Brief Report

Epsin 1 Undergoes Nucleocytosolic Shuttling and its Eps15 Interactor NH₂-terminal Homology (ENTH) Domain, Structurally Similar to Armadillo and HEAT Repeats, Interacts with the Transcription Factor Promyelocytic Leukemia Zn²⁺ Finger Protein (PLZF)

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Abstract. Epsin (Eps15 interactor) is a cytosolic protein involved in clathrin-mediated endocytosis via its direct interactions with clathrin, the clathrin adaptor A P-2, and Eps15. The NH₂-terminal portion of epsin contains a phylogenetically conserved module of unknown function, known as the ENTH domain (epsin NH₂-terminal homology domain). We have now solved the crystal structure of rat epsin 1 ENTH domain to 1.8 Å resolution. This domain is structurally similar to armadillo and Heat repeats of β-catenin and karyopherin-β, respectively. We have also identified and characterized the interaction of epsin 1, via the ENTH domain, with the transcription factor promyelocytic leukemia Zn²⁺ finger protein (PLZF). Leptomycin B, an antifungal antibiotic, which inhibits the Crm1-dependent nuclear export pathway, induces an accumulation of epsin 1 in the nucleus. These findings suggest that epsin 1 may function in a signaling pathway connecting the endocytic machinery to the regulation of nuclear function.

Key words: clathrin • Eps15 homology domain • catenin • karyopherin • endocytosis

Introduction

Epsin (Eps15 interactor)1 proteins were recently identified as interacting partners for the EH (Eps15 homology) domains of Eps15 (Chen et al., 1998; Rosenthal et al., 1999). Both epsin 1 and 2 interact with components of the clathrin coat, partially localize at clathrin-coated pits, and are

Joel Hyman and Hong Chen contributed equally to this work.

The coordinates and structure factors have been deposited with the Research Collaboratory for Structural Bioinformatics (RSCB), accession number 1edu, and will also be available at http://atb.csb.yale.edu

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1Abbreviations used in this paper: CNS, Crystallography & NMR System; EH, Eps15 homology; ENTH domain, epsin NH₂-terminal homology domain; epsin, Eps15 interactor; MAD, multiwavelength anomalous dispersion; NPF motifs, asparagine-proline-phenylalanine motifs; PLZF, promyelocytic leukemia Zn²⁺ finger protein; RMSD, root mean square difference; SeMet, selenomethionine.
Table I. Crystallographic Data Collection Statistics

|          | Unique refl. | Redundancy | Completeness | I/σ | Rmerge* |
|----------|--------------|------------|--------------|-----|--------|
| SeMet λ1 (1.06883 Å) | 24057 | 5.44 (2.03) | 93.6 (68.3) | 25.3 (2.8) | 5.6 (20.2) |
| SeMet λ2 (0.97984 Å) | 25278 | 5.82 (3.07) | 98.4 (88.2) | 20.5 (2.2) | 7.1 (40.0) |
| SeMet λ3 (0.97961 Å) | 25300 | 5.89 (3.05) | 98.4 (89.0) | 20.8 (2.3) | 7.2 (40.5) |
| SeMet λ4 (0.92526 Å) | 25598 | 6.01 (3.83) | 99.6 (97.1) | 17.0 (1.9) | 8.3 (57.2) |

Values in parentheses are for the high resolution bin (1.86–1.80 Å).

*Rmerge = Σl[I( h) − <I( h)>]/ΣlI( h) where I( h) is the i-th measurement and <I( h)> is the mean of all measurements of I( h) for Miller indices h.

Table II. Crystallographic Phasing Statistics

| Wavelength | Overall isomorphous and dispersive differences* | Phasing power as defined in CNS† |
|------------|-----------------------------------------------|---------------------------------|
| λ1 (remote) | 0.0427                                        | F1 Friedel mate: 0.51           |
| λ2 (infection) | 0.0766                                         | F1 Friedel mate: 2.79           |
| λ3 (peak) | 0.0916                                         | F1 Friedel mate: 3.31           |
| λ4 (remote) | 0.0569                                         | F1 Friedel mate: 1.06           |

*MAD phasing power is defined as |max | Fw - Fc | | / | Fw | P(0)(|Fw| + |Af| - |Fc|) | [db] | where P(0) is the experimental phase probability distribution, i is any wavelength, Fw and Fc correspond to a Bijvoet pair of structure factors. ΔF is the anomalous scatter structure factor difference for a particular lack of closure expression.

Rosenthal et al., 1999. Epsin 1 is very abundant in brain and probably participates in membrane recycling at the synapse (Chen et al., 1998).

Yeast homologues of epsin, Ent1p and Ent2p, were shown to be involved in endocytosis and actin function (Wendland et al., 1999). The NPF motifs of the Ent proteins bind the EH domains of Panl, which shares functional similarities with Eps15 (Wendland et al., 1999). Based on genetic studies, the ENTH domain represents the functionally most important portion of the Ent proteins. Whereas a strain harboring a disruption of both the functionally most important portion of the Ent proteins (Wendland et al., 1999). The NPF motifs of the Ent proteins are lacking this domain, was sufficient to rescue the lethal phenotype (Wendland et al., 1999)

So far, the function of the ENTH domain is unknown. To gain insight into its function, we have determined the X-ray crystal structure of the ENTH domain of rat epsin 1 and investigated its protein-binding properties. Our findings suggest an unexpected dual function of epsin in the cytoplasm and in the nucleus.

Materials and Methods

ENTH Expression for Structural Determination

ENTH of rat epsin 1 was expressed as an NH2-terminal GST fusion using the pGEX-6-1 expression vector (Amerham Pharmaica Biotech). The plasmid was introduced into B834(DE3) cells (Novagen) and grown in defined M9 media supplemented with 50 μg/ml selenomethionine (SeMet; Lehay et al., 1994) and 0.1 mg/ml ampicillin. Cells were grown to an optical density of 2 measured at 600 nm, at which time the culture was induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside and allowed to continue growing for ~5 h. Cells were harvested by centrifugation at 46,000 g of frozen cells resuspended in 200 ml of 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 10 mM DTT. GST-ENTH beads with bound ENTH were then resuspended in 15 ml of wash buffer with ~100 μg bovine thrombin and incubated overnight with agitation. As a note, although pGEX-6-1 is a rTEV cleavable GST fusion vector, we found that rTEV cleavage was not effective. However, thrombin cleaves quite well at an internal site 10 amino acids into the ENTH domain. Crystals of lesser quality have been obtained with rTEV cleaved protein, but this protein showed significant degradation in crystal trays, so studies with these crystals were not pursued. Cleaved ENTH protein was separated from glutathione-Sepharose beads and injected onto a Superdex 75 16/60 size-exclusion column (Amerham Pharmaica Biotech) at 0.5 ml/min in wash buffer. Fractions containing ENTH as determined by UV absorption and SDS-PAGE were pooled and dialyzed into 20 mM Na-Hepes, pH 7.5, 20 mM NaCl, and 10 mM DTT. ENTH was then concentrated to ~7.5 mg/ml (Miliopore ultrafilters and stored at ~80°C.

Crystallization and Data Collection

Initial crystals were obtained by hanging drop vapor diffusion at 20°C. 1 μl of protein solution at ~7.5 mg/ml was mixed with 1 μl of mother liquor containing 100 mM Na-Hepes, pH 7.5, 10% polyethylene-glycol (PEG) 1000, and 8% ethylene glycol on a siliconized glass coverslip, and equilibrated against 0.5 ml of mother liquor. Crystals typically grew to full size (220 × 220 × 100 μm) in 30–40 h. Crystals were cryoprotected by sequential transfer into mother liquor containing 5% increasing increments of ethylene glycol to a final concentration of 30%. The crystals were then flash frozen in liquid nitrogen-cooled liquid propane that was subsequently frozen in liquid nitrogen. ENTH crystallized in space group P3211 with one molecule per asymmetric unit, and a solvent content of 40% as determined by the Matthews coefficient (Matthews, 1968). Diffraction data were collected at four wavelengths, and processed using DENVIZ/ scalepack (Otwinski and Minor, 1997: Table I).

Structure Determination

Four of the five expected selenium sites were located in an anomalous difference Patterson map calculated at the peak wavelength using an automated Patterson heavy-atom search method (Grosse-Kunstleve and Bricogne, 1997) implemented in the Crystallography & NMR System (CNS; Brunger et al., 1998). The final site was located after initial multivavelength anomalous dispersion (MAD) phasing in CNS using a log likelihood gradient map (Bricogne, 1997). The MAD phasing statistics were ex-
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Table III. Crystallographic Figure-of-Merit

| Bragg Spacing Limits (Å) | Overall (43.14–1.79) | 3.58 | 2.84 | 2.48 | 2.26 | 2.09 | 1.97 | 1.87 | 1.79 |
|-------------------------|-----------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Figure-of-Merit (FOM)   | 0.72                  | 0.92  | 0.91  | 0.85  | 0.77  | 0.69  | 0.58  | 0.46  | 0.33  |

MAD phasing power is defined as $\frac{|F_{\text{obs}} - F_{\text{calc}}^2|}{\sigma(F_{\text{calc}})}$ where $F_{\text{obs}}$ is the experimental phase probability distribution, $F_{\text{calc}}$ is any wavelength, $F_{\text{obs}}$ and $F_{\text{calc}}$ correspond to a Bijvoet pair of structure factors. AF is the anomalous scatterer structure factor difference for a particular lack of closure expression.

Model Building and Refinement

A noninitial model was built with the program O (Jones et al., 1991), using the electron density map obtained by M A D phasing and subsequent density modification. All of the ENTH domain could be unambiguously traced except for residues 1–5, 20–23, 149, and 150. Model refinement was accomplished in CNS using the M L H L target (Pannu and Read, 1998) against the diffraction data at the low-energy remote wavelength. Final model statistics are given in Table IV and were calculated using CNS (Brunger et al., 1998) and D enzo (Otwinski and Minor, 1997). The final model contains residues 16–160 of the ENTH domain, 65 ordered water molecules, and 3 ethylene glycol molecules.

Yeast Two-hybrid Screen

The ENTH domain of rat epsin 1 (amino acids 1–160 of rat epsin 1; Chen et al., 1998) fused to the LexA DNA-binding domain was used as bait to screen a rat brain Matchmaker activation domain library in pGAD10 (C L O N T E C H Laboratories, Inc.). The bait was transformed along with the ENTH-LexA DNA-binding domain, were restriction mapped and sequenced. A pair of primers specific to the pGAD10 vector: 5′-CAC GAT GCA CAG TTG AAG TGA ACT TGC-3′ were used in the leptomycin B studies. Deletion mutants of rPLZFΔPOZ were generated by PCR amplification of the desired fragments, which were subcloned into the pG A D 424 activation domain vector (C L O N T E C H Laboratories, Inc.).

Affinity Purification

For affinity-purification of rat brain proteins, the same ENTH domain used for crystallographic studies was coupled to UltraLink Biosupport Medium (Pierce Chemical Co.) according to the manufacturer’s instructions. A Triton X-100 extract of a rat brain total homogenate (10 mg/ml) was incubated with immobilized ENTH domain at 4°C and the bound material was analyzed by SDS-PAGE and Western blotting. Wild-type and mutant GST-ENTH fusion proteins were used for affinity purifications from Triton X-100 extracts of cells transfected with flag epitope-tagged rPLZFΔPOZ or full-length human promyelocytic leukemia Zn2+ finger protein (PLZF) according to standard procedures (Nemoto et al., 1997).

Generation of Mutant ENTH Domains

The mutant ENTH domains were obtained using PCR-based site-directed mutagenesis (Chen et al., 1999). The sequences of these mutant ENTH domains were confirmed by standard double-strand sequencing.

Leptomycin B Incubations

Leptomycin B (10 ng/ml; kind gift of Dr. M. O yoshiba; Kudo et al., 1999) was added to the culture medium 16 h after transfection and cells were incubated for additional 3.5 h before fixation. A full-length GFP-tagged rat epsin 1 clone was constructed as described (Rosenthal et al., 1999) and used in the leptomycin B studies.

Antibodies and Miscellaneous cDNAs

Rabbit polyclonal antibodies against human PLZF and epsin were prev-

Table IV. Crystallographic Refinement

| Resolution range | Overall | 500–1.8 Å | 500–3.88 Å | 3.08 Å | 2.69 Å | 2.44 Å | 2.27 Å | 2.13 Å | 2.03 Å | 1.94 Å | 1.86 Å | 1.80 Å |
|------------------|---------|-----------|-----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Resolution (Å)   | 500–1.8 Å | 500–3.88 Å | 3.08 Å | 2.69 Å | 2.44 Å | 2.27 Å | 2.13 Å | 2.03 Å | 1.94 Å | 1.86 Å | 1.80 Å |
| R-value*          | 20.6     | 20.8      | 20.4     | 21.3   | 18.1   | 20.7   | 20.1   | 20.2   | 20.5   | 23.6   | 25.2   |
| Rfree*           | 22.5     | 22.5      | 22.0     | 22.3   | 19.0   | 22.8   | 23.4   | 23.0   | 24.2   | 27.4   | 24.6   |
| Luzzati coordinate error | 0.21 Å   | 0.21 Å    | 0.21 Å   | 0.21 Å | 0.21 Å | 0.21 Å | 0.21 Å | 0.21 Å | 0.21 Å | 0.21 Å | 0.21 Å |
| Cross-validated Luzzati coordinate error | 0.23 Å   | 0.23 Å    | 0.23 Å   | 0.23 Å | 0.23 Å | 0.23 Å | 0.23 Å | 0.23 Å | 0.23 Å | 0.23 Å | 0.23 Å |
| Bond-length deviation | 0.005 Å  | 0.005 Å   | 0.005 Å  | 0.005 Å | 0.005 Å | 0.005 Å | 0.005 Å | 0.005 Å | 0.005 Å | 0.005 Å | 0.005 Å |
| Bond-angle deviation | 1.12°   | 1.12°     | 1.12°    | 1.12°  | 1.12°  | 1.12°  | 1.12°  | 1.12°  | 1.12°  | 1.12°  | 1.12°  |
| Improper angle deviation | 0.68°   | 0.68°     | 0.68°    | 0.68°  | 0.68°  | 0.68°  | 0.68°  | 0.68°  | 0.68°  | 0.68°  | 0.68°  |
| Dihedrals        | 18.0°    | 18.0°     | 18.0°    | 18.0°  | 18.0°  | 18.0°  | 18.0°  | 18.0°  | 18.0°  | 18.0°  | 18.0°  |
| Average B-factor  | 20.9 Å2  | 20.9 Å2   | 20.9 Å2  | 20.9 Å2 | 20.9 Å2 | 20.9 Å2 | 20.9 Å2 | 20.9 Å2 | 20.9 Å2 | 20.9 Å2 | 20.9 Å2 |
| RMSD for bonded main chain atoms | 1.87 Å2  | 1.87 Å2   | 1.87 Å2  | 1.87 Å2 | 1.87 Å2 | 1.87 Å2 | 1.87 Å2 | 1.87 Å2 | 1.87 Å2 | 1.87 Å2 | 1.87 Å2 |
| RMSD for bonded side chain atoms | 3.32 Å2  | 3.32 Å2   | 3.32 Å2  | 3.32 Å2 | 3.32 Å2 | 3.32 Å2 | 3.32 Å2 | 3.32 Å2 | 3.32 Å2 | 3.32 Å2 | 3.32 Å2 |
| Minimum B-factor  | 7.46 Å2  | 7.46 Å2   | 7.46 Å2  | 7.46 Å2 | 7.46 Å2 | 7.46 Å2 | 7.46 Å2 | 7.46 Å2 | 7.46 Å2 | 7.46 Å2 | 7.46 Å2 |
| Maximum B-factor  | 62.3 Å2  | 62.3 Å2   | 62.3 Å2  | 62.3 Å2 | 62.3 Å2 | 62.3 Å2 | 62.3 Å2 | 62.3 Å2 | 62.3 Å2 | 62.3 Å2 | 62.3 Å2 |
| % Residues in core $\phi$-$\psi$ region | 91.1     | 91.1      | 91.1     | 91.1   | 91.1   | 91.1   | 91.1   | 91.1   | 91.1   | 91.1   | 91.1   |
| % Residues in disallowed regions | 0.0      | 0.0       | 0.0       | 0.0    | 0.0    | 0.0    | 0.0    | 0.0    | 0.0    | 0.0    | 0.0    |

* R = \sum |F_{\text{calc}}|kF_{\text{calc}}|/2|F_{\text{calc}}|.$

Free R value is the R value obtained for a test set of reflections, consisting of a randomly selected 10% subset of the diffraction data, not used during refinement.
Rabbit polyclonal antibodies against rat PLZF were obtained using GST-rPLZF<sub>D</sub>POZ as the immunogen. The resulting serum was first depleted of anti-GST antibodies by incubation with GST beads and then affinity-purified with GST-rPLZF<sub>D</sub>POZ fusion protein. A cDNA encoding GFP-β-galactosidase fusion protein in pHM830 was a kind gift of Dr. T. Stamminger (Institute fur Klinische und Molekulare Urologie, Universitat Erlangen-Nurnberg, Erlangen, Germany). Xpress-tagged epsin 1 cDNA was previously described (Chen et al., 1998). A human PLZF cDNA in pcDNA3 was a kind gift of Dr. P.G. Pelicci (European Institute of Oncology, Milano, Italy). The cDNA encoding GFP-rat epsin 1 was generated by subcloning the epsin 1 cDNA into the pEGFP-C1 vector (CLONTECH Laboratories, Inc.).

**Miscellaneous Procedures**

Cell transfection, immunoprecipitation, immunofluorescence, GST-fusion protein production, SDS-PAGE, Western blotting, and protein assay were carried out as previously described (Chen et al., 1998).

**Results**

**Structure Determination**

The ENTH domain of rat epsin 1 (residues 1–160) was expressed as a GST-fusion protein in E. coli. After cleavage of the fusion protein and purification, crystals of the ENTH domain were obtained by hanging drop vapor diffusion in a polyethylene-glycol condition at 4°C. SeM-i substituted ENTH domain crystallized in a similar condition, and these crystals were used for further study. Diffraction data were collected for a MAD experiment (Hendrickson, 1991) using synchrotron radiation at four wavelengths around the selenium K absorption edge. Crystals belonged to space group p3<sub>2</sub>1<sub>2</sub>, with unit cell parameters: a = b = 49.81 Å, c = 99.97 Å, α = β = 90°, and γ = 120°. The crystals contained ~40% solvent and diffracted to a minimum Bragg spacing of 1.8 Å. The electron density map obtained from density-modified MAD phases was superb, and easily traceable (Fig. 1). The final refined model has a free R value (Brunger, 1992) of 22.5% for all reflections with a Bragg spacing >1.8 Å.

**General Features of the Structure**

The ENTH domain forms a compact globular domain (Fig. 2, A and B) with a solvent accessible surface area of ~8,200 Å. The structure is composed of eight α helices connected by loops of varying length. The general topology of the structure is determined by three helical hairpins (α1–2, α3–4, and α6–7) that are stacked consecutively with a right-handed twist. This stacking gives the ENTH domain a rectangular appearance when viewed face on (Fig. 2, A i and B i). The two primary faces, one concave and one convex, are made up of parallel α helices (Fig. 2 A).
The COOH-terminal α helix (α8) folds back across the domain, framing the bottom edge perpendicular to the central hairpin (α3–4). From the opposite edge, loops that connect α helices 1–2, 3–4, and 6–7 form a conical projection, lending a wedge-like appearance (Fig. 2, A i).

The ENTH domain family contains several highly conserved residues (Chen et al., 1998; Kay et al., 1999; Rosenthal et al., 1999; Fig. 2 C). The most highly conserved residues fall roughly into two classes: internal residues that are involved in packing and therefore are necessary for structural integrity, and solvent accessible residues that may be involved in protein–protein interactions. Two large regions of high conservation emerge on the molecular surface: a larger patch formed primarily from residues on loop 2 and α helix α4, and a smaller patch was found near loop 7 (Fig. 2, A, B, and C).

**Structural Alignment and Comparison**

A structural similarity search using DALI (Holm and...
Sander, 1993) identified proteins that are structurally similar to ENTH, but not related by primary sequence. The ENTH structure is most similar to an armadillo repeat segment from β-catenin (Huber et al., 1997; Fig. 3, A and B), the HEAT repeat unit from karyopherin-β (importin-β; Chook and Blobel, 1999; Cingolini et al., 1999; Fig. 3, C and D), and to the scaffolding subunit of protein phosphatase 2A (Groves et al., 1999). 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 (Huber et al., 1997; Chook and Blobel, 1999; Cingolini et al., 1999; Groves et al., 1999). Structural superimposition of the ENTH domain with either type of repeat aligns their concave faces (Figs. 2 and 3), which contain the largest patch of conserved surface residues (Vriend, 1990). The Cα superposition of ENTH with karyopherin-β has a root mean square difference (RMSD) of 1.5 Å, whereas the Cα superposition with β-catenin produces an RMSD of 1.8 Å.

**Isolation of an ENTH Domain Binding Protein, PLZF**

The structural similarity of the ENTH domain to armadillo and HEAT repeats suggests that the ENTH domain may interact with other proteins. We searched for potential binding partners of the ENTH domain of rat epsin 1 (residues 1–160) using the yeast two-hybrid method. A bait construct consisting of the ENTH domain fused to the DNA-binding domain of LexA was used to screen a pGAD10 rat brain cDNA library. 20 of the clones isolated by the screen were all identical, and encoded an open reading frame highly homologous (96% identity) to the COOH-terminal portion of human PLZF (corresponding to amino acids 262–673 of the human protein). PLZF is a transcription factor containing an NH2-terminal BTB/POZ domain and nine C2H2 Zn2+ finger motifs (Li et al., 1997; Fig. 4 A).

The identification of a transcription factor as an interaction partner for the ENTH domain of epsin 1 seemed surprising at first, given the known function of epsin 1 at the cell periphery. However, β-catenin family proteins, which contain a structurally related module, have a dual function in the cell cortex and in the nucleus (Barth et al., 1997; Willert and Nusse, 1998). Furthermore, catenin p120, which contains armadillo repeats, binds to a POZ/BTB-Zn2+ finger containing transcription factor, Kaiso, that is homologous to PLZF (Daniel and Reynolds, 1999). The interaction of catenin p120 with Kaiso is mediated by the armadillo repeat region of p120, and by the COOH-terminal portion of Kaiso, corresponding to the region of PLZF selected by the yeast two-hybrid screen. In addition, based on the binding properties of protein fragments, the Zn2+ finger region and upstream sequences of both Kaiso and PLZF are required for the interaction with catenin p120 and ENTH, respectively (Daniel and Reynolds, 1999; and Fig. 4 A).

The similarity of these two interactions is likely
to reflect evolutionary conservation. We hypothesized that epsin and PLZF are physiological partners, and carried out further biochemical and functional studies to validate this interaction.

**Biochemical Interaction of Epsin and PLZF**

To verify the interaction of PLZF and epsin, affinity purification experiments were performed using immobilized ENTH domain. The GST-ENTH domain, but not GST alone, specifically retained a flag-tagged- rPLZFΔPOZ fragment (the protein fragment of PLZF lacking the POZ domain, corresponding to the fragment isolated by the yeast two-hybrid screen) from extract of transfected CHO cells (Fig. 4 B). In addition, purified GST-free ENTH domain conjugated to beads, but not beads alone, interacted with endogenous PLZF from a rat brain extract (Fig. 4 C).

To determine whether epsin and PLZF interact in vivo, immunoprecipitation experiments from rat brain extracts were carried out. An anti-epsin antibody, but not control IgGs, coprecipitated PLZF, but not p53 (a negative control; Fig. 4 D). Conversely, anti-PLZF antibody, but not preimmune IgGs, coprecipitated epsin (Fig. 4 E), but not p53 (not shown). While epsin is present in the cytosol and further concentrated at the synapse, PLZF was shown to be primarily localized in the nucleus (Ruthardt et al., 1998). However, a cytosolic pool of PLZF was observed by immunofluorescence in brain (not shown).

To further assess the specificity of the binding of the ENTH domain to PLZF, highly conserved residues of the ENTH domain were altered by site directed mutagenesis: T28 to A, R72 to A, and G88 to S. Mutation at position 88 was previously found to confer temperature sensitivity to the function of yeast Ent1p (Wendland et al., 1999). Wild-type and mutant ENTH domains were expressed as GST fusion proteins and used to affinity purify flag-tagged human PLZF expressed in CHO cells. As shown by Fig. 4 F, wild-type, but not mutant ENTH domains, pulled down PLZF from transfected cell extracts. A control protein, clathrin, was not retained by any of these fusion proteins.

**PLZF Can Recruit Epsin to the Nucleus**

Based on immunofluorescence, most epsin is found in the cytosol, even after overexpression (Chen et al., 1998; Rosenthal et al., 1999; Fig. 5 A). The interaction of epsin 1 with PLZF raises the possibility that epsin may be found in the nucleus under certain conditions. Enriched expression of PLZF may increase the fraction of epsin 1 localized in the nucleus and allow the visualization of a nuclear epsin pool by immunofluorescence. When either full-length human PLZF or rPLZFΔPOZ were overexpressed by transfection, both proteins partially accumulated in the nucleus (Fig. 5 A, and not shown). In cells cotransfected with epsin 1 and PLZF, a nuclear pool of epsin was also observed (the concentration of endogenous epsin is below detectability with our antibodies; Chen et al., 1998).

We next investigated whether epsin physiologically shuttles between the nucleus and the cytoplasm. The antifungal antibiotic, leptomycin B, was recently shown to block the Crm1-dependent nuclear export pathway and to induce a nuclear accumulation of several proteins which shuttle between the nucleus and the cytoplasm (Nishi et al., 1994; Kudo et al., 1999; Van Hengel et al., 1999). We examined whether leptomycin B affected the intracellular distribution of epsin in CHO cells transiently transfected with either full-length human PLZF or rPLZFΔPOZ. An extract of CHO cells transfected with flag-tagged rPLZFΔPOZ was affinity-purified on either GST or a GST-ENTH domain fusion protein. The starting material and the bead-bound material were analyzed by Western blotting using antibody directed against the flag epitope. C, ENTH domain affinity purification from a rat brain extract. A Triton X-100 extract of a rat brain total homogenate was affinity-purified on ENTH domain conjugated to beads or control beads. The starting material and the bead-bound material were analyzed by Western blotting for PLZF. D and E, PLZF and epsin can be coprecipitated from a rat brain Triton X-100 extract. D, Immuno-precipitates generated by anti-PLZF antibodies or control IgGs were analyzed by Western blotting with anti-rat PLZF or anti-p53 (used as control) antibodies. E, Immuno-precipitates generated by anti-PLZF antibodies or control IgGs were analyzed by Western blotting using antiepsin 1 antibodies. F: Mutant ENTH domains fail to interact with PLZF. A n extract of CHO cells transfected with flag-tagged human PLZF was affinity-purified on wild-type or mutant (T28A, R72A, and G88S) GST-ENTH domain fusion proteins. The starting material, the bead-unbound and the bead-bound material were analyzed by Western blotting using antibody directed against the flag epitope or anti-clathrin antibody as a control. B, Bead-bound; c, control IgG s; e, anti-epsin antibodies; P, pellet; S, supernatant; SM, starting material; U, bead-unbound.
with either GFP-epsin 1 or Xpress-tagged epsin 1. As shown by Fig. 5 B, both epsin 1 fusion proteins accumulated in the nucleus after incubation with leptomycin B, but not in control cells. A GFP-β-galactosidase fusion protein used as a control did not accumulate in the nucleus regardless of the presence of leptomycin B.

**Discussion**

The crystal structure of the ENTH domain of epsin 1 revealed a relationship to other well characterized protein modules that could not have been predicted by primary amino acid sequence analysis. The ENTH domain adopts a fold strikingly similar to that of armadillo and HEAT repeats, two domains found in β-catenin and karyopherin, respectively. Structural similarity often indicates functional similarity. Accordingly, we have identified properties of epsin suggesting that this similarity is functionally meaningful.

β-Catenin was first discovered as a component of a cytoskeletal scaffold underlying the plasma membrane and is thought to play an important role in the function of the cortical cytoskeleton (Tao et al., 1996; Bullions and Levine, 1998). β-Catenin was subsequently found to be part of a signaling pathway from the cell surface to the nucleus, the so-called Wnt signaling pathway. Activation of the pathway leads to decreased phosphorylation and degradation of β-catenin and to its accumulation in the nucleus, where it regulates transcription (Barth et al., 1997; Willert and Nusse, 1998; Peifer and Polakis, 2000). Mammalian epsin was identified as an accessory factor in clathrin-mediated endocytosis (Chen et al., 1998; Kay et al., 1999; Rosenthal et al., 1999) and its homologues in yeast, Ent1p and Ent2p, were implicated both in endocytosis and actin function (Wendland et al., 1999). The predominant pool of epsin is localized in the cytosol (Chen et al., 1998; Rosenthal et al., 1999). However, our present results demonstrate that epsin binds a transcription factor, PLZF, and that it can enter the nucleus. Moreover, specifically, the nuclear accumulation of epsin produced by leptomycin B illustrates that epsin shuttles physiologically between the cytoplasm and the nucleus. Leptomycin B blocks the Crm1-dependent nucleocytoplasmic export pathway (Nishi et al., 1994; Kudo et al., 1999; Van Hengel et al., 1999), thus implying that nuclear accumulation of epsin results from an imbalance between nuclear import and nuclear export. An attractive possibility is that a regulated interaction of epsin with cytosolic components, possibly mediated by its phosphorylation (Chen et al., 1999), may control the nuclear pool of epsin and, via PLZF, the transcription of specific genes. This regulation may occur in response to external stimuli that activates endocytosis. This scenario defines a new regulatory pathway from the endocytic machinery to the nucleus.

These findings are convergent with recent studies of the epsin binding protein Eps15. As in the case of epsin, the role of Eps15 in clathrin-mediated endocytosis is well established and the bulk of Eps15 is localized in the cytosol (Salcini et al., 1997; Benmerah et al., 1998). However, an additional role of Eps15 in the regulation of nuclear functions has emerged. The same EH domain containing region of Eps15 that binds epsin also interacts with Numb and RAB/Hrb (Salcini et al., 1997). Numb binds Mdm2, a negative regulator of p53 (Juven-Gershon et al., 1998; Juven-Gershon and Oren, 1999), demonstrating a connection between Eps15 and a transcription factor. RAB/Hrb is a cofactor for export from the nucleus of the HIV protein Rev, and the EH domains of Eps15 cooperates with RAB/Hrb in the function of the Rev export pathway (Doria et al., 1999). This export pathway, in turn, is Crm1-dependent (Ullman et al., 1997). The role of Eps15 as a cofactor in nucleocytoplasmic export raises the possibility that even the similarity of the ENTH domain of epsin to the
karyophorins may reflect some functional relationship. The karyophorins are proteins implicated in cargo transport and recognition across the nuclear envelope via their HEAT repeat domains (Radu et al., 1995; Görlich, 1998; Pemberton et al., 1998; Nakielny and Dreyfuss, 1999).

Based on genetic studies, the ENTH domain is the most important functional domain of the yeast epsins, Ent1p and Ent2p (Wendland et al., 1999). Since the residues of the Ent proteins that interact with yeast EH domains and clathrin are localized outside the ENTH domain, the essential role of this domain may reflect its implication in nuclear functions. This possibility is consistent with our observation that at least one of the residues important for the function the ENTH domain of Ent1p is also required for the binding of rat epsin 1 ENTH domain to PLZF. Although an obvious orthologue of PLZF is not present in Saccharomyces cerevisiae, proteins with multiple Zn$^{2+}$ finger motifs similar to those of PLZF are expressed by this organism. The similarity of PLZF to Kaiso, another transcription factor previously shown to bind the armadillo repeat region of catenin p120 (Daniel and Reynolds, 1999), indicates that the interaction of armadillo-repeat domains with this family of transcription factors is evolutionary conserved, and therefore likely to be physiologically important. The interaction of the ENTH domain with a transcription factor is not mutually exclusive with potential interactions with other proteins. Based on the property of the structurally related armadillo and HEAT repeats to bind multiple partners (Barth et al., 1997; Huber et al., 1997; Willert and Nusse, 1998; Chook and Blobel, 1999; Cingolini et al., 1999; Groves et al., 1999), it is likely that even the ENTH domain may have multiple physiological interactors. Pull-down experiments from rat brain have shown that tubulin, and to a lower extent coatomer (Rothman and Wieland, 1996), can bind ENTH domain of epsin 1 (our unpublished observations). However, the possible significance of these interactions remains unclear.

ENTH domains are present not only in proteins of the epsin family, but also in proteins which differ substantially from epsin outside this domain (Chen et al., 1998; Kay et al., 1999; Rosenthal et al., 1999). It is of interest that certain motifs are frequently found in such proteins. Besides NPF motifs that bind EH domains (Salcini et al., 1997), A P-2 and clathrin motifs, as well as FG motifs, are often present in them (Kay et al., 1999). FG repeats are signature motifs of nucleoporins (Radu et al., 1995), further emphasizing a potential connection of the ENTH domain to nuclear function. While epsin 1 does not contain FG repeats, most other epsin family members do (three or more such repeats are present in yeast Ent1 and Ent 2, in mammalian epsin 2, and in the epsin-like protein D79993.1). It is therefore possible that a dual function at the cell surface and in the nucleus may be a general property of most, possibly all, ENTH containing proteins.

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Note A died in Prof. M. et al. (Mao, Y.; A. Nickitenko, X. Duan, T. E. Lloyd, N.N. Wu, H. Bellen, and F.A. Quiocio. 2000. Cell. 100:447–456) have recently reported the structure of VH5 and FYVE tandem domains from the hepatocyte growth factor-regulated tyrosine kinase substrate. The VH5 and ENTH domains align with an RMSD of ~1.8 Å, indicating an extremely similar fold. This is especially interesting because HRS is involved in membrane trafficking and signal transduction, perhaps indicating that this fold is a common motif in these areas.

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