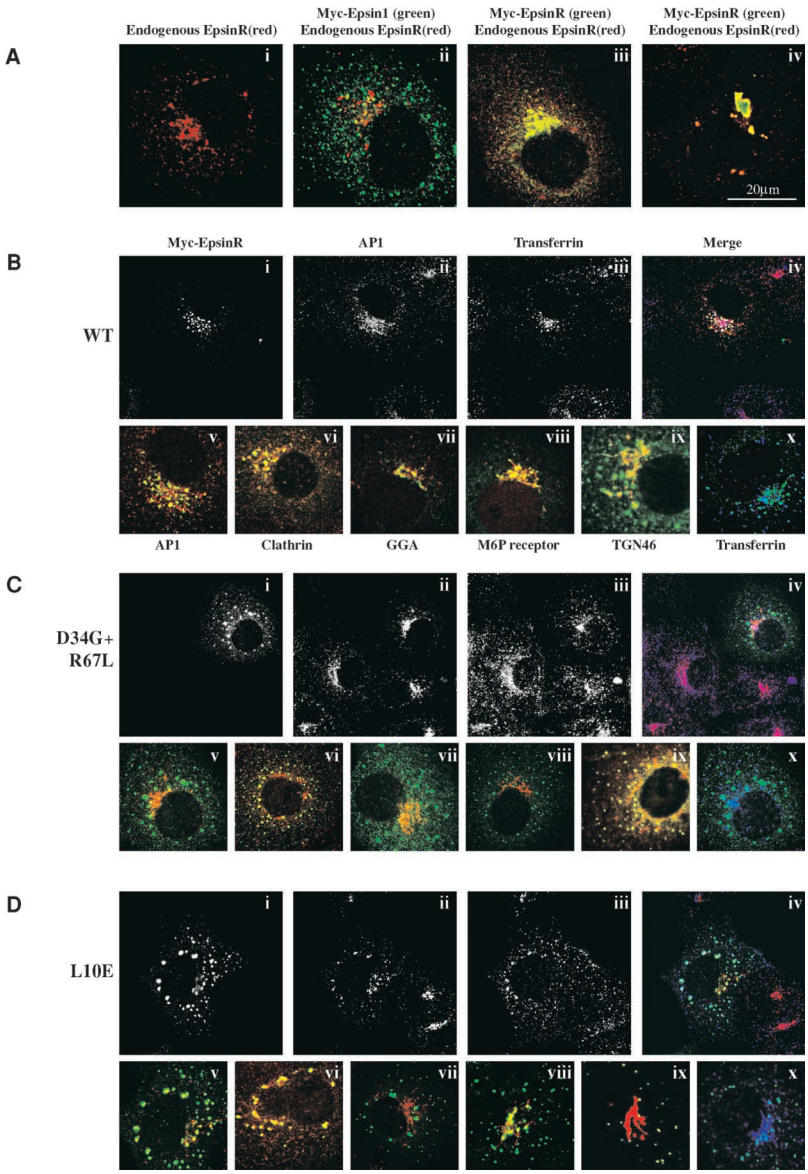

Figure 6. **Subcellular localization of epsinR.** As a convention throughout the figure, myc-epsinR and mutants are labeled green and the endogenous proteins are labeled red. (A) Endogenous epsinR shows a perinuclear enrichment (Ai) with a very different distribution to Myc-epsin1 (green in Aii), but it colocalizes with overexpressed Myc-epsinR (green in Aiii). Colocalization is orange/yellow. We observed that the epsinR perinuclear compartment was frequently enlarged, and in extreme examples much of the overexpressed epsinR was accumulated there (Aiv). Endogenous epsinR is also accumulated in this compartment (Aiv). (B) Colocalization of Myc-epsinR with endogenous AP1 (ii and v), transferrin (iii and x), clathrin (vi), GGA (vii), cation-independent mannose-6-phosphate receptors (M6P, viii), and TGN46 (ix). Panels v–x are closer views of the perinuclear regions of cells stained for overexpressed epsinR and the indicated marker. There is much orange/yellow color in the perinuclear region of the cell implying a great deal of colocalization, but precise colocalization is hard to define in this region because of the accumulation of so many compartments. Transferrin (panel x) is labeled blue and colocalization with AP1 is cyan. (C and D) Colocalization of various markers with the D34G + R67L and the L10E mutants of epsinR, respectively. Labeling is the same as in B. Links to original images and extra data can be found at http://www.jcb.org/cgi/content/full/jcb.200208023/DC1.

To investigate AP interactions further, we made peptides for direct interaction studies (see Fig. 1 D for peptide locations). The P1 peptide contains the first DLF motif. It did not bind to the α-, β-, or γ-appendages (Fig. 5), and mutagenesis of D325 and D328 had no effect on AP1 binding (Fig. 4 B). Peptides P2, P3, and P4 are overlapping; P2 and P3 contain the second DLF motif and P3 and P4 contain the first DFG sequence. All three peptides bound weakly to the γ-appendage, thus excluding the possibility that the DFG alone is responsible for the interaction. P3 and P4 both interacted with the β-appendage but only P3 with the α-appendage domain. The P5 peptide containing the second DFG sequence bound γ-appendage with the highest affinity (48 μM) although still much weaker than the full N3 construct. P5 also showed a weak interaction with the β- but not the α-appendage domain. Looking at the two peptides that bound with highest affinity to the γ-appendage domain (P3 and P5), we concluded that Fxx[F/W] was a potential binding sequence. Using the P3 and P5 peptide consensus, we searched for homologous sequences in the protein database and found γ-synergin. Next, we made a peptide of one of the homologous sequences from γ-synergin, and found that it bound with an affinity of 13 μM. This was the highest affinity for any of our peptides. We now recompared the similarity between our two highest affinity peptides, γ-synergin-P1 and epsinR-P5, and came up with the consensus DFxD[F/W]. The highest scoring protein in the database is now γ-synergin, with three copies of this motif. γ-synergin has already been characterized as a γ-adaptin binding partner (Page et al., 1999) and thus, with this work, we identify a binding site within the protein for the γ-appendage. Rabaptin5 (Nogi et al., 2002) and Eps15 (Kent et al., 2002) are also proposed to interact directly with the γ-appendage domain. Both these proteins contain homologous sequences to the above consensus. Looking through the protein database for other potential γ-adaptin appendage binding partners, we get a high score for an uncharacterized EF-hand protein (NP_060127) with three copies of this motif. This protein also contains potential clathrin-binding motifs and multiple DDF motifs, and thus, this is a future candidate to join the ranks as a γ-appendage interacting protein.

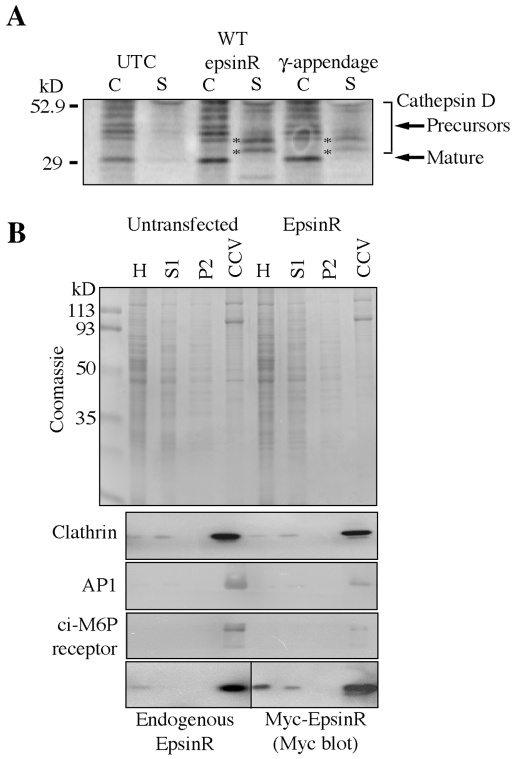

Figure 7. **Disruption of CCV trafficking from the TGN.** (A) Overexpression of Myc-epsinR caused mistargeting of pro-cathepsin D. COS cells, either untransfected (UTC) or transfected with WT Myc-epsinR or Myc γ-appendage domain were pulse-chased with ^{35}S, and both-cell associated (C) and -secreted (S) cathepsin D were immunoprecipitated and gels were phosphorimaged. The secreted forms are indicated by the asterisks. (B) Reduced incorporation of the cation-independent M6P receptor into purified CCVs in epsinR transfected COS cells.

Clathrin assembly

EpsinR is enriched in CCVs (Fig. 3 B). Clathrin binding to the truncations and mutants of N3 imply that there may be multiple clathrin-interacting motifs, including the one centered on D422. If this were the case, then it might be expected that epsinR will promote clathrin cage assembly by linking clathrin triskelia together. We and others have previously shown that epsin1 binding to membranes can promote clathrin recruitment and polymerization, and that this is due to the presence of multiple clathrin-binding motifs (Ford et al., 2002; Kalthoff et al., 2002a; and unpublished data). By analogy, epsinR might be expected to assemble clathrin. We tested this in comparison to AP180, which strongly exhibits this activity at neutral pH and with the β-appendage + hinge domain, which exerts this effect more strongly at a lower pH (Owen et al., 2000; Ford et al., 2001). Under these conditions, the N3 domain does not promote clathrin cage assembly, and even antagonizes the limited assembly by β-appendage + hinge domain at neutral pH. This does not exclude an assembly activity of epsinR, as the N3 construct does not include all the clathrin-binding activity (Fig. 4 A), but the N1 construct gave similar results. We conclude that clathrin assembly is not a primary function of this domain.

Subcellular localization of EpsinR

Epsin1 is brain-enriched, whereas epsinR is ubiquitous in its tissue distribution (Fig. 3), and homologues are found in both *C. elegans* and *Drosophila*. Thus, we used epsinR antibodies to detect endogenous protein in COS cells. It showed a perinuclear concentration with additional puncta distributed throughout the cytoplasm (Fig. 6 Ai). These puncta were completely distinct from those observed with overexpressed epsin1 (Fig. 6 Aii). Overexpressed Myc-epsinR showed the same expression pattern, although with a greater perinuclear concentration (Fig. 6 Aiii). Overexpressed myc-epsinR also overlapped with staining for other endogenous proteins (Fig. 6 B); AP1, perinuclear clathrin, GGA, M6P receptors and TGN46 (but not the medial Golgi marker GM130 and the lysosomal marker Lamp1; see http://www.jcb.org/cgi/content/full/jcb.200208023/DC1). Strong overexpression of WT epsinR led to enlarged perinuclear compartments (Fig. 6 Aiv). We have previously shown that overexpression of epsin1 leads to an inhibition of AP2/clathrin-coated vesicle formation from the plasma membrane (see Fig. 3 d in Ford et al., 2002), likewise, these enlarged epsinR positive compartments may be due to a budding deficiency (Fig. 7 B). This phenotype became stronger over time, thus, in these studies, we have been careful not to prolong the overexpression.

The D34G + R67L mutation in the ENTH domain of epsinR greatly weakened its binding to lipids (Fig. 2). These same mutations in full-length epsinR resulted in the redistribution away from the perinuclear region into irregularly sized puncta (Fig. 6 C). Thus, the ENTH domain of epsinR is important for localization. These puncta were not aggregates of epsinR, as they costained for the membrane protein TGN46 and perinuclear clathrin also redistributed to these spots. The amount of clathrin and TGN46 in the large puncta was directly proportional to the amount of epsinR, judging from the equivalent intensities of the stainings. We did not detect any direct interaction of epsinR and the cytoplasmic tail of TGN46 in vitro (unpublished data). AP1, GGAs, early endosome antigen 1 (EEA1), transferrin, and M6P receptors did not follow the mutant and were not redistributed (links to original images and extra data can be found at http://www.jcb.org/cgi/content/full/jcb.200208023/DC1). Fractionation studies showed that the D34G mutation resulted in the loss of Golgi epsinR enrichment (Fig. S1).

Mutation of the L6 residue on helix zero of epsin1 has a profound effect on lipid curvature (Ford et al., 2002). The equivalent residue on epsinR is L10, and this was mutated to a hydrophilic residue (L10E) and the protein was overexpressed (Fig. 6 D). The TGN enrichment was severely reduced. Thus, like epsin1, the hydrophobicity of helix zero is important for the protein's normal distribution. The phenotype was distinguished by large puncta around the nucleus and in the cytoplasm, which cannot clearly be classified as a known compartment. AP1 was found in these epsinR puncta as a very diffuse stain, but we did not detect GGAs. We also did not find EEA1, Lamp1, GM130, or M6P receptors in the puncta (links to original images and extra data can be found at http://www.jcb.org/cgi/content/full/jcb.200208023/DC1). These puncta colocalized with clathrin and a minor component of TGN46, but most of the TGN46 was still in its perinuclear compartment. Further-

more, the intensities of epsinR and clathrin/TGN46 puncta were equivalent, and there was a disruption of the perinuclear clathrin organization (Fig. 6 D). Given the partial redistribution of TGN46 with WT epsinR, D34G + R67L, and the L10E mutants, we speculate that trafficking of this transmembrane protein is affected by epsinR. Further epsinR mutants are found at http://www.jcb.org/cgi/content/full/jcb.200208023/DC1.

EpsinR is involved in vesicle budding between the TGN and endosomes

Given the enrichment of endogenous epsinR in Golgi fractions and the distribution by immunofluorescence, we would predict that at least one trafficking step in which epsinR is involved is AP1/clathrin-mediated budding from the TGN. Numerous proteins are incorporated into AP1/clathrin-coated vesicles from the TGN, including the cation-independent M6P receptor and the lysosomal hydrolase cathepsin D. In mice deficient in the μ1A subunit of AP1, the trafficking of both the cation-independent M6P receptor and cathepsin D is perturbed (Meyer et al., 2000). As epsinR has a high affinity for AP1, we tested whether overexpression of epsinR could perturb cathepsin D trafficking. Cathepsin D is synthesized as an inactive 53-kD glycosylated protein (pro-cathepsin) in the Golgi and trafficked to lysosomes, via late endosomes (direct pathway) or via the cell surface (indirect pathway/secretion and reuptake). Pro-cathepsin D is de-glycosylated and further cleaved in lysosomes to generate the active 30-kD mature form. In mice deficient in the μ1A subunit of AP1, there is an increased secretion of glycosylated cathepsin D into the medium due to a disruption of its trafficking to the lysosome. We found that overexpression of epsinR caused a >10-fold increase ($n = 4$) in the quantity of cathepsin D precursors secreted into the medium versus untransfected control cells (Fig. 7 A). Overexpression of the γ-appendage domain also perturbed normal trafficking, though not to the same extent (Fig. 7 A), whereas epsin1 and the ENTH domain of epsinR had no effect (unpublished data). Thus, WT epsinR overexpression disrupts this AP1-dependent process in line with our observations of the enlarged perinuclear compartments with WT protein overexpression. The cation-independent M6P receptor also traffics from the TGN via CCVs (Meresse and Hoflack, 1993; Meyer et al., 2000), and is enriched in purified liver CCV fractions (Fig. 3 B). We were not able to detect a bulk change in the distribution by immunofluorescence, but there was a reduction in receptor incorporation in purified CCVs from COS cells transfected with epsinR (Fig. 7 B). This reinforces the argument that epsinR functions in CCV budding from the TGN.

Discussion

Previously, we have proposed that epsin1 has a fundamental role in generating the curvature of the clathrin-coated pit (Ford et al., 2002). Epsin1 binds to clathrin and AP2 adaptors and to plasma membrane PtdIns(4,5)P_2. By sequence homology, epsin2 and 3 are also likely to act in clathrin-mediated endocytosis from the plasma membrane. In this paper, we show that epsinR is involved in clathrin-mediated budding from internal compartments. Consistent with this function, it binds to clathrin and AP1 adaptors and to PtdIns(4)P, and its distribution in cells did not overlap with that of epsin1. In fact, most of the clathrin puncta in transfected COS cells are accounted for by a combination of epsin1 and epsinR puncta (see http://www.jcb.org/cgi/content/full/jcb.200208023/DC1).

The clathrin/adaptor-binding domain of epsinR contains multiple clathrin-binding sites and multiple DxF motifs that are predicted to bind to adaptor appendage domains. It is now generally assumed that these regions in the various endocytic proteins are unfolded and the motifs act like hooks on a fishing line to maximize the potential for ligand binding (Kalthoff et al., 2002a). The simultaneous binding of multiple β- and/or γ-appendages to N3 supports this fishing line model. By CD spectroscopy, the N3 construct is largely unstructured and we did not detect any major change on binding of γ-appendage (Fig. S2). However, our results imply that limited folding gives rise to the high affinity interactions. First, separate point mutants of the two γ-appendage binding sites in N3 (D349R and D371R) both result in a reduction of the high affinity interaction. Second, the peptide binding data do not reproduce the high affinities achieved with the N3 construct.

In this paper, we have identified for the first time a γ-appendage binding motif, [D/E]FxD[F/W], present in epsinR, γ-synergin, and an uncharacterized EF-hand protein, NP_060127, that will be the subject of future investigations. This motif should bind to a basic patch on the surface of the γ-appendage (Kent et al., 2002; Nogi et al., 2002), and thus, the introduction of a negative charge into this patch (L762E) disrupted the interaction (Fig. 4 C). The *Drosophila* epsinR homologue has two copies of EFxDF, whereas the *Drosophila* epsin1 homologue has none. The *C. elegans* epsinR homologue conserves the GFxDF as present in epsinR-P3.

To enable the study of epsinR function, we modeled its ENTH domain on the structure of epsin1 ENTH bound to Ins(1,4,5)P_3. Mutations reduced the binding to lipids and to purified Golgi membranes (Fig. 2 B and Fig. S1), and showed the ENTH domain primarily determines the location of the protein in cells, just as for epsin1 (Fig. 6). We found that the lipid binding of epsinR was not strong enough to enable us to do more biochemistry on the protein to assay for clathrin recruitment and liposome tubulation. However, this weaker affinity points to the importance of other factors in the localization of the protein, perhaps multimerization and/or the presence of other proteins. Arf could well play a role in recruitment, as it does in the localization of the AP1 complex, OSPB and PtdIns 4-kinase to the Golgi (Godi et al., 1999; Levine and Munro, 2002). Consistent with a role for oligomerization, we noted that the N3 construct of epsinR binds to full-length epsinR (Fig. 4 D). This is not due to N3 self-association (Fig. S3; ultracentrifugation data).

Another key feature of the epsin1 ENTH domain is the amphipathic nature of helix zero with an exposed ridge of hydrophobic residues. This is preserved in epsinR and is likely extended at the NH_2 terminus for another turn of the helix (Fig. 1 B and yellow shading in Fig. 1 C). We would propose that epsinR, like epsin1, will fold this helix on binding to PtdInsP and insert the amphipathic helix between the lipid head groups, aiding membrane curvature during clathrin cage formation.

In this paper, we also propose that epsinR is functionally equivalent to epsin1, but in CCV budding from the TGN/

endosomes rather than from the plasma membrane. We observe a trafficking defect in cathepsin D like that found in the μ1A-deficient cell line (Meyer et al., 2000). A budding defect from the TGN is supported by the observation of enlarged compartments on overexpression of epsinR (and especially with the lipid-binding mutants, for example L10E) and the disruption of M6P receptor incorporation into CCVs on overexpression of epsinR. Our data strongly suggest that these budding defects involve clathrin/AP1 trafficking pathways.

Having submitted this paper, other manuscripts have been published or are in press where epsinR has been found using a proteomics approach on purified CCVs (Wasiak et al., 2002; Hirst et al., 2003) and by homology searches for ENTH domain proteins (Kalthoff et al., 2002b). The authors give various names to the same protein "enthoprotin," epsinR, and "clint," respectively, and find a similar localization in cells and clathrin/adaptor interactions. Also, a yeast epsin–related protein has been shown to be required for Golgi–endosome traffic (Duncan et al., 2003).

Materials and methods

Protein expression and purification

The cDNA for human epsinR (KIAA0171) was a gift from the Kazusa DNA Research Institute, Chiba, Japan. Human epsinR ENTH (residues 1–169), N1, N2, and N3 domains and their mutants, as well as full-length epsin1, epsin1 N488, AP180 COOH terminus (residues 530–915), Eps15 COOH terminus (residues 529–898), GGA1 appendage (residues 468–639), GGA2 appendage (residues 454–613), and the α-adaptin appendage domain (residues 701–938) were expressed as NH_2-terminal GST fusion proteins (in pGex4T2) in BL21 cells after IPTG induction overnight at 22°C. All GST fusion proteins were purified from bacterial extracts by incubation with glutathione-Sepharose beads, followed by extensive washing with 20 mM Hepes, pH 7.4, 150 mM NaCl, and 1 mM DTT. The proteins were cleaved by incubation for 2 h with thrombin, and further purified by passage over a Q-Sepharose column. For ITC experiments, the protein was additionally passed down a Superdex™ 75 gel filtration column and dialyzed into 100 mM Hepes, pH 7.4, 50 mM NaCl, and 2 mM DTT. Myc-tagged proteins (in pCMV-myc) were used for COS-7 expression. Baculovirus expression and purification of full-length AP180 was described previously (Ford et al., 2001). The appendage domains of α-, β-, and γ-adaptins (h-β2 appendage, residues 700–937; h-β2 appendage + hinge, residues 616–937; h-γ appendage, residues 704–822) and the GFP-oxysterol binding protein (OSBP, a gift of S. Munro, MRC-LMB, Cambridge, UK) were expressed in BL21 cells as NH_2-terminal 6xHis fusion proteins (in pET or pTrcHis, respectively) and purified by passage over nickel-NTA and then Q-Sepharose. Human $p40^{phox}$ PX domain protein was a gift of Olga Perisic, MRC-LMB. Purification of CCVs and clathrin from rat brain and liver is described on http://www.jcb.org/cgi/content/full/jcb.200208023/DC1. This preparation does not exclude the presence of trace amounts of AP2 and AP180. COS cell CCVs were purified from cracked cells passed through a 23-gauge needle and then processed as described previously (Gaffet et al., 1997).

Transfections and antibodies

COS-7 cells were transfected using GeneJuice™ (Novagen) according to the manufacturer's protocol. 28 h after transfection, cells were transferred to serum-free medium and incubated with biotinylated transferrin for 15 min before fixation with 4% PFA. EpsinR-transfected cells were detected using either a polyclonal anti-Myc antibody (green in merged images; Cell Signaling), or an mAb (9E10; Sigma-Aldrich). AP1 distribution was followed using a γ-adaptin mAb (100–3, red in merged images), and biotinylated transferrin was detected with labeled streptavidin (blue in merged images). Other antibodies used were as follows: polyclonal Epsin1 DPW domain (Ra14), monoclonal clathrin (X22, a gift of B. Pearse, MRC-LMB), monoclonal EEA1 (Transduction Laboratories), polyclonal cation-independent M6P receptor (1001, a gift of P. Luzio, Cambridge Institute for Medical Research, Cambridge, UK), and polyclonal GGA1 and 2 antibodies (a gift of J. Hirst and M. Robinson, Cambridge Institute for Medical Research). Cells were mounted and imaged using a confocal system (Radiance; Bio-Rad Laboratories). The Ra43 antibody raised against an NH_2-terminal epsinR construct crossreacts with a band of 180 kD (likely AP180, which has a homologous NH_2-terminal domain). By immunofluorescence, it does not recognize overexpressed epsin1, its closest homologue (Fig. 6 A), and the distribution of the overexpressed epsinR is the same as the endogenous protein, even in cells where there is an enlarged perinuclear compartment. The antibody works better on purified fractions than in total homogenates. We also raised another antibody against epsinR N3 that showed the same staining pattern. Biosynthetic labeling of cathepsin D was as described previously (Davidson, 1995), except that ProMix at 5 μl/ml of medium (Amersham Biosciences) was used for labeling.

Phospholipid binding

Protein binding to phospholipids was investigated in a protein lipid overlay assay using PIP Strips™ (Echelon Biosciences Incorporated) according to the manufacturer's protocol. In brief, the membrane was blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20 [vol/vol] + 3% BSA [wt/vol]) for 1 h at RT. Protein was added at 0.5 μg/ml in TBST + BSA and incubated for 1 h at RT. The membrane was washed three times for 10 min in TBST + BSA. Proteins were detected by incubation with a 1/1,000 dilution of our polyclonal rabbit antibody Ra30 against the epsin1 ENTH domain, with a 1/1,000 dilution of our polyclonal rabbit antibody Ra43 for wild-type and mutant epsinR ENTH and with a 1/1,000 dilution of a monoclonal mouse anti–GFP antibody (CLONTECH Laboratories, Inc.) for detection of His-GFP-OSBP in TBST + BSA, followed by incubation with anti–rabbit or anti–mouse HRP conjugate (Bio-Rad Laboratories) in a 1/10,000 dilution and by ECL (Amersham Biosciences). Lipid specificity was tested with a liposome pelleting assay as described on http://www.jcb.org/cgi/content/full/jcb.200208023/DC1. Proteins were detected with Coomassie stain.

Isothermal titration calorimetry

Binding of epsinR constructs and peptides to α-, β-, and γ-adaptin appendage domains were investigated by ITC (Wiseman et al., 1989) using a VP-ITC (MicroCal, Inc.). All experiments were performed in 100 mM Hepes, pH 7.4, 50 mM NaCl, and 2 mM DTT at 10°C. The peptides or proteins were injected from a syringe in 40–50 steps up to a 3–4-fold molar excess. The cell contained 1.4 ml protein solution, and typically, the ligand was added in steps of 4–8 μl every 3.5 min. Concentrations were chosen so that α-, β-, and γ-adaptin appendage domains were at least fivefold higher than the estimated dissociation constant, if possible. The epsinR protein or peptide ligands were again at least 10-fold more concentrated than the ear domains. The heat of dilution of the ligand was subtracted from the data before fitting. Titration curves were fitted to the data using the ORIGIN program (MicroCal, Inc.) yielding the stoichiometry N, the binary equilibrium constant K_A (= K_D^{-1}) and the enthalpy of binding. The entropy of binding $\Delta S°$ was calculated from the relationship $\Delta G° = -RT \ln K_A$ and the Gibbs-Helmholtz equation.

Clathrin assembly and sedimentation assays

Purified clathrin was dialyzed into 0.1 M MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM DTT, and centrifuged at 100,000 *g* for 25 min to remove aggregates. Clathrin was incubated with proteins or an equivalent volume of buffer at 4°C for 10 min and spun at 100,000 *g* in a rotor (model TLA100; Beckman) for 25 min. Final protein concentrations were as follows: clathrin, 0.3 μM; epsinR N3, 50 μM; γ-appendage + hinge domain, 40 μM; AP180, 0.3 μM.

Online supplemental material

Fig. S1 shows the enrichment of epsinR in Golgi membrane fractions along with the cation-independent M6P receptor. It also shows that the epsinR lipid-binding mutant, D34G + R67L, no longer associates with Golgi membrane fractions. Fig. S2 shows that the N3 construct of epsinR is largely unstructured according to CD spectroscopy. Fig. S3 shows that epsinR is a monomer at all concentrations tested by analytical ultracentrifugation. Table SI supplies the detailed thermodynamic parameters measured by isothermal calorimetry for binding of the N3 construct of epsinR to various ligands. Original immunofluorescence images are presented as web pages that allow the reader to see individual channels and larger fields of cells. Additional antibody stains and mutants are also presented. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200208023/DC1.

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