Molecular mechanism of NPF recognition by EH domains

Article *in* Nature Structural Biology · December 2000

DOI: 10.1038/80924 · Source: PubMed

7 authors, including:

Montarop Yamabhai
Suranaree University of Technology
16 PUBLICATIONS **783** CITATIONS

SEE PROFILE

Brian K. Kay
University of Illinois at Chicago
201 PUBLICATIONS **8,989** CITATIONS

SEE PROFILE

Michael Overduin
University of Alberta
145 PUBLICATIONS **6,368** CITATIONS

SEE PROFILE

Experimental Cell Research **253,** 186–209 (1999)
Article ID excr.1999.4694, available online at http://www.idealibrary.com on IDEAL®

The EH Network

Elisa Santolini,* Anna Elisabetta Salcini,* Brian K. Kay,† Montarop Yamabhai,† and Pier Paolo Di Fiore*,‡,[1]

**Department of Experimental Oncology, European Institute of Oncology, Milan, Italy; †Department of Pharmacology, University of Wisconsin–Madison, 1300 University Avenue, Madison, Wisconsin 53706-1532; and ‡Istituto di Microbiologia, University of Bari, Italy*

The EH domain is an evolutionary conserved protein–protein interaction domain present in a growing number of proteins from yeast to mammals. Even though the domain was discovered just 5 years ago, a great deal has been learned regarding its three-dimensional structure and binding specificities. Moreover, a number of cellular ligands of the domain have been identified and demonstrated to define a complex network of protein–protein interactions in the eukaryotic cell. Interestingly, many of the EH-containing and EH-binding proteins display characteristics of endocytic "accessory" proteins, suggesting that the principal function of the EH network is to regulate various steps in endocytosis. In addition, recent evidence suggests that the EH network might work as an "integrator" of signals controlling cellular pathways as diverse as endocytosis, nucleocytosolic export, and ultimately cell proliferation.

Key Words: **EH; domains; Eps15; endocytosis.**

INTRODUCTION

Many cellular functions, including proliferation, differentiation, cytoskeleton organization, and apoptosis, are regulated through a complex intracellular network of signal transducers. Specialized protein domains, such as the SH2, SH3, PTB, or WW domain, mediate this vast array of interactions by binding to specific sequences located in target proteins (for reviews see [1–4] and references therein). Within individual transducing proteins, protein interaction domains have evolved to recognize specific consensus sequences in which some positions must be occupied by given amino acids while others can vary. The list of specialized binding domains, and the definition of their binding partners, are continuously growing, due to the exploitation of powerful techniques such as yeast two-hybrid, cDNA expression screening, and screening of phage-displayed combinatorial peptide libraries. In addition, increased genomic sequencing, coupled to computational tools, allows the prediction of additional novel protein domains, many of which can be reasonably expected to be protein–protein interaction domains.

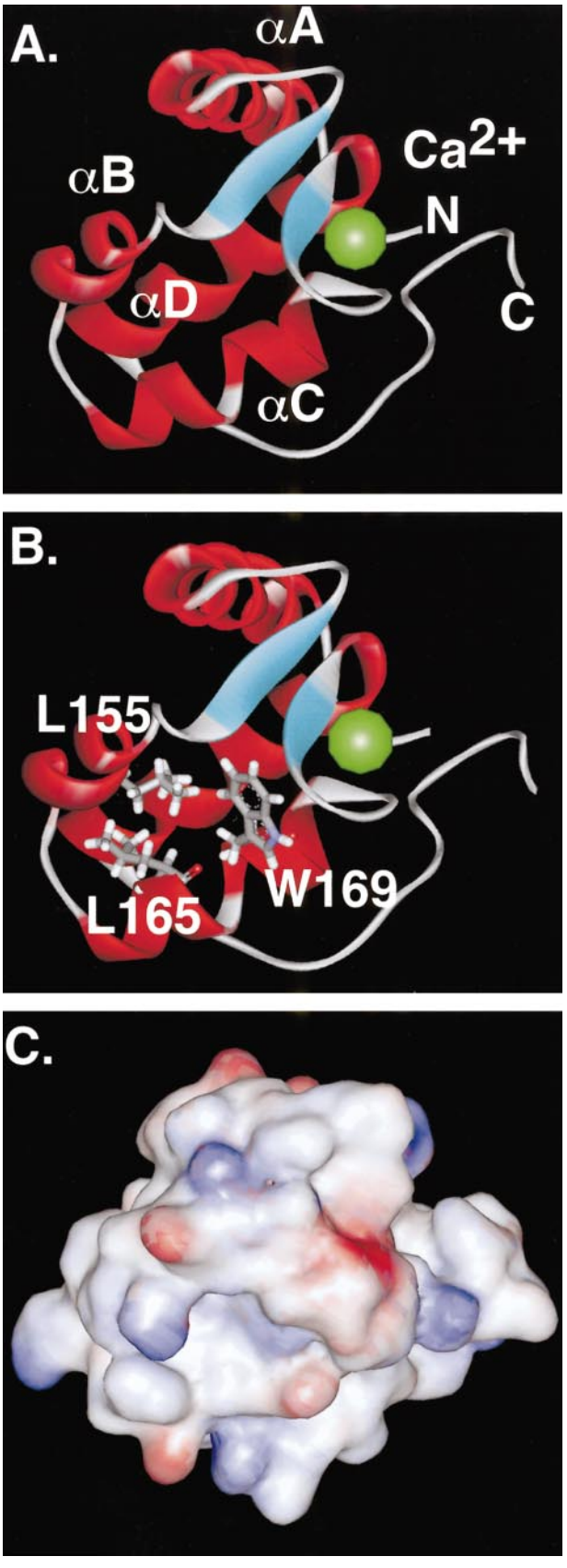
Many intracellular signaling proteins display a modular organization comprising several independently folded binding domains. Each of these domains is able to recognize specific sequences on diverse target proteins, thus allowing the formation of multimeric complexes. The domains contribute to the function of the protein, by targeting proteins to specific cellular compartments, and for enzymes, by recruiting protein substrates. The relationship between a protein's function and its modular make-up is so informative that in many cases the biological function of a protein can be partially inferred from the analysis of the domains themselves. A nice example of such a correlation is given by the recently identified EH domain, the subject of this review. The identification of a number of EH-containing and EH-binding proteins, together with functional data about the role of these proteins, has revealed the existence of an intracellular EH-based protein–protein interaction network. This interaction network is involved in cellular functions as diverse as endocytosis, nucleocytosolic export, and ultimately in the control of cell proliferation.

THE EH DOMAIN: STRUCTURE AND BINDING SPECIFICITY

The EH domain was initially identified as a repeated sequence present in three copies in the NH_2-termini of Eps15 and of the related molecule Eps15R, two substrates of the epidermal growth factor receptor (EGFR) tyrosine kinase [5–7]. The EH domain has a protein–protein interaction surface, as demonstrated by its ability to interact with proteins from different cell types in filter binding assays [7]. Since then, EH domains have been observed in proteins in yeast, nematodes, frogs, and mammals. The typical EH domain in these various organisms is ~100 amino acids long with ~50% of the positions showing >50% similarity. EH domains are frequently, but not obligatorily, present in multiple copies and they may include calcium-binding domains of the EF hand type (for recent reviews see [8, 9]).

[1] To whom correspondence and reprint requests should be addressed at Istituto Europeo di Oncologia, Via Ripamonti 435, 20141 Milan, Italy. Fax: 39-02-57489851. E-mail: pdifiore@ieo.cilea.it.

0014-4827/99 $30.00



Recently the structure of the second EH domain of human Eps15 has been solved by heteronuclear magnetic resonance spectroscopy [10] (Fig. 1). It contains two closely associated helix-loop-helix motifs connected by a short antiparallel β-sheet; the two loops are close to each other on one side of the structure, whereas the NH_2- and COOH-termini of the domain are together at the opposite side. The predicted amino acid sequence of the second EH domain of human Eps15 revealed the presence of two calcium-binding motifs of the EF hand type, although NMR showed that only a single calcium ion is bound by the second EF hand (Fig. 1). The tight association with calcium suggests a structural role for this ion and predicts that physiological calcium variations may not play a regulatory role in the EH domain function (Fig. 1). Limited experimentation has shown that the binding of the central EH domain to filter-blotted proteins is not calcium-dependent [7]. The structures of the N-terminal domain of Eps15 and the solitary EH domain of POB1 have also been solved [11, 12].

A variety of approaches has been employed to determine the target sequences that bind to EH domains. Salcini *et al.* [13] used the EH domains of Eps15 and Eps15R to screen a phage-displayed combinatorial peptide library, which led to the identification of the motif asparagine–proline–phenylalanine (NPF) as optimal ligand preference for the EH domain. Consistent with this finding, screening a human fibroblast expression library with the EH domains of Eps15 yielded several cDNA clones, representing bona fide EH-binding proteins. Among these are (i) Numb, the human homologue of *Drosophila* Numb; (ii) a Numb-related protein, Numbl (Human Gene Nomenclature Committee-approved symbols are used throughout this review); (iii) Hrb, a protein involved in nucleocytosolic transport; and (iv) a Hrb-related protein, Hrbl (see below for details). These proteins displayed no sequence similarities among themselves, except for the presence of one or more copies of the NPF motif, which were experimentally demonstrated to be sufficient for binding to Eps15 as short peptides [13]. In addition, mutagenesis of the unique NPF motif of Numb to NAA (asparagine–alanine–alanine) severely impaired interaction with Eps15 *in vivo,* clearly demonstrating that the motif is required for interaction with EH domains. In contrast, mutational analysis of the NPF-containing

FIG. 1. The three-dimensional structure of the central EH domain of Eps15. In A, the secondary structure of one representative structure of the domain (Protein Database No. 1EH2), which was deduced by NMR [10], is shown. The four α helices are labeled in red (i.e., αA–αD) and the β-sheets are labeled in blue. The calcium ion is shown in green. In B, three critical residues are highlighted. These residues are highly conserved among EH domains and form a hydrophobic pocket on the surface of the domain (C).

peptide of Hrb revealed a more complex pattern of interactions. Even though mutation of individual amino acids of the NPF motif of Hrb completely abolished binding to Eps15 *in vitro*, alanine scanning of amino acids surrounding the NPF motif revealed that positions −1, −2, and + 1 also contributed to the interaction.

The molecular specificity of EH binding was further investigated by Paoluzi *et al.* [14], who tested 11 different EH domains from yeast and mammalian proteins for binding to phage-displayed peptides. Not only did they confirm that the NPF motif is the core sequence of most EH-binding peptides, they also showed that individual EH domains prefer NPF motifs within specific contexts. These data demonstrated that the NPF motif is necessary, but not sufficient, for binding to the majority of the EH domains and suggested that surrounding amino acids are also required, possibly to modulate the fine specificity of the interactions. Furthermore, some EH domains, such as the C-terminal EH domain of Eps15 and the two N-terminal domains of the yeast YBL47c protein, recognize peptides characterized by the presence of aromatic and hydrophobic di- and tripeptides, such as FW, WW, or SWG. These ligands are designated as class II peptides, whereas NPF-containing peptides are referred to as class I ligands. A third class of ligands, H(S/T)F, was selected from the phage libraries with the EH domain of the yeast protein End3p. It is currently unknown if ligands other than NPF represent bona fide ligands or whether they represent mimotopes (i.e., short peptides with different primary but similar tertiary structures).

The NMR structure of the central EH domain of Eps15, complexed to a peptide derived from the C-terminal region of Hrb [10], has been obtained. The structure revealed that the NPF-containing peptides fit into a hydrophobic pocket formed by conserved residues in helices αB and αC of the domain (Fig. 1). Three conserved residues, Leu155, Leu165, and Trp169, form the bottom of the hydrophobic pocket and contact directly the NPF residues in the ligand, whereas other residues around the edge of the pocket likely contribute to the specificity of binding to peptide ligands (Fig. 1). The importance of these conserved residues within the EH domain has been experimentally proven. Mutation of some of these residues to Ala completely abolished binding, whereas conservative mutations had a less dramatic effect [10, 14]. The orientation of the peptide on the hydrophobic surface could not be defined due to the low affinity of the peptide for the domain. This problem might be overcome in the future by use of cyclic NPF-containing peptides, which display a higher affinity for EH domains than their linear counterparts [15], possibly by mimicking a turn conformation that is likely to be adopted by asparagine–proline dipeptides within proteins [16].

EH-CONTAINING PROTEINS

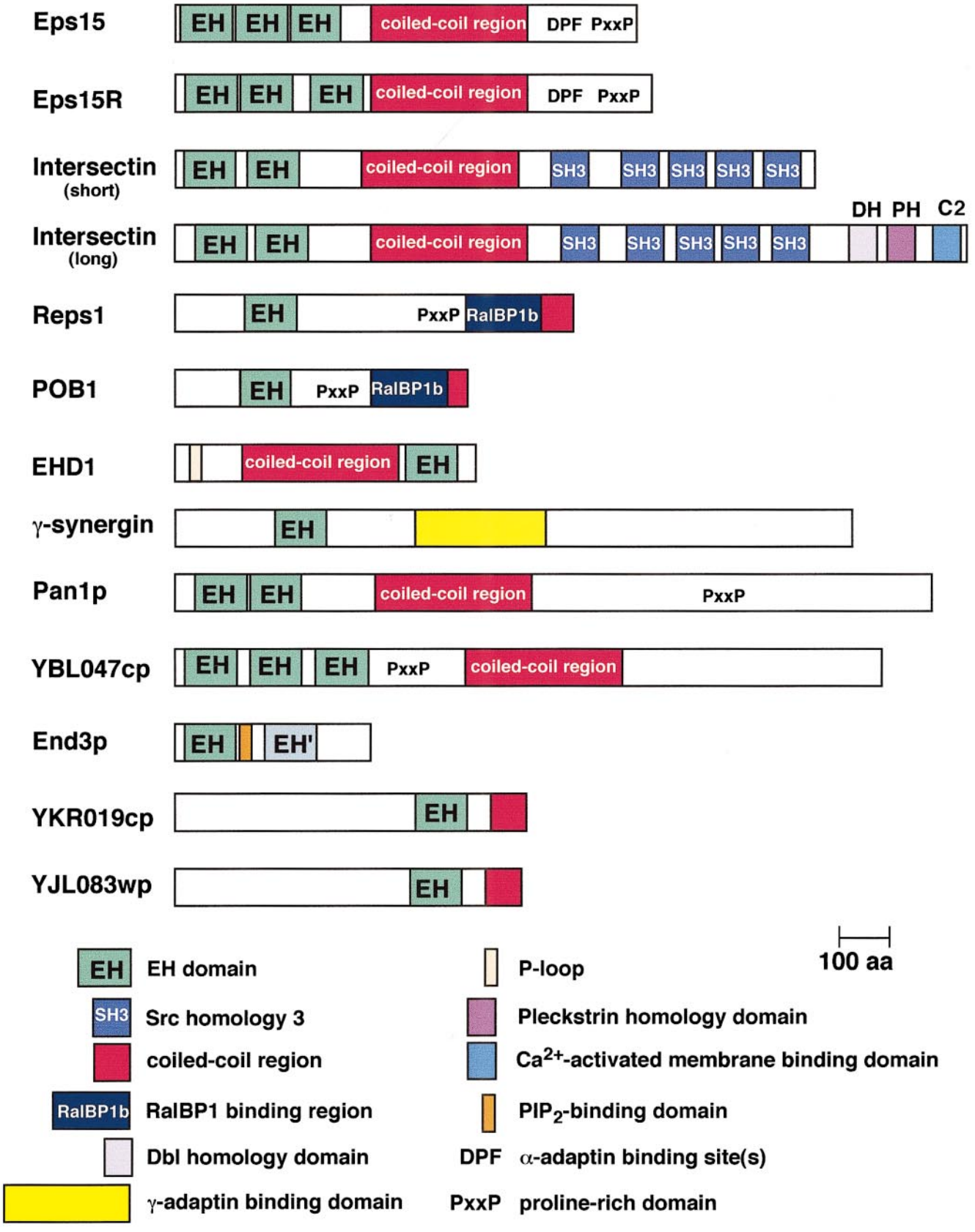
Eps15 and Eps15R

Eps15 cDNAs were originally cloned from mouse and human and encode for proteins of 897 and 896 amino acids, respectively. A different gene encodes for Eps15R (Eps15 Related), a highly related protein of 907 amino acids [7, 17]. A homologue of Eps15, named EHS-1 (Eps15 Homologue Sequence 1), is present in the nematode *Caenorhabditis elegans*, indicating evolutionary conservation. In each case, these proteins contain three copies of the EH domain in the NH_2-terminal region, a central coiled-coil region, and a COOH-terminal region, which is characterized by repeated DPF (aspartic acid–proline–phenylalanine) tripeptides (Fig. 2).

The central coiled-coil region of Eps15 is important for homodimerization of Eps15 and heterodimerization with other proteins, including Eps15R [17] and Intersectin [18]. Cross-linking and gel-filtration experiments have demonstrated that essentially all of the Eps15 in the cell is in macromolecular complexes [19]. Electron microscopy of rotary shadowed recombinant Eps15 molecules [20] has revealed Eps15 to be an elongated molecule with a globular head (corresponding to the EH domains), a "stalk" (corresponding to the coiled-coil region), and a "kink" (corresponding to the C-terminal region). Eps15 can form homodimers, where two molecules are arranged parallel to each other, as well as homotetramers, in which dimers are associated in an antiparallel fashion. The close apposition of the NH_2- and COOH-terminal regions of Eps15 in tetrameric complexes requires close contacts between the EH domains of one dimer and the C-terminal regions of the other dimer.

The C-terminal regions of Eps15 and Eps15R contain multiple copies of the DPF tripeptide that bind to α-adaptin, a subunit of the clathrin adaptor complex, AP-2 (Fig. 3). AP-2 plays a major role in clathrin-mediated endocytosis by recruiting several structural and regulatory protein components of the endocytic machinery to ligand-activated receptors in the plasma membrane [21–25] (Fig. 3). In the cell, most of the Eps15 or Eps15R molecules are complexed with AP-2; however, since AP-2 is more abundant than Eps15, more than 90% of the AP-2 molecules are not associated with Eps15 [26].

Mapping of the surfaces involved in the interaction has revealed that α-adaptin binds to Eps15 through one of its appendage domains, the so-called "α-ear" [27–29]. This region is believed to coordinate the spatial and temporal recruitment of several regulatory components of the endocytic machinery such as amphiphysin I, AP-180, auxillin, Epsins, Eps15 and Eps15R, and possibly dynamin I [25, 30–34]. The interaction surface on Eps15 has been mapped to amino


Eps15
EH
EH
EH
coiled-coil region
DPF PxxP
Eps15R
EH
EH
EH
coiled-coil region
DPF PxxP
Intersectin (short)
EH
EH
coiled-coil region
SH3
SH3
SH3
SH3
SH3
Intersectin (long)
DH PH C2
EH
EH
coiled-coil region
SH3
SH3
SH3
SH3
SH3
Reps1
EH
PxxP
RalBP1b
POB1
EH
PxxP
RalBP1b
EHD1
coiled-coil region
EH
γ-synergin
EH
Pan1p
EH
EH
coiled-coil region
PxxP
YBL047cp
EH
EH
EH
PxxP
coiled-coil region
End3p
EH
EH'
YKR019cp
EH
YJL083wp
EH
100 aa
EH EH domain
SH3 Src homology 3
coiled-coil region
RalBP1b RalBP1 binding region
Dbl homology domain
γ-adaptin binding domain
P-loop
Pleckstrin homology domain
Ca2+-activated membrane binding domain
PIP2-binding domain
DPF α-adaptin binding site(s)
PxxP proline-rich domain

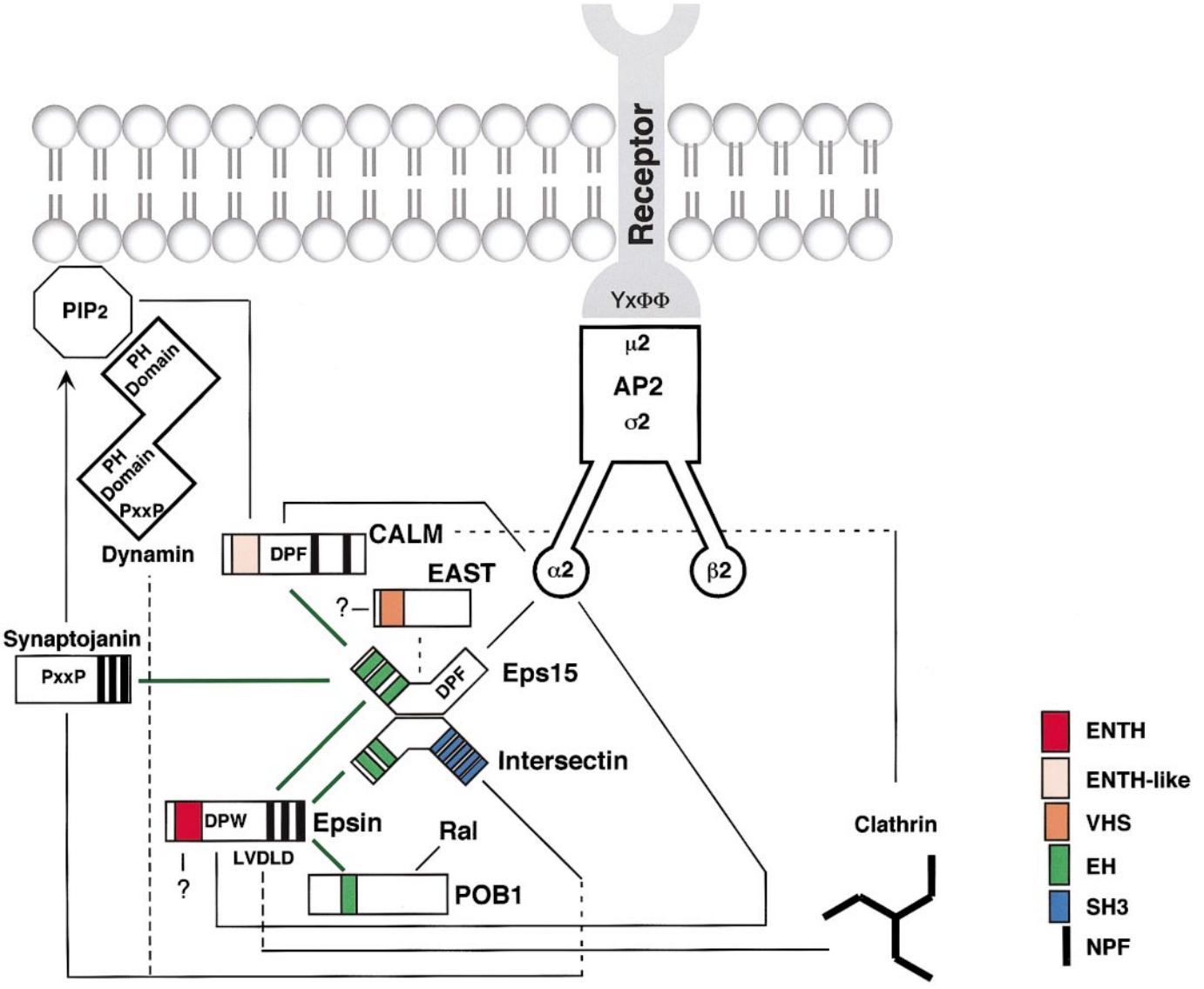


FIG. 3. Molecular interactions in endocytosis. The AP-2 complex (α2, β2, σ2, and μ2) is shown complexed with the sorting signal (YxΦΦ) of a transmembrane receptor. The AP-2 complex recruits a variety of proteins to the site of clathrin-coated pit formation. This diagram highlights the involvement of EH, SH3, ENTH, ENTH-like, and VHS domain-containing proteins in receptor-mediated endocytosis in mammals. Eps15 and Intersectin are shown heterodimerized, presumably through the formation of coiled-coils [18]. Molecular interactions between proteins involving EH domains (green) and their NPF tripeptide ligands (bars) are shown as green lines. In some cases (i.e., ENTH domain, VHS domain), the nature of the molecular interaction(s) is unknown.

FIG. 2. The architecture of EH domain-containing proteins. The EH domains are shown in green. The positions of SH3, DH, PH, and C2 domains are denoted in the proteins, along with potential coiled-coil regions. Potential ligand sites for the α-ear of AP-2 and SH3 domains are shown as DPF and PxxP, respectively. The yeast protein End3p contains a second region of similarity to the EH domain that is denoted as EH′. The proteins are drawn to scale with the bar corresponding to 100 amino acids. A color-coded legend is also provided. Other EH-containing proteins, identified so far in different organisms, include (in parentheses are the GenBank accession numbers) in *Homo Sapiens*, HPAST (AF001434.1) and its rat and *D. melanogaster* homologues MGEPS (AF081251.1) and PAST-1 (U70135.1); in *C. elegans*, the Eps15 orthologue EHS-1 (U29244.1) and the predicted proteins of genes Y116A8C.36 (AL117204.1) and R10E11.6 (P34550); in *S. pombe*, the predicted products of genes SPA1687.09 (AL035064.1), SPAC27F1.01c (Z69368.1), and SPBC83.01 (AL035536.1); in *P. falciparum*, sarcalumenin (U84395.1); in *Emericella nodulans*, SAGA (U28804.1); and in *Arabidopsis thaliana*, the product of gene F8K7.4 (AC007727.2).

acids 623–750 of mouse Eps15 [28, 29]. This region of Eps15 contains several copies of the DPF motif, which has been recently shown to be a target sequence for the appendage domain of α-adaptin *in vitro* [25, 34]. Thus, binding of Eps15 and a number of other proteins to α-ear is likely to be mediated through their DPF repeats. However, as indicated by mapping studies [28, 29], other sequences in the C-termini of Eps15 and Eps15R likely influence binding.

The C-terminal region of Eps15 and Eps15R also contains a proline-rich segment that is a potential target for SH3 domain-containing proteins. Indeed, the SH3 domains of Crk bind to this proline-rich region, both in Eps15 and in Eps15R [35]. The functional meaning of this interaction, which has only been demonstrated *in vitro,* remains to be elucidated.

Eps15 is a cytosolic protein, as assessed by fractionation studies [5]. By indirect immunofluorescence (IF), Eps15 and Eps15R can be found in punctate structures scattered throughout the cytoplasm and concentrated in the perinuclear region [17, 26, 36]. A small amount of Eps15R has also been detected in the nucleus, by both biochemical fractionation and IF experiments [17]. Co-immunoprecipitation experiments have revealed that Eps15 is associated with the protein components of coated pits and vesicles, including, not surprisingly, AP-2 [26–29, 36]. While these observations originally supported the hypothesis that Eps15 is a component of the endocytic machinery, it was yet observed that Eps15 (and Eps15R) localization did not change appreciably following EGF-stimulated receptor internalization [26, 36]. More recently, however, by surface-labeling cells with EGF at 4°C, then inducing endocytosis at 37°C, it has been possible to demonstrate a dramatic relocalization of Eps15 to the plasma membrane [37].

Additional support for a role of Eps15 in endocytosis has come from ultrastructural analysis in which Eps15 has been localized to several organelles of the endocytic route, including coated pits, vesicles, and endosomes [26, 37]. Eps15 is restricted to the rim of the budding coated pit, which is the growing part of a forming pit, whereas it is absent from the deeper part of the invaginated pit [26]. This observation was not immediately reconcilable with its interaction with AP-2, which is present on the entire profile of a coated pit. Elegant *in vitro* studies by Kirchhausen's group, however, have demonstrated that the interaction between Eps15 and AP-2 is disrupted when clathrin binds to the latter [38]. In an AP-2-dependent *in vitro* coat assembly assay, the polymerization of clathrin only occurred with the exclusion of Eps15. Thus, during assembly of the coat *in vivo,* the only site where Eps15 remains associated with AP-2 is the growing part of the pit, the rim, where no clathrin polymerization has taken place yet. Accordingly, it was demonstrated that while the cytosolic pool of Eps15 is associated with AP-2 with an almost 1:1 stoichiometry, the two molecules co-immunoprecipitate much less efficiently from the plasma membrane-associated fraction [26].

Eps15 appears to be almost totally absent from clathrin-coated vesicles as analyzed by electron microscopy [37] or by biochemical characterization of vesicles isolated from brain [38]. Its association with later organelles of the endocytic route, i.e., uncoated vesicles and endosomes, is however readily detectable [37]. Presumably Eps15 is retargeted to endocytic organelles, once clathrin has been shed from the vesicles, suggesting multiple functions for this molecule at different steps of the endocytic route.

A formal demonstration of the functional involvement of Eps15 and Eps15R in endocytosis came from studies in which Eps15 function was perturbed by either overexpression/microinjection of truncated forms of Eps15 or microinjection of anti-Eps15 antibodies [39, 40]. Introduction of the C-terminal region of Eps15, encompassing the AP-2 binding site, acts as a dominant negative in blocking the endocytosis of EGF, transferrin, or Sindbis virus. The inhibition of EGF and transferrin uptake, observed upon microinjection of neutralizing antibodies, demonstrates that the effect was not merely due to titration of the AP-2 adaptor, but rather indicates that Eps15 is actively required for the endocytic process [39]. Data obtained with the *C. elegans* homologue of Eps15 support a similar role in lower eukaryotes. Depletion of EHS-1 results in a temperature-sensitive uncoordinated phenotype in nematodes (A. E. Salcini and P. P. Di Fiore, unpublished) similar to that observed for dynamin mutants, a GTPase involved in the fission of coated vesicles from the plasma membrane into the cytoplasm [41].

Eps15 and Eps15R undergo multiple posttranslational modifications, including monoubiquitination [42] and tyrosine phosphorylation [5, 17], that are triggered by EGFR activation. *In vitro,* Eps15 is a substrate for the EGFR kinase, supporting the notion that it might be directly phosphorylated by activated EGFR. However, the functional meaning of tyrosine phosphorylation of Eps15 remains unknown. It is less clear how activation of the EGFR might stimulate monoubiquitination of Eps15. In addition, although several studies point to a function of ubiquitination in the internalization process [43–45], the role for this modification in endocytosis is still unresolved.

Other evidence supports a role for Eps15 serine–threonine phosphorylation in synaptic vesicle recycling, a process whose molecular machinery largely overlaps with that of "classic" endocytosis. Eps15 is highly expressed in nerve terminals where it is subject to constitutive serine–threonine phosphorylation. Interestingly, upon stimulation of nerve terminals, Eps15 undergoes rapid dephosphorylation [46] and

shows an increased affinity for AP-2. These biochemical events are a general feature of endocytic proteins in nerve terminals [47], as calcium-stimulated dephosphorylation favors the endocytosis of synaptic vesicle membranes after a stimulation-dependent burst of exocytosis. Moreover, Eps15 undergoes phosphorylation during mitosis [46], an event that might contribute to the well-known block of clathrin-mediated endocytosis during this phase of the cell cycle. All these data clearly identify Eps15 as a crucial molecule for the clathrin-mediated endocytic process, both in presynaptic nerve terminals and in nonneuronal tissues.

In addition to the established role for Eps15 and Eps15R in endocytosis, some other observations indicate additional functions of these proteins in mammalian cells. Human Eps15 has been mapped to chromosome 1p31-p32, a region displaying nonrandom chromosomal abnormalities, including deletions in neuroblastoma and translocations in acute lymphoblastic and myeloid leukemias [6]. In two translocations t(1;11) (p32;q11), described in rare cases of myeloid leukemias, the Eps15 gene was found fused to the MLL (also known as HRX/ALL-1) gene [48], resulting in MLL mislocalization and possible alteration of its function [49]. This finding, together with the ability of overexpressed Eps15 to transform NIH3T3 cells, albeit with low efficiency [5], indicates that subversion of Eps15 function might lead to alterations in cell proliferation.

Intersectins

Intersectins comprise another family of EH-containing proteins (Fig. 2). Intersectin was originally identified in a screening of a *Xenopus laevis* oocyte expression library, with SH3 peptide ligands as probes [15]. Frog Intersectin, as well as the two murine homologues, known as Ese1 and Ese2 [18], and the two rat homologues, known as EHSH1 and EHSH2 [50], contain two EH domains in the N-terminal region, a central coiled-coil domain, and five C-terminal SH3 domains. A human form of Intersectin (ITSN) has also been identified through genomic analysis of chromosome 21 [51]. The C-terminal regions of the mammalian Intersectins differ due to alternative splicing. Alternative splicing in the stop codon of all these genes gives rise to extended isoforms containing Dbl homology (DH), Pleckstrin homology (PH), and C2 domains [18, 50–52] (Fig. 2). The long form is predominantly expressed in neurons, whereas the short form is expressed in glial and other nonneuronal cells [18, 52].

Intersectin colocalizes with clathrin at the plasma membrane in both cultured hippocampal neurons and COS-7 cells [15, 52]. Intersectin has also been shown to interact with a variety of endocytic proteins (Fig. 5). It binds to dynamin and synaptojanin through its SH3 domains [15, 18] and to epsin (see below) through its EH domains and is constitutively associated to Eps15 and Eps15R via its coiled-coil region [18]. Moreover, the central region of rat Intersectin interacts with SNAP-25, a membrane protein involved in exocytosis of synaptic vesicles [50]. The *Drosophila* homologues of Intersectins, Dap-1 and Dap-2 (for Dynamin-Associated Protein), contain four, instead of five, SH3 domains, bind to dynamin and synaptojanin as well, and colocalize with dynamin at sites of synaptic vesicle recycling at nerve terminals [53].

Because of their interactions with multiple protein components of the endocytic machinery, Intersectins were originally believed to take part in the endocytic process. Experimental validation of this hypothesis came from overexpression of mouse Intersectin in cultured cells, where it blocked clathrin-mediated endocytosis, likely through perturbation of the many proteins that may assemble onto scaffold created by the Intersectin/Eps15 heterodimer [18]. In particular, in an *in vitro* reconstitution assay Intersectin SH3 domains have been shown to act early in clathrin-coated vesicle formation [175].

In addition to Intersectin's general function in endocytosis, the long isoforms of Intersectin might be expected to have specialized functions. DH domains act as guanine nucleotide exchange factors (GEFs) on Rho-like GTPases and regulate both the actin cytoskeleton and several signal transduction pathways; PH domains mediate the interaction with inositol phospholipids, and C2 domains mediate calcium-dependent phospholipid binding. The roles of the long isoforms of Intersectins, which are likely to be exerted in neuronal cells where they are expressed, remain to be explored.

Reps1/POB1

Two other EH domain-containing proteins were recently identified, based on their interaction with RalBP1/RLIP/RLP [54–56], a GTPase-activating protein for CDC42 and Rac that acts downstream of Ras. The two proteins were designated Reps1, for RalBP1-associated Eps-homology protein, and POB1, for Partner of RalBP1 [57, 58] (Fig. 2). Reps and POB1 were cloned from mouse and human, respectively. They display around 40% amino acid similarity and most likely represent two distinct genes, isolated in different species, rather the murine and human orthologues of the same gene. The two proteins have, however, remarkable colinear topology, displaying, from the NH_2 to the COOH-terminus, one EH domain, two proline-rich regions, a RalBP1-interacting domain, and a putative coiled-coil region (Fig. 2). Both Reps1 and POB1 are tyrosine phosphorylated in response to EGF stimulation. POB1 interaction with the EGF receptor is most likely mediated by an adaptor, such as Grb2; interac-

tions with the SH3 domains of Grb2 and Crk (this latter one only in the case of Reps1) have been demonstrated [57, 58]. Finally, the solution structure of the EH domain of POB1 has been solved by nuclear magnetic resonance spectroscopy [12].

To explore what proteins may interact with the EH domain of POB1, potential binding partners were purified from bovine brain membrane extracts on a GST–POB1-EH column (Fig. 3). Two interacting proteins were identified, the NPF-containing protein Epsin and, surprisingly, Eps15R [59, 60]. To establish whether POB1 was interacting with Eps15 or Eps15R directly or indirectly, filter overlay assays were performed with bacterially purified proteins. The finding that the EH domain of POB1 interacts directly with the C-terminal region of Eps15, which does not contain NPF motifs [59], supports the observation that EH domains may recognize motifs other than NPF. Further analysis will clarify whether the EH domain of Reps1/POB1 binds to one of the class II peptides identified by Paoluzi *et al.* [14] or to a new motif.

In keeping with a general function for the EH network in endocytosis, overexpression of POB1 deletion mutants has been shown to inhibit internalization of EGF and insulin. In particular, both a mutant containing only the EH domain of POB1 and a deletion mutant lacking the EH domain acted as dominant negatives on ligand-stimulated endocytosis, although constitutive transferrin uptake was not impaired by these mutants [59]. POB1 may function to transmit signals from activated receptors to the endocytic machinery, possibly through Eps15, Epsin, and the AP-2–clathrin complex, thereby regulating EGF- or insulin-dependent endocytosis. If this turns out to be true, it is temping to speculate that such complex formation might be regulated in a phosphorylation-dependent manner.

Other Mammalian EH-Containing Proteins

Another protein that has been isolated from mammals, which contains a copy of the EH domain, is EHD1 (EH Domain-containing protein 1) (Fig. 2). It is conserved in mammals, *Drosophila,* and nematodes [61]. The predicted protein sequences contain one EH domain at the COOH-terminus, a central coiled-coil structure, and a nucleotide binding consensus site at the NH_2-terminus. In cultured cells, EHD1 is localized to transferrin-containing endocytic vesicles [61]. Thus, its predicted protein structure, as well as its subcellular localization, points to a possible role for EHD1 in receptor-mediated endocytosis, although further details are required to establish the molecular interactions linking this protein to the endocytic machinery.

In addition to proteins involved in the internalization process, another EH-containing protein was found associated with the γ subunit of the adaptor complex AP-1, extending the EH-mediated network to events associated to budding from the Golgi compartment. This protein, named γ-synergin (Fig. 2), binds directly to the ear domain of γ-adaptin, but not to α-adaptin, and is found associated to AP-1 in the TGN and in the cytosolic fraction; in addition, it is enriched in clathrin-coated vesicles. A chimeric construct, in which the ear appendage of γ-adaptin is substituted for the homologous domain of α-adaptin, targets to the plasma membrane. Upon overexpression of this construct, γ-synergin is also rerouted to the membrane, suggesting that it follows AP-1 onto membranes, rather than directing it there. The role and the function(s) of γ-synergin remain to be elucidated [62].

EH Domain-Containing Proteins in Yeast

As the entire nucleotide sequence of *Saccharomyces cerevisiae* is currently available, computer analysis has revealed the existence of five EH domain-containing proteins, End3p, Pan1p, YBL047cp (Pan1p-like), YJL083wp, and YKR019cp [8, 14, 63] (Fig. 2). Pan1p and YBL047cp contain two and three NH_2-terminal EH domains, respectively. End3p contains a single bona fide EH domain in its NH_2 terminus and a second region of limited similarity (EH-like) in its central portion (Fig. 2). YJL083wp and YKR019cp are homologous proteins of unknown function, with a single C-terminal EH domain.

End3p was cloned by complementation of a temperature-sensitive mutant, *end3,* defective in the internalization step of the α-factor endocytosis [64, 65]. End3p was subsequently shown to be required for the internalization of several other plasma membrane proteins, such as Ste6p [66] and the uracil and inositol permeases [67, 68]. In addition, End3p is required for the correct organization of the actin cytoskeleton [65]. In addition to its EH and EH-like domains, End3p contains a consensus sequence for the binding of phosphatidylinositol 4,5-biphosphate (PIP_2) in the central region and two short repeats in the C-terminal region. Structure–function studies have demonstrated that the EH domain and the repeated regions in the C-terminus are required for protein function.

As with End3p, Pan1p is required for both the endocytic process and the normal organization of the cortical actin cytoskeleton. Mutants of the PAN1 gene exhibit defects in both fluid-phase and receptor-mediated endocytosis [69] and accumulate vesicles and tubulovesicular structures as well as plasma membrane invaginations [63]. Moreover, loss of activity or overproduction of Pan1p results in abnormal distribution of the actin cytoskeleton at the cell cortex and *pan1* mutants exhibit abnormal bud growth and severe defects in cytokinesis. In immunofluorescence studies, Pan1p was found localized with cortical actin patches [70]. At

the structural level, Pan1p has several similarities with Eps15; it contains two EH domains in the N-terminal region, a central region with a putative coiled-coil structure, and a proline-rich region at the C-terminus. Similarly to Eps15, Pan1p fractionates, in gel-filtration experiments, in a range consistent with high-molecular-weight complexes, possibly through coiled-coil-mediated interactions, either with itself or with other coiled-coil-containing proteins [71]. It is not known whether Pan1p represents a true yeast orthologue of Eps15. However, it is worth mentioning that another yeast protein, YBL047cp, displays a higher degree of amino acid sequence similarity and conserved topology with Eps15 than Pan1p.

End3p and Pan1p associate with each other and act as a complex *in vivo* [69]. Several experiments support the biological relevance of this interaction. Not only do both the *end3* and *pan1* mutants display similar phenotypes, but, in the *end3* mutant, Pan1p localization is altered, resulting in a diffuse cytoplasmic staining. This observation suggests that End3p might be required either for the targeting or the stabilization of Pan1p to the cortical actin cytoskeleton.

The finding that Pan1p-stained membrane structures are not always associated with actin patches argues against the hypothesis that the End3p/Pan1p complex acts on endocytosis as a consequence of its role on the actin cytoskeleton [63, 69]. Definitive proof that it is indeed the case was obtained when the association between Pan1p and several endocytic proteins was reported. The EH domains of Pan1p were found to interact directly with yAP180A and yAP180B, the two yeast homologues of AP180, a mammalian clathrin assembly protein that exhibits *in vitro* clathrin cage assembly activity (see below). Furthermore, a genetic interaction was demonstrated between Pan1p and Sjl1, one of the three yeast homologues of mammalian synaptojanin, also implicated in endocytosis ([71], see also below). Finally, a genetic interaction was identified between Pan1p and Rsp5, a ubiquitin–protein ligase required for endocytosis [72]. One of Pan1p's functions might be to bring Rsp5p in proximity to the cytosolic tails of certain plasma membrane proteins that are destined for ubiquitination and endocytosis. Alternatively, Pan1p might be ubiquitinated by Rsp5p, just as Eps15 is ubiquitinated in mammalian cells, upon ligand-induced endocytosis of receptors. These results demonstrate that the Pan1p/End3p complex is a multivalent adaptor that coordinates interactions between proteins required for the internalization process and actin cytoskeleton organization.

In *S. cerevisiae* the sequence NPFXD has been observed to act as an endocytic signal [73]. This motif occurs in the cytoplasmic tails of the Kex2p membrane protein and the α-factor receptor Ste3p. Internalization of Kex2p is abolished when the N, P, or F residues are converted to alanine, and mutations in this sequence prevent pheromone-stimulated internalization of Ste3p. Moreover, a NPFSD motif was sufficient to restore the uptake when added to the C-terminus of an endocytosis-defective Ste2p mutant. Given the overlap of the NPF and the NPFXD motifs, it is intriguing to speculate that endocytosis of these and other yeast receptors harboring NPFXD motifs is mediated by the Pan1p/End3p complex or other yeast EH domain-containing proteins.

EH-BINDING PROTEINS

In searching for cellular ligands that interact with EH domain-containing proteins, a number of approaches have been utilized, including screening of cDNA expression libraries, affinity purification, and noting the presence of multiple NPF motifs in the primary structures of certain proteins. These efforts have led to the identification of several interacting proteins that help define the functions and the relevance of the EH-mediated network of interactions within the cell.

Numb and Numbl

By direct screening of an expression library with the EH domains of Eps15, several putative interactors were isolated [13]. Among these were Numb, a mammalian homologue of the developmentally regulated Numb gene of *Drosophila*, and Numbl (also known as Nbl or Numb-R), a related protein [13, 74–78]. Mammalian Numb and Numbl as well as *Drosophila* Numb (dNumb) contain a phosphotyrosine-binding (PTB) domain in their NH_2-termini and NPF motifs (Fig. 4). *Drosophila* Numb is a membrane-associated protein that determines cell fate in several lineages during both nervous and muscle system development [75, 79, 80]. In all these lineages the Numb protein undergoes asymmetric partitioning into one of the sibling cells during mitosis of the precursor [75, 81–83]. The biological function of Numb is to negatively regulate the Notch signaling pathway [77, 84–88]; Notch is a conserved transmembrane receptor that plays a key role in cell fate establishment in a variety of tissues and organisms through local cell interactions (reviewed in [89–91]). Numb physically interacts with the cytosolic tail of the Notch receptor via its PTB domain [77, 86, 88].

There are several lines of evidence that suggest that mammalian Numb (mNumb) may play a role in mammalian development similar to that of the *Drosophila* protein. First, ectopically expressed mNumb is asymmetrically partitioned in dividing *Drosophila* progenitor cells and can rescue the dNumb mutant phenotype [76, 77]. Second, in the mouse embryo, Numb is ex-

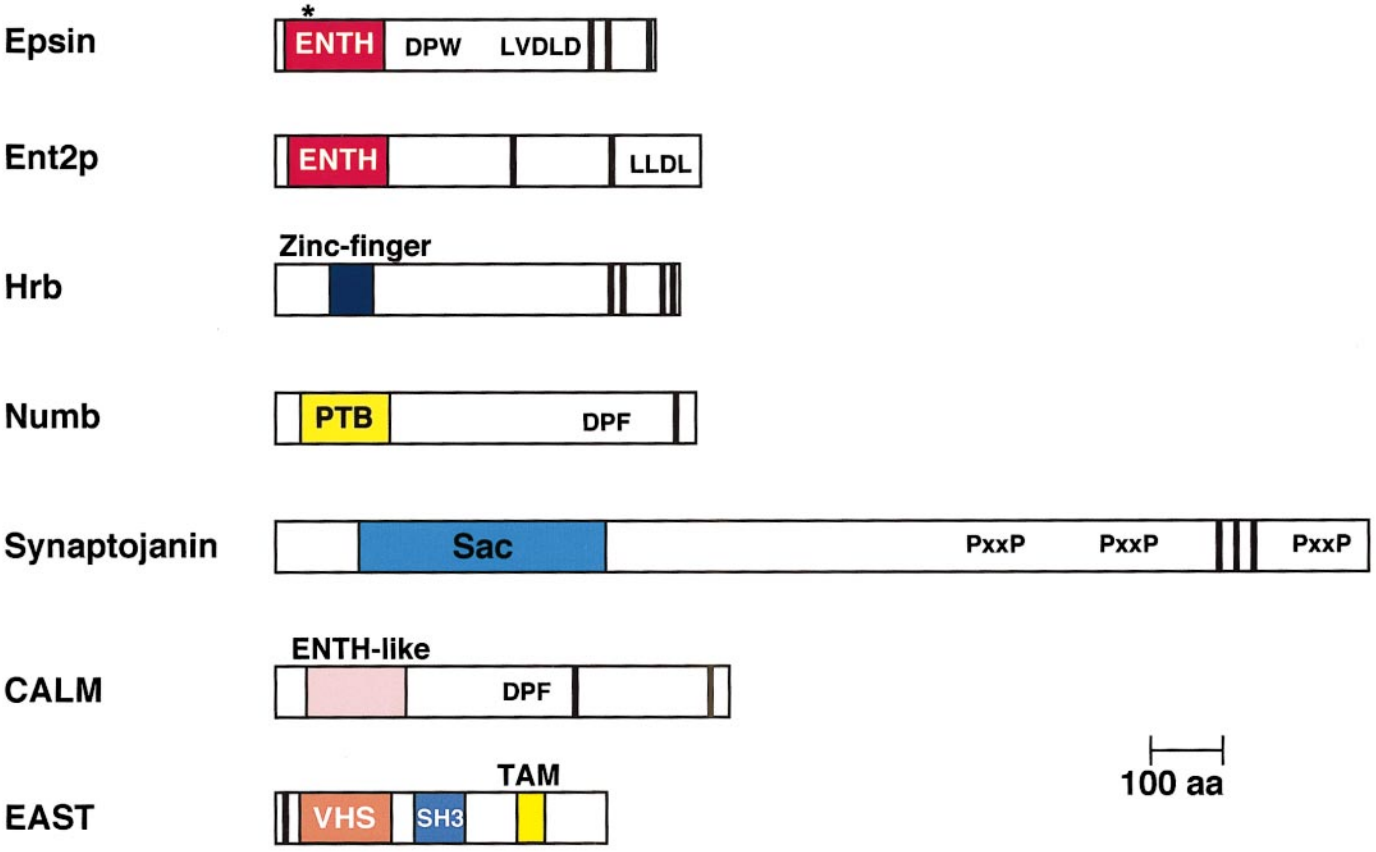


FIG. 4. The architecture of several cellular ligands of the EH domain-containing proteins. The relative locations of the tripeptide motif, NPF, are shown in the proteins with black bars. The locations of potential ligand sites for the α-ear of AP-2, SH3 domains, and heavy chain of clathrin are shown as DPF/W, PxxP, and LVDLD and LLDL-COOH, respectively. One DPW tripeptide is denoted in the ENTH domain with an asterisk. CALM contains an ENTH-like domain at its N-terminus [110]. EAST contains a tyrosine-based activation motif (TAM [143]). The positions of other domains, such as Sac1, PTB, and Zn finger domains, are also indicated. The proteins are drawn to scale with the bar corresponding to 100 amino acids.

pressed in the ventricular zone of the developing brain, where dividing neuroblasts are located [76, 77], and the ectopic expression of some isoforms of Numb protein in multipotent cell lines biases precursors toward the neuronal phenotype [76, 92]. Finally, mammalian Numb has been shown to interact with the corresponding Notch homologues [77].

In spite of extensive genetic studies, little is known about the biochemical function of Numb. The molecular mechanism(s) underlying the antagonistic role of Numb on Notch signaling is likewise obscure. The participation of Numb in the EH network, however, provides a working hypothesis to investigate a potential role of Numb in endocytosis. In this regard, it is noted that Numbl and Numb contain one and two copies of the DPF motif, respectively, which is a known ligand for the appendage domain of α-adaptin [34]. We have also demonstrated that Numb directly binds to the α-ear of AP-2 (E. Santolini and P. P. Di Fiore, unpublished). It will be of interest to determine whether Numb is part of the endocytic machinery and whether this putative function is linked to its role in development.

Hrb and Hrbl

The Rev-associated binding/Rev-interacting protein Hrb (also known as RAB or Rip) [93, 94] and the related Hrbl (also known as RAB-R) [13] were isolated from the screening of both a human expression library with the EH domains of Eps15 [13] and a mouse expression library with the EH domains of frog Intersectin [15]. Both Hrb and Hrbl display a zinc-finger region in their NH_2-termini and contain several FG (phenylalanine–glycine) repeats, which are characteristic, although not predictive, of nucleoporins. The two proteins also contain four NPF motifs, located toward the COOH-terminus of the proteins (Fig. 4).

In both human and mouse Hrb, deletion or mutation of the four NPFs completely abolished binding to the

EH-interacting proteins, whereas fusion of the NPF-containing region to GST resulted in strong binding to the EH domains ([15], M. Doria and P. P. Di Fiore, unpublished). Moreover when a GST-fusion protein containing the last NPF motif of Hrb was tested, Yamabhai *et al.* [15] found that binding to Intersectin was progressively impaired upon introduction of one or more residues at the C-terminus of the fusion protein. This result underscores the contribution of residues flanking the NPF motif in binding to certain EH domains.

Hrb is a cellular cofactor of the HIV-1 Rev protein. Rev shuttles between the nucleus and the cytoplasm of an infected cell, directing cytoplasmic export of specific unspliced and partially spliced transcripts of the viral genome that carry Rev-binding nucleotide sequences, termed the Rev response element (RRE). Rev is believed to function by adapting the viral machinery to a cellular pathway that normally exports endogenous RNAs, such as the 5S rRNA and the U1 snRNA, as well as several proteins which, like Rev, contain a nuclear export signal (NES) ([95–98], see [99] for a review). This cellular pathway is now operationally referred to as the Rev export pathway.

Initial efforts to identify cellular cofactors mediating Rev export, using yeast two-hybrid screens with the Rev NES as bait, led to the isolation of Hrb and of the distantly related yeast Rip1p protein [100]. Due to the presence of FG repeats in Hrb and Rip1p, the hypothesis was put forward that they might function as nucleoporins, with Rev recruiting RRE-containing RNAs to the nuclear pore, through the interaction with Hrb or other nucleoporins.

This simple model has been recently challenged, based on a number of observations. First, the interaction between Rev and Hrb is not direct [97, 101, 102]. Crm1, a shuttling protein that belongs to the transport receptor family, serves as a bridging factor between Hrb and Rev [103–107]. Second, the FG-containing region of Rip1p was shown to make only limited contributions to Rev-mediated export [108]. Finally, there is uncertainty as to whether Hrb is an authentic constituent of the nuclear pore complex [93, 94]. Thus, while overexpression of Hrb modestly enhances Rev activity, suggesting a role for Hrb in the Rev export pathway, the exact nature of this role remains to be determined.

The finding that Hrb and Hrbl are components of the EH network provides new insights in dissecting the function of Hrb in nucleocytosolic shuttling. In this regard, it was recently shown, in one of our laboratories, that perturbation of the Eps15–Hrb interaction alters Rev function (M. Doria and P. P. Di Fiore, unpublished). While the molecular mechanisms of this effect remain to be defined, these results focus the attention on an unexpected convergence of molecular machinery involved in endocytosis with that of nucleocytoplasmic transport.

Epsins

Epsin (for Eps15-interacting protein, now called Epsin-1) was originally isolated in a search for nerve-terminal binding partners of the EH domains of Eps15 and Eps15R [33]. There are now two known related genes defining the Epsin family, Epsin-1 and Epsin-2 (also identified as Ibp1 and Ibp2, standing for Intersectin binding proteins 1 and 2). They were independently isolated in several labs, based on their ability to interact with the EH domains of Eps15 and Eps15R, Intersectins, and POB1 ([15, 18, 33, 59], Rosenthal *et al.*, submitted). A frog homologue of Epsin, MP90, is also known [109]. The overall structure of Epsins is shown in Fig. 4.

The N-terminal region of Epsins contains a 140-amino-acid domain that is highly conserved among Epsins of different organisms and is present in proteins otherwise very divergent from Epsins. This new protein module has been named ENTH for Epsin N-terminal homology domain ([110], see also below). The central region, downstream of the ENTH domain, is characterized by several aspartate–proline–tryptophan (DPW) motifs that are required for interaction with the AP-2 adaptor [34] (Fig. 3). The C-terminal region of Epsin-1 and Epsin-2 contains three NPF motifs that account for the association with Eps15 [33, 111] (Fig. 3). Indeed, their association with the EH domain-containing proteins Eps15, Intersectin, and POB1 has been confirmed both *in vitro* and *in vivo* [18, 33, 52, 60]. Furthermore, Epsin-2 can interact, at least *in vitro*, with the N-terminus of the clathrin heavy chain via a putative clathrin-binding site located in the C-terminal region of the protein [15, 52] (Figs. 3 and 5). These data suggest that Epsins might take part in the internalization process, either through the formation of a multimeric protein complex or, alternatively, through sequential interaction with a variety of structural and regulatory endocytic proteins. As with Eps15, Eps15R, and Intersectin, Epsin-1 is not a major intrinsic component of the coat, but rather may participate as an "accessory" protein in the dynamic rearrangements responsible for the assembly, invagination, and fission of the clathrin-coated vesicles.

Epsin-1 is concentrated together with Eps15 in presynaptic nerve terminals where it colocalizes with clathrin-coated vesicles; a similar pattern can also be observed in cultured cells [33]. It was shown that, like Eps15, Epsin undergoes depolarization-dependent dephosphorylation in nerve terminals and that dephosphorylation enhances the association with AP-2 in brain extracts [46]. In addition, the *Xenopus* homologue MP90 was originally isolated as a mitotic phos-

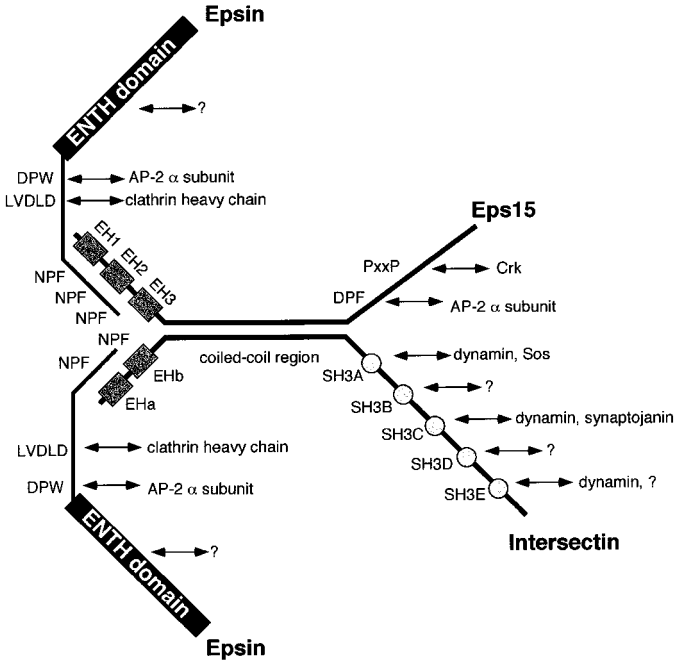


FIG. 5. Protein–protein interactions on the scaffold generated by the Eps15/Intersectin heterodimer. The two proteins have been demonstrated to form a heterodimer in cells [18], presumably through the association of their central coiled-coil regions; the proteins are shown in a parallel association, although this has not been rigorously tested. Various protein–protein interactions, which have been mapped on the scaffold, are denoted with arrows.

phoprotein [109]. A cdc2 phosphorylation site is conserved in both MP90 and Epsin-1. Consistent with these observations, Epsin has been shown to be a mitotic phosphoprotein [46]; its phosphorylation might be a mechanism by which endocytosis is inhibited at mitosis.

When Epsin function was disrupted by either overexpression of the region of the protein rich in the DPW motifs or microinjection of neutralizing antibodies, clathrin-mediated endocytosis was blocked, demonstrating that it participates in clathrin-dependent internalization processes, including endocytosis at the synapse [33].

Two yeast proteins, Ent1p and Ent2p (corresponding to the open reading frames YDL161w and YLR206w, respectively), have also been recently described, with a high degree of similarity to mammalian Epsins [176]. Ent1p and Ent2p contain an ENTH domain at their NH_2-termini along with multiple NPF motifs, which mediate their interaction with the EH domains of Pan1p. Ent1p and Ent2p are likely to represent functional yeast homologues of Epsins, despite the lack of homology in their central and COOH-terminal regions and, most notably, the absence of a DPF/W region [176]. Disruption of either gene in *S. cerevisiae* does not result in any obvious phenotype; however, double mutants are nonviable, thus indicating redundancy in their function. Analysis of temperature-sensitive alleles of the *ENT1* gene has demonstrated that Ent1p, and in particular its ENTH domain, is required for proper endocytosis and actin cytoskeleton organization. Finally, Ent1p has been shown to bind to clathrin, through its C-terminal eight amino acids (RGYTLIDL) [176].

Synaptojanins

Another cellular ligand for an EH domain-containing protein is synaptojanin, which has been shown to bind to Eps15 [112] (Fig. 4). There are two known mammalian synaptojanins, synaptojanins 1 and 2 [113–115]. Synaptojanins have a tripartite structure: the NH_2-terminus contains a region of homology to yeast Sac1p, a protein implicated in phospholipid metabolism and actin cytoskeleton organization, the central region contains a catalytic inositol 5-phosphatase domain and the C-terminal domain which is variable in length due to RNA splicing (Fig. 4). Alternative splicing of an exon containing a stop codon generates protein isoforms of different length [115, 116].

In the central region of synaptojanin is the inositol 5-phosphatase catalytic domain that dephosphorylates inositol polyphosphates and inositol phospholipids at the 5′-position of the inositol ring. In agreement with the multiple functions of inositol metabolites, synaptojanins have been implicated in a variety of cellular processes such as intracellular signaling, cytoskeletal function, and vesicular trafficking (reviewed in [117, 118]). Consistent with a crucial function in actin cytoskeleton organization, synaptojanin overexpression leads to a massive rearrangement of actin filaments and to the formation of multinuclear cells [119].

Within the C-terminal region of synaptojanins are a number of proline-rich sequences that are ligand sites for the SH3 domains of Grb2 [119, 120], amphiphysin I and II [31, 113, 121], the SH3p4/SH3p8/SH3p13 (endophilin) family of proteins [122, 123], and syndapin I [124] (Fig. 3). Furthermore, within the C-terminal region of the long isoform of synaptojanin 1 are three NPF motifs that are responsible for the interaction with Eps15 (Fig. 3). The association of synaptojanins with a variety of endocytic proteins indeed supports a general role for the synaptojanin family members in endocytosis. Consistent with this hypothesis, the short isoforms of synaptojanin 1 are highly enriched in brain and are concentrated at clathrin-coated endocytic intermediates in nerve terminals [120]. Interestingly, the C-terminal regions of synaptojanins are subject to developmentally regulated and tissue-specific alternative splicing events and differ in their SH3 ligand specificities, subcellular localization, and tissue distribu-

tion. Thus, one additional function of this region may be to target specific synaptojanin family members to distinct subcellular sites [115, 116].

Three synaptojanin-like genes are found in *S. cerevisiae*, SJL1, SJL2, and SJL3 [125], also known as INP51, INP52, and INP53 [126], respectively. None of these genes is essential for growth; however, different combinations of double mutants for these genes exhibit defects in receptor-mediated and fluid-phase endocytosis and a triple deletion strain is inviable [126, 127]. In addition, the severity of the endocytic impairment correlates well with the severity of actin and polarity defects. Moreover, as already mentioned, genetic interference has been reported between the EH domain-containing proteins Pan1p and Sjl1p [71]. These observations, besides supporting a direct function in endocytosis, provide further evidence for multiple roles for synaptojanins in endocytosis and organization of the actin cytoskeleton.

AP180s

A yeast two-hybrid screen performed with the EH domains of Pan1p identified yAP180A and yAP180B [71], the two yeast homologues of mammalian AP180, a clathrin assembly protein [128–131], as interacting proteins. Both yAP180A and yAP180B bind to Pan1p through NPF sequences repeated five times in the C-terminal half of these proteins. Both proteins also bind to clathrin, share putative inositol polyphosphate- and polyphosphoinositide-binding sites with mammalian AP180, and localize with Pan1p to punctate spots at the cell periphery, where several other endocytic proteins are concentrated [71].

AP180 is highly conserved throughout evolution and its function has been examined *in vivo* in different organisms. In mutant flies lacking LAP, the *Drosophila* homologue of AP180, clathrin is diffusely distributed, and synaptic vesicle endocytosis is severely impaired leading to a dramatic depletion of synaptic vesicles in nerve terminals and to large invaginations formed by excess plasma membrane. In mutant flies the mean size of synaptic vesicle is increased, and fewer vesicles are formed [132]. Mammalian AP180 (also known as AP3, F1-20, pp155, or NP185) is a neuronal-specific synaptic phosphoprotein that localizes to clathrin-coated pits in nerve terminals [130, 131, 133, 134]. It binds to clathrin and promotes its assembly into clathrin cages *in vitro* [128, 135, 136]. AP180′s ability to promote clathrin assembly can be inhibited by inositol polyphosphates, consistent with a role of inositol polyphosphates and inositol lipids as direct regulators of the protein [137, 138]. These data demonstrate a crucial role for AP180 in promoting cage formation and limiting the vesicle size both *in vitro* and *in vivo*.

Initially it was believed that AP180 had a unique function in the brain. However, a related and ubiquitously expressed human protein, CALM (for clathrin assembly lymphoid myeloid leukemia gene), has been cloned, suggesting a widespread role in membrane trafficking [139, 140] (Fig. 4). Somewhat surprisingly, neuronal AP180 lacks the NPF motifs, whereas the more divergent C-termini of the other family members (CALM, yAP180s, *Drosophila* LAP, and *C. elegans* AP180) all contain multiple NPFs (Fig. 4). The meaning of the NPF motifs being absent or present in the neuronal or nonneuronal variants of AP180 awaits future experimentation. It is reasonable to expect that the differences, among family members, in lipid-binding domains and ligand sites (i.e., DPF and NPF motifs) affect both the subcellular localization and the regulation of AP180, thus allowing specialized roles in different tissues (Fig. 3).

CONNECTIONS BETWEEN THE EH-BASED NETWORK AND OTHER PUTATIVE PROTEIN–PROTEIN NETWORKS IN THE CELL

Recently two superfamilies of proteins, each individually characterized by the presence of evolutionarily conserved domains, have been linked to members of the EH network and to the endocytic process in general. The two superfamilies in question are characterized by the presence of the ENTH and VHS domains [110, 141] (Fig. 4). The function(s) of these two domains are not known; however, there is evidence that the ENTH domain can act as a protein–protein interaction surface (B. K. Kay and P. S. McPherson, unpublished). This raises the possibility that separate networks of protein–protein interactions in the cell are coordinated toward regulation of endocytosis, intracellular sorting of vesicles, and possibly other cellular phenotypes.

The ENTH Domain

The ENTH (Epsin N-terminal homology) domain is a ~140-amino-acid-long homology domain shared by several proteins in budding and fission yeast, oat, nematode, frog, and mammals [110]. It was originally identified in Epsin-1 [33] and subsequently in Epsin-2, MP90 (a frog homologue of Epsins), and the yeast Epsins Ent1p and Ent2p. ENTH domains are generally located at the N-terminus and their identity ranges from 40 to 90%. Interestingly, a region of AP180 also displays weak homology to ENTH domains, around 22% similarity. Proteins of the ENTH superfamily share various other motifs involved with protein–protein interactions in the endocytic/sorting pathway, including EH-binding consensus (NPFs), α-adaptin appendage binding sites (DPF/Ws), and clathrin binding sites [L(L/I/V) (D/E/G/N) (L/F) (D/E/Q) or LIDL-COOH] [110].

The VHS Domain

The VHS (VPS-27, Hrs, and STAM) domain was originally identified by Ponting *et al.* (C. Ponting, J. Schultz, and P. Bork, submission to SMART database, EMBL; see also [142]) based on database screenings for proteins with regions of homology. Attention to the VHS domain, and to its potential implications in the sorting pathway, was drawn by Lohi and Lehto [141] who identified the domain in EAST [143], a chicken protein which probably represents an orthologue of human STAM2 (Gene Bank Accession No. NM_005843).

EAST (EGFR-associated protein with SH3 and TAM domains) is a protein containing a VHS domain in its NH_2-terminus followed by an SH3 domain and a tyrosine-based activation motif (TAM) in its COOH-terminus (Fig. 4). EAST is tyrosine phosphorylated *in vivo* and *in vitro* by activated EGFR and associates with the receptor. It also partially colocalizes with clathrin by indirect immunofluorescence. EAST co-immunoprecipitates with Eps15 and contains an NPF motif in its NH_2-terminus. However, mutagenesis of the NPF to DPF, which has been shown to abrogate the interaction of Eps15 with other EH-binding proteins, did not affect the co-immunoprecipitation between Eps15 and EAST. It is not clear, therefore, whether EAST represents a bona fide cellular ligand of EH domain-containing proteins [143].

The VHS domain is ~140 amino acids long and resides, invariably so far, in the NH_2-termini of harboring proteins [141]. Members of the VHS superfamily have in general been implicated with endocytosis/vesicular transport and also with signal transduction. They include VPS27, a *S. cerevisiae* protein that controls membrane trafficking in the prevacuolar/endosomal compartment [144]; Hrs, a substrate of the hepatocyte growth factor, which is localized to early endosomes [145]; and STAM, a protein involved in cytokine signaling, which has also been implicated in control of sorting through its association with Hrs [146, 147]. Several other less-characterized proteins also display a VHS domain [141].

Proteins belonging to the VHS superfamily can also display other signaling/sorting domains, such as SH3 and TAM domains and NPF motifs. VPS-27 and Hrs also display a FYVE finger domain, which has recently been shown to bind to PtdIns(3)P, the phosphorylated product of PI3-kinase. FYVE fingers are required for binding of early endosome antigen 1 (EEA1) to endosomes and are believed to play a general role in endosomal targeting [148–151].

FUNCTIONS OF THE EH NETWORK

EH Network and Coated Pits Formation

Despite a wealth of biological information about the role of the EH network in endocytosis, a number of questions remain about the specific biochemical function of particular protein–protein interactions. This issue is compounded by the fact that many of these proteins are likely to exert multiple functions (Fig. 3). However, since many of the EH domains and their cellular ligands have the characteristics of accessory endocytic proteins, that is being present in coated pits and/or vesicles in substoichiometric amounts, presumably their function(s) is regulatory in nature (Fig. 3).

The recent finding that Eps15 and Intersectin interact through their coil-coiled regions [18] suggests that they might work together as a scaffolding complex that might be required to coordinate recruitment of other accessory components during the formation of clathrin-coated pits (Fig. 5). Such a complex displays remarkable binding and scaffolding properties. Eps15 (or Eps15R) possess a minimum of six protein–protein interaction surfaces, including three EH domains, a coiled-coil domain, an α-ear binding region, and proline-rich SH3 domain-binding site. Intersectins display, in their short versions, eight binding modules, including two EH domains, a coiled-coil region, and five SH3 domains. If one takes into account that Eps15 can dimerize and tetramerize [20] and that the long isoforms of Intersectins contain additional interaction surfaces (such as a DH, a PH, and a C2 domain [18, 50–52]), there is potential for an enormous number of proteins recruited to the scaffold created by the Eps15: Intersectin heterodimer (Fig. 5). However, this does not imply that all the proteins that bind to the Eps15: Intersectin complex must be simultaneously recruited. Indeed, an interesting possibility is that the scaffolding complex might be "loaded" with different proteins, whose interactions might also be mutually exclusive, at different phases of the endocytic process or in different regions of a forming pit.

Two protein–protein interactions within this scaffolding complex are better characterized and provide, at least, testable working hypotheses for future experiments.

One such interacting molecule is dynamin which binds, through its proline-rich region, to the SH3 domains of Intersectin [15, 18, 50, 52, 53]. Since Intersectin and Eps15 are present in clathrin-coated pits, they may possibly influence the recruitment of dynamin to these structures. There is a complex relationship among Eps15, Intersectin, and dynamin, in terms of localization. It has been shown that Intersectin influences the subcellular localization of Eps15, a situation reminiscent of the End3p:Pan1p interaction in yeast, where End3p controls the localization of Pan1p [69]. Overexpression of Intersectin recruits dynamin to vesicular-like subcellular structures, as well as Eps15. One of Eps15's functions might be to finely tune the interaction between dynamin and Intersectin. Two observations in support of this hypothesis are that simul-

taneous overexpression of Intersectin and of a COOH-terminally truncated version of Eps15 results in mislocalization of the two proteins, even though they still remain partially associated to each other [18], and that overexpression of a NH_2-truncated mutant of Eps15 results in mislocalization of dynamin [152]. Thus, while most of the molecular mechanisms remain obscure, it appears as if an Eps15:Intersectin heterodimer might serve as a scaffold to recruit dynamin. If this were true one should expect alterations of dynamin function(s) upon perturbation of the scaffold. While evidence to this regard is lacking, we have recently uncovered a genetic interaction between Eps15 and dynamin in *C. elegans* (Salcini *et al.*, unpublished). The availability of dynamin-related genetic tools in lower organisms should therefore greatly aid the elucidation of the relationships among Intersectin, Eps15, and dynamin in the dynamics of clathrin-coated pit formation.

Another well-characterized protein–protein interaction of Eps15 is that with α-adaptin. Cytosolic Eps15 is stoichiometrically associated with AP-2, through its α-adaptin subunit [27]. Upon stimulation of receptor internalization, Eps15 and AP-2 are recruited to the plasma membrane into forming clathrin-coated pits [37]. The question that arises from these observations is "who takes whom to the nascent pit?" (Fig. 3).

There is reason to believe that one of Eps15's functions is to recruit AP-2 to the clathrin-coated pit. Molecular determinants have been identified in the intracellular domains of internalizable receptors, defined as "endocytic codes," i.e., amino acid sequences capable by themselves of sustaining receptor internalization [153]. Endocytic codes are believed to be cryptic in the unstimulated EGFR and to be unmasked by conformational changes that follow receptor activation and autophosphorylation [154]. In other types of receptors, such as cargo receptors, endocytic codes are probably continuously exposed, thus determining constitutive internalization. In many cases endocytic codes contain a critical tyrosine residue, thus being known as "tyrosine-based" signals (reviewed in [155]), and are interchangeable among receptors [153]. There is ample evidence that tyrosine-based signals, in the EGFR and in other receptors, bind directly to AP-2 and, in particular, to its μ subunit [22, 24, 156–159] (Fig. 3).

The simple model that a direct interaction of endocytic codes with AP-2 is responsible for the recruitment of the latter to a nascent pit has been recently challenged. Indeed, removal of the tyrosine-based signal, responsible for AP-2 binding, in the EGFR did not appreciably affect internalization kinetics [157], at least at low levels of receptor expression [158]. In cells expressing a mutant EGFR lacking the tyrosine-based signal, AP-2 and Eps15 were still recruited to the plasma membrane [37], thus suggesting that Eps15 might serve as the primary localizer of AP-2 to the membrane. Studies from Van Delft *et al.* [36] also supported this notion: clathrin and AP-2 could be extracted from coated pits containing membranes attached to glass slides, without distorting the staining pattern of Eps15. This result indicates that the presence of Eps15 in coated pits is independent of AP-2 and clathrin. Finally, Benmerah *et al.* [152] demonstrated that overexpression of a NH_2-truncated mutant of Eps15 disrupted the proper subcellular localization of AP-2 and clathrin. All of these observations suggest an important role of Eps15 in directing AP-2 complexes to the plasma membrane.

The interaction between Eps15 and α-ear might have additional roles in the dynamics of pit formation, as well. Recent structural studies have demonstrated that there is a single binding site in the α-ear of α-adaptin, which binds to peptides containing the consensus sequence DPF/W (aspartate–proline–phenylalanine/tryptophan). This interaction provides a molecular basis for understanding the binding of various proteins (including Eps15, Eps15R, Epsins, AP180, amphiphysin I, Numb, and possibly dynamin I) to the appendage domain of α-adaptin. The presence of a single binding site within the appendage domain predicts that the α-ear can bind to a variety of accessory molecules in a forming pit. However, it is not known whether recruitment of these proteins to the α-ear occurs simultaneously, by the numerous AP-2 molecules present in a coated pit, or sequentially. While the two possibilities need not be mutually exclusive, in the former case α-ear would work as a spatial organizer and in the latter as a temporal one (Fig. 3).

The presence of a single binding site also predicts competition among accessory proteins for binding to the AP-2 complex. Some of the proteins, such as Eps15, Eps15R, and Epsins, might bind avidly to a clathrin-coated pit with its dense concentration of AP-2 complexes, due to the presence of multiple binding motifs (DPF/W) within their sequences. This prediction is confirmed, at least in the case of competition between Eps15 and Numb, by the demonstration that Eps15 binds more strongly to the α-ear than Numb (Santolini *et al.*, unpublished). As noted above, Kirchhausen's group has demonstrated that polymerization of clathrin progressively displaces Eps15 from AP-2. Thus, one might envision a model in which Eps15 has the additional role of saturating the α-ear binding sites, under conditions in which interactions with other proteins are not desired, and that upon pit formation Eps15 is displaced, thus allowing the coordinated recruitment of other accessory molecules. Other putative high-affinity cellular ligands, such as Eps15R or the Epsins, might exert similar functions.

Finally, proteins of the EH network might differentially regulate internalization of different categories of

membrane receptors. There is evidence of specificity in the endocytic machinery as shown by the findings that EGFRs compete with themselves, but not with the transferrin receptor for endocytosis [160–163]. These findings indicate that the two molecular machineries are, at least in part, distinct. In addition, endocytosis of EGFR is a second-order saturable process, likely involving competition among EGFRs for a downstream component of the endocytic machinery [164]. Evidence has been provided that a tyrosine kinase substrate, different from EGFR itself, is needed for efficient recruitment into coated pits of ligand-activated EGFR [165].

Both POB1 and Eps15 possess characteristics that fit this role, since each is tyrosine phosphorylated in response to EGF stimulation. Interestingly, interference with POB1 function inhibits EGF and not transferrin receptor internalization. In addition, we have mapped and mutagenized the tyrosine phosphorylation site on Eps15 (S. Confalonieri and P. P. Di Fiore, unpublished) and found that this mutant inhibits endocytosis of EGFR, but not of the transferrin receptor. Thus, components of the EH network might differentially regulate endocytosis of different surface receptors.

EH Network in Other Steps of the Endocytic Pathway

At least in the case of Eps15, there is evidence for its involvement in the endocytic pathway, at steps other than the formation of a coated pit. As already reviewed, Eps15 is progressively disengaged from a forming pit and undetectable in coated vesicles [38]. After clathrin shedding, though, Eps15 is retargeted to uncoated vesicles and early and late endosomes, thus suggesting a role for it in these late stations of the endocytic route [37].

Some functional evidence supports this contention. Overexpression of a dominant negative mutant comprising only the AP-2 binding region of Eps15 inhibited internalization of EGFR and establishment of successful infection by Sindbis virus [39]. This latter assay allows for a more comprehensive analysis of the endocytic route, since establishment of successful infection requires both coated pit-mediated internalization of the virus and its delivery to late endosomes (reviewed in [166]). Surprisingly, an Eps15 mutant encompassing only its three EH domains had no effect on EGFR internalization, but completely inhibited Sindbis infection [39]. One possible explanation for this discrepancy is that the inhibition by EH domains is exerted at a step in the endocytic pathway other than internalization, as for example in the transport from early endosomes to the more acidic late endosomes. This result also draws attention to EH-binding proteins as potential effectors of Eps15 in the late phases of endocytosis.

The EH Network and the Cytoskeleton

In recent years, evidence has pointed to the involvement of the actin-based cytoskeleton in endocytosis. Both genetic and biochemical experiments have demonstrated in yeast that the actin cytoskeleton plays a central role in endocytosis, even though the precise details are unknown [167]. For example, actin and fimbrin are essential for the internalization of receptors [168], and cells expressing mutant forms of the actin filament depolymerizing protein cofilin display an endocytosis-defective phenotype [169]. Conversely, a number of genes isolated in screens for defects in endocytosis have been shown to be involved in the regulation of the yeast cortical actin cytoskeleton [170].

Pharmacological and biochemical experiments have demonstrated that the cytoskeleton is likely just as important to endocytosis in mammalian cells. Treatment of cultured cells with latrunculin A, a drug that sequesters actin monomers, inhibits receptor-mediated endocytosis [171]. Recently, observations on the formation of clathrin-coated pits in real-time, utilizing a clathrin light chain fused to the green fluorescent protein, have revealed that coated pits form repeatedly at defined sites at the plasma membrane [172]. The cytoskeleton is implicated in fixing the spatial locations of clathrin-coated pit formation, as the coated pits remain attached to a detergent-resistant cytoskeleton, even after Triton X-100 removal of the plasma membrane [172].

Cytoskeleton-associated proteins that might interface between the mammalian cytoskeleton and endocytosis include profilin, the human Wiskott–Aldrich syndrome protein (WASP), and syndapin. Profilin, which binds to proline-rich peptide sequences as well as actin, can form *in vitro* protein complexes with various regulators of endocytosis and actin assembly [173]. WASP, an actin-depolymerizing protein that regulates cytoskeletal rearrangement, co-immunoprecipitates with the activated EGFR after exposure to EGF [174] and has been shown to interact with syndapin, a protein that binds to dynamin and colocalizes with it at vesicular structures in primary neurons [124]. Syndapin has been functionally implicated in endocytosis, as addition of the SH3 domain of syndapin to a cell-permeabilized assay system can block uptake of transferrin [175].

EH domain-containing proteins and their cellular ligands may play a dual role in endocytosis and the cytoskeleton. In yeast, Pan1p and End3p are required for endocytosis and for proper formation of the actin cytoskeleton [65, 69]. The cellular ligands for the EH domains of Pan1p, Ent1p and Ent2p, may play similar roles; temperature-sensitive alleles of the Entps have defects in endocytosis and the actin cytoskeleton [176]. In *Drosophila*, the Intersectin family member Dap160 is localized in nerve terminals to the cytoskeleton that

surrounds the active zones of synaptic vesicle exocytosis and endocytosis [53]. Finally, upon EGFR activation, Eps15 fails to move to perinuclear regions in cells upon exposure to the microtubule-disrupting agent nocodazole [37].

The EAST protein may also serve to link the two cellular processes. As mentioned above, EAST was originally cloned as the SH3 domain-containing protein from chicken brain [177] and later shown to contain a single NPF tripeptide motif, a VHS domain, an SH3 domain, and a TAM motif [143] (Fig. 4). Not only does EAST interact with the EGFR and Eps15 in the cell [143], thus qualifying it as a potential component of the endocytic machinery, but immunofluorescence studies have revealed it to be colocalized with focal adhesions and actin filaments [141]. In addition, overexpression of the N-terminal (aa 1–205, including the VHS domain), but not the C-terminal (aa 260–469, including the TAM motif) segments of EAST in HeLa, MDCK, and HER-14 cells led to an increase in the number of microspikes and the formation of large protrusions [141]. Furthermore, the N-terminal segment (aa 4–188) could associate with actin *in vitro*, as revealed in an actin cosedimentation assay. Thus, one of EAST's functions may be to serve at the interface of endocytosis and the actin cytoskeleton.

Other Putative Functions of the EH Network

Some of the characteristics of EH network proteins are not immediately reconcilable with an exclusive role in endocytosis. Reps/POB1, for instance, is likely to exert some function in the regulation of Rho-like small GTPases, which are in turn part of mitogenic signaling pathways. The long isoforms of Intersectins, due to the presence of signaling domains such as the DH domain, might also exert functions in the regulation of small GTPases. In addition, there is an emerging body of literature that connects endocytic machinery to proliferation control and its subversion in cancer (recently reviewed in [178, 179]). In the following two paragraphs, we will review the evidence for a role of EH network proteins in cancer, nucleocytoplasmic shuttling, and cell cycle control.

Proteins of the EH Network Are Altered in Human Leukemias

The Eps15 gene is involved in balanced translocations with the MLL gene, which maps to 11q23, in rare cases of acute myeloid leukemias. Translocations result in fusion proteins in which the N-terminal domain of MLL is fused in frame to almost the entire open reading frame of Eps15, with the exclusion of the first few amino acids [48]. Abnormalities of the 11q23 region, involving the MLL gene, are frequent in acute leukemias. To date, a dozen translocation partners of MLL have been identified (reviewed in [180]). Furthermore, in-frame duplications of the MLL gene have been detected which result in altered proteins, in the absence of a balanced translocation [181]. This latter finding, together with the molecular heterogeneity of the MLL translocation partners, led to the hypothesis that the functional truncation of the MLL protein, achieved in the various fusion proteins, is the important molecular event in the pathogenesis of leukemias. However, other evidence points to the relevance of the fusion partners, in that truncation of MLL, or its fusion to irrelevant proteins, does not cause leukemias, whereas the fusion proteins present in the natural leukemogenic process are able to do so [182].

Another EH network protein involved in leukemias is CALM, the nonneuronal form of the AP180 adaptor protein. A specific rearrangement between the CALM and the AF-10 genes was originally discovered in the t(10;11) (p13;q24) of U937 cells [139]. Interestingly, AF-10, which encodes a potential transcription factor, has been observed to be a partner of the MLL gene in t(10;11) (p13;q23) translocations as well, providing further support for the concept that fusion partners such as endocytic proteins play a role in leukemogenesis [183, 184]. Recently, t(10;11) translocations involving AF-10 and CALM have also been identified in fresh samples from acute leukemia patients, further suggesting important roles in the leukemogenesis process for both components of the fusion proteins [185].

Alterations in EH network proteins (or in other components of the endocytic machinery) might result in unattenuated proliferative signals, due to decreased clearance of stimulatory receptors from the cell surface. This is, for instance, the case of oncogenic conversion of c-Cbl, a protein involved in EGFR downregulation, into v-Cbl, an oncogene that interferes with this process [186]. Other mechanisms might, however, be envisioned. Abnormal forms of sorting/targeting proteins can misdirect interacting proteins to aberrant locations or alter their functioning. Neomorphic functions might result from the fusion of functionally unrelated proteins, which might lead to aberrant connections of metabolic pathways, resulting in their disregulations. Finally, alterations of sorting proteins could lead to profound disregulations in the integration of pathways connecting various functions necessary for cell growth, as will be proposed in the next paragraph.

Is the EH Network an "Integrator" of Different Signaling Pathways Connected to the Control of Cell Proliferation?

Shuttling of proteins in and from the nucleus relies on transporters of the importin family and is regulated by the Ran GTPase (reviewed in [187]). In particular,

CRM1 (importin-1) translocates proteins containing a nuclear export signal (NES) out of the nucleus. As already mentioned, this branch of the pathway is also operationally defined as the Rev export pathway. The importinβ/importinα complex serves to translocate into the nucleus proteins containing a nuclear localization signal (NLS). The oncoprotein mdm2 (hdm2 in *Homo sapiens*) shuttles (due to the presence of both a NES and a NLS) in and out of the nucleus. In its way out, it mediates the export into the cytosol of nuclear p53, leading to p53 degradation by cytosolic proteasomes. This event accounts, in part, for the mechanism by which mdm2 regulates p53 activity [98, 188].

In its way into the nucleus, mdm2 appears to bind to Numb, although it is unclear which protein directs nuclear translocation of the other [189]. Interestingly, nuclear uptake of Numb is associated with its degradation; thus, one of mdm2′s function(s) might be to direct Numb to its proper site of degradation by the nuclear proteasome. The transforming abilities of mdm2 might, thus, be due in part to the enhanced degradation of Numb, which would then have tumor suppressor gene activity. In addition, since Numb and p53 bind to the same site on mdm2 [189], one can also postulate that Numb may control cell proliferation by competing the interaction of mdm2 with p53. This would result in the lack of mdm2-mediated p53 degradation or, alternatively, in the lack of direct inhibition of p53 function exerted by mdm2 (for recent reviews, see [190–192]).

Intriguingly, in both branches of the mdm2 shuttling pathway, proteins belonging to the EH network appear to be involved, i.e., Numb and Hrb (through its connection with Rev). Thus, elements of the EH network might link the endocytic/sorting machinery to the nucleocytoplasmic export pathway and, possibly, to mechanisms of protein degradation.

CONCLUSIONS

As our understanding of the cellular molecular machinery progresses, complex levels of regulation of such machinery are coming into focus. The process of endocytosis constitutes a paradigmatic case. The structural requirements for the formation of a coated pit are being elucidated, and a three-dimensional, high-resolution picture of the clathrin coat is emerging [24, 34, 193–195]. While we start to understand the mechanisms of assembly of the “hardware” of a pit, the discovery of an increasing number of accessory molecules underscores the requirement for the “software” that finely regulates spatial and temporal recruitment of pit components. The EH network constitutes a major component of this network of regulatory interactions. Not surprisingly, and given the fundamental importance of endocytosis to cellular homeostasis, the control of endocytic pathways needs to be coordinated and integrated with many other cellular functions. The EH network appears also to fulfill the requirements for such a role and might contribute to connect intracellular signaling mechanisms as diverse as endocytosis, nucleocytosolic shuttling, and possibly protein degradation and control of cell proliferation.

Studies of E.S., A.E.S., and P.P.D.F. are supported by the AIRC (Associazione Italiana Ricerca Sul Cancro), Ministero Sanita' (AIDS 1997, 1998), the Armenise-Harvard Foudation, the Ferrero Foundation, Telethon, and the European Community (BIOMED-2). M.Y. and B.K.K. acknowledge support from the Graduate and Medical Schools of University of Wisconsin–Madison.

REFERENCES

1. van der Geer, P., and Pawson, T. (1995). The PTB domain: A new protein module implicated in signal transduction. *Trends Biochem. Sci.* **20,** 277–280.
2. Cohen, G. B., Ren, R., and Baltimore, D. (1995). Modular binding domains in signal transduction proteins. *Cell* **80,** 237–248.
3. Musacchio, A., Wilmanns, M., and Saraste, M. (1994). Structure and function of the SH3 domain. *Prog. Biophys. Mol. Biol.* **61,** 283–297.
4. Pawson, T., and Scott, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278,** 2075–2080.
5. Fazioli, F., Minichiello, L., Matoskova, B., Wong, W. T., and Di Fiore, P. P. (1993). eps15, a novel tyrosine kinase substrate, exhibits transforming activity. *Mol. Cell. Biol.* **13,** 5814–5828.
6. Wong, W. T., Kraus, M. H., Carlomagno, F., Zelano, A., Druck, T., Croce, C. M., Huebner, K., and Di Fiore, P. P. (1994). The human eps15 gene, encoding a tyrosine kinase substrate, is conserved in evolution and maps to 1p31-p32. *Oncogene* **9,** 1591–1597.
7. Wong, W. T., Schumacher, C., Salcini, A. E., Romano, A., Castagnino, P., Pelicci, P. G., and Di Fiore, P. (1995). A protein-binding domain, EH, identified in the receptor tyrosine kinase substrate Eps15 and conserved in evolution. *Proc. Natl. Acad. Sci. USA* **92,** 9530–9534.
8. Di Fiore, P. P., Pelicci, P. G., and Sorkin, A. (1997). EH: A novel protein–protein interaction domain potentially involved in intracellular sorting. *Trends Biochem. Sci.* **22,** 411–43.
9. Mayer, B. J. (1999). Endocytosis: EH domains lend a hand. *Curr. Biol.* **9,** R70–R73.
10. de Beer, T., Carter, R. E., Lobel-Rice, K. E., Sorkin, A., and Overduin, M. (1998). Structure and Asn-Pro-Phe binding pocket of the Eps15 homology domain. *Science* **281,** 1357–1360.
11. Whitehead, B., Tessari, M., Carotenuto, A., van Bergen en Henegouwen, P. M., and Vuister, G. W. (1999). The EH1 domain of Eps15 is structurally classified as a member of the S100 subclass of EF-hand-containing proteins. *Biochemistry* **38,** 11271–11277.
12. Koshiba, S., Kigawa, T., Iwahara, J., Kikuchi, A., and Yokoyama, S. (1999). Solution structure of the Eps15 homology domain of a human POB1 (partner of RalBP1). *FEBS Lett.* **442,** 138–142.
13. Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P. G., and Di Fiore, P. P. (1997). Binding specificity and *in vivo* targets of the EH

domain, a novel protein–protein interaction module. *Genes Dev*. **11,** 2239–2249.

14. Paoluzi, S., Castagnoli, L., Lauro, I., Salcini, A. E., Coda, L., Fre, S., Confalonieri, S., Pelicci, P. G., Di Fiore, P. P., and Cesareni, G. (1998). Recognition specificity of individual EH domains of mammals and yeast. *EMBO J*. **17,** 6541–6550.
15. Yamabhai, M., Hoffman, N. G., Hardison, N. L., McPherson, P. S., Castagnoli, L., Cesareni, G., and Kay, B. K. (1998). Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. *J. Biol. Chem*. **273,** 31401–31407.
16. Wilson, D. R., and Finlay, B. B. (1997). The 'Asx-Pro turn' as a local structural motif stabilized by alternative patterns of hydrogen bonds and a consensus-derived model of the sequence Asn-Pro-Asn. *Protein Eng.* **10,** 519–529.
17. Coda, L., Salcini, A. E., Confalonieri, S., Pelicci, G., Sorkina, T., Sorkin, A., Pelicci, P. G., and Di Fiore, P. P. (1998). Eps15R is a tyrosine kinase substrate with characteristics of a docking protein possibly involved in coated pits-mediated internalization. *J. Biol. Chem*. **273,** 3003–3012.
18. Sengar, A. S., Wang, W., Bishay, J., Cohen, S., and Egan, S. E. (1999). The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15. *EMBO J*. **18,** 1159–1171.
19. Tebar, F., Confalonieri, S., Carter, R. E., Di Fiore, P. P., and Sorkin, A. (1997). Eps15 is constitutively oligomerized due to homophilic interaction of its coiled-coil region. *J. Biol. Chem*. **272,** 15413–15418.
20. Cupers, P., ter Haar, E., Boll, W., and Kirchhausen, T. (1997). Parallel dimers and anti-parallel tetramers formed by epidermal growth factor receptor pathway substrate clone 15. *J. Biol. Chem*. **272,** 33430–33434.
21. Gallusser, A., and Kirchhausen, T. (1993). The beta 1 and beta 2 subunits of the AP complexes are the clathrin coat assembly components. *EMBO J*. **12,** 5237–5244.
22. Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995). Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* **269,** 1872–1875.
23. Boll, W., Ohno, H., Songyang, Z., Rapoport, I., Cantley, L. C., Bonifacino, J. S., and Kirchhausen, T. (1996). Sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes. *EMBO J*. **15,** 5789–5795.
24. Owen, D. J., and Evans, P. R. (1998). A structural explanation for the recognition of tyrosine-based endocytotic signals. *Science* **282,** 1327–1332.
25. Traub, L. M., Downs, M. A., Westrich, J. L., and Fremont, D. H. (1999). Crystal structure of the alpha appendage of AP-2 reveals a recruitment platform for clathrin-coat assembly. *Proc. Natl. Acad. Sci. USA* **96,** 8907–8912.
26. Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M., and Kirchhausen, T. (1996). Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits. *J. Biol. Chem*. **271,** 28727–28730.
27. Benmerah, A., Gagnon, J., Begue, B., Megarbane, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1995). The tyrosine kinase substrate eps15 is constitutively associated with the plasma membrane adaptor AP-2. *J. Cell Biol*. **131,** 1831–1838.
28. Benmerah, A., Begue, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1996). The ear of alpha-adaptin interacts with the COOH-terminal domain of the Eps 15 protein. *J. Biol. Chem*. **271,** 12111–12116.
29. Iannolo, G., Salcini, A. E., Gaidarov, I., Goodman, O. B., Jr., Baulida, J., Carpenter, G., Pelicci, P. G., Di Fiore, P. P., and Keen, J. H. (1997). Mapping of the molecular determinants involved in the interaction between eps15 and AP-2. *Cancer Res*. **57,** 240–245.
30. Wang, L. H., Sudhof, T. C., and Anderson, R. G. (1995). The appendage domain of alpha-adaptin is a high affinity binding site for dynamin. *J. Biol. Chem*. **270,** 10079–10083.
31. David, C., McPherson, P. S., Mundigl, O., and de Camilli, P. (1996). A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc. Natl. Acad. Sci. USA* **93,** 331–335.
32. Wigge, P., Vallis, Y., and McMahon, H. T. (1997). Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain. *Curr. Biol*. **7,** 554–560.
33. Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P., and De Camilli, P. (1998). Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* **394,** 793–797.
34. Owen, D. J., Vallis, Y., Noble, M. E., Hunter, J. B., Dafforn, T. R., Evans, P. R., and McMahon, H. T. (1999). A structural explanation for the binding of multiple ligands by the alpha-adaptin appendage domain. *Cell* **97,** 805–815.
35. Schumacher, C., Knudsen, B. S., Ohuchi, T., Di Fiore, P. P., Glassman, R. H., and Hanafusa, H. (1995). The SH3 domain of Crk binds specifically to a conserved proline-rich motif in Eps15 and Eps15R. *J. Biol. Chem*. **270,** 15341–15347.
36. van Delft, S., Schumacher, C., Hage, W., Verkleij, A. J., and van Bergen en Henegouwen, P. M. (1997). Association and colocalization of Eps15 with adaptor protein-2 and clathrin. *J. Cell Biol*. **136,** 811–821.
37. Torrisi, M. R., Lotti, L. V., Belleudi, F., Gradini, R., Salcini, A. E., Confalonieri, S., Pelicci, P. G., and Di Fiore, P. P. (1999). Eps15 is recruited to the plasma membrane upon epidermal growth factor receptor activation and localizes to components of the endocytic pathway during receptor internalization. *Mol. Biol. Cell* **10,** 417–434.
38. Cupers, P., Jadhav, A. P., and Kirchhausen, T. (1998). Assembly of clathrin coats disrupts the association between Eps15 and AP-2 adaptors. *J. Biol. Chem*. **273,** 1847–1850.
39. Carbone, R., Fre, S., Iannolo, G., Belleudi, F., Mancini, P., Pelicci, P. G., Torrisi, M. R., and Di Fiore, P. P. (1997). eps15 and eps15R are essential components of the endocytic pathway. *Cancer Res*. **57,** 5498–5504.
40. Benmerah, A., Lamaze, C., Begue, B., Schmid, S. L., Dautry-Varsat, A., and Cerf-Bensussan, N. (1998). AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J. Cell Biol*. **140,** 1055–1062.
41. Clark, S. G., Shurland, D. L., Meyerowitz, E. M., Bargmann, C. I., and van der Bliek, A. M. (1997). A dynamin GTPase mutation causes a rapid and reversible temperature-inducible locomotion defect in *C. elegans*. *Proc. Natl. Acad. Sci. USA* **94,** 10438–10443.
42. van Delft, S., Govers, R., Strous, G. J., Verkleij, A. J., and van Bergen en Henegouwen, P. M. (1997). Epidermal growth factor induces ubiquitination of Eps15. *J. Biol. Chem*. **272,** 14013–14016.
43. Roth, A. F., and Davis, N. G. (1996). Ubiquitination of the yeast a-factor receptor. *J. Cell Biol*. **134,** 661–674.
44. Strous, G. J., van Kerkhof, P., Govers, R., Ciechanover, A., and Schwartz, A. L. (1996). The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *EMBO J*. **15,** 3806–3812.

45. Hicke, L., and Riezman, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand- stimulated endocytosis. *Cell* **84,** 277–287.

46. Chen, H., Slepnev, V. I., Di Fiore, P. P., and De Camilli, P. (1999). The interaction of epsin and Eps15 with the clathrin adaptor AP-2 is inhibited by mitotic phosphorylation and enhanced by stimulation-dependent dephosphorylation in nerve terminals. *J. Biol. Chem.* **274,** 3257–3260.

47. Slepnev, V. I., Ochoa, G. C., Butler, M. H., Grabs, D., and Camilli, P. D. (1998). Role of phosphorylation in regulation of the assembly of endocytic coat complexes. *Science* **281,** 821–824.

48. Bernard, O. A., Mauchauffe, M., Mecucci, C., Van den Berghe, H., and Berger, R. (1994). A novel gene, AF-1p, fused to HRX in t(1;11) (p32;q23), is not related to AF-4, AF-9 nor ENL. *Oncogene* **9,** 1039–1045.

49. Rogaia, D., Grignani, F., Carbone, R., Riganelli, D., LoCoco, F., Nakamura, T., Croce, C. M., Di Fiore, P. P., and Pelicci, P. G. (1997). The localization of the HRX/ALL1 protein to specific nuclear subdomains is altered by fusion with its eps15 translocation partner. *Cancer Res.* **57,** 799–802.

50. Okamoto, M., Schoch, S., and Sudhof, T. C. (1999). EHSH1/Intersectin, a protein that contains EH and SH3 domains and binds to dynamin and SNAP-25. A protein connection between exocytosis and endocytosis? *J. Biol. Chem.* **274,** 18446–18454.

51. Guipponi, M., Scott, H. S., Chen, H., Schebesta, A., Rossier, C., and Antonarakis, S. E. (1998). Two isoforms of a human Intersectin (ITSN) protein are produced by brain-specific alternative splicing in a stop codon. *Genomics* **53,** 369–376.

52. Hussain, N. K., Yamabhai, M., Ramjaun, A. R., Guy, A. M., Baranes, D., O'Bryan, J. P., Der, C. J., Kay, B. K., and McPherson, P. S. (1999). Splice variants of Intersectin are components of the endocytic machinery in neurons and nonneuronal cells. *J. Biol. Chem.* **274,** 15671–15677.

53. Roos, J., and Kelly, R. B. (1998). Dap160, a neural-specific Eps15 homology and multiple SH3 domain-containing protein that interacts with Drosophila dynamin. *J. Biol. Chem.* **273,** 19108–19119.

54. Cantor, S. B., Urano, T., and Feig, L. A. (1995). Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases. *Mol. Cell. Biol.* **15,** 4578–4584.

55. Jullien-Flores, V., Dorseuil, O., Romero, F., Letourneur, F., Saragosti, S., Berger, R., Tavitian, A., Gacon, G., and Camonis, J. H. (1995). Bridging Ral GTPase to Rho pathways. RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. *J. Biol. Chem.* **270,** 22473–22477.

56. Park, S. H., and Weinberg, R. A. (1995). A putative effector of Ral has homology to Rho/Rac GTPase activating proteins. *Oncogene* **11,** 2349–2355.

57. Yamaguchi, A., Urano, T., Goi, T., and Feig, L. A. (1997). An Eps homology (EH) domain protein that binds to the Ral-GTPase target, RalBP1. *J. Biol. Chem.* **272,** 31230–31234.

58. Ikeda, M., Ishida, O., Hinoi, T., Kishida, S., and Kikuchi, A. (1998). Identification and characterization of a novel protein interacting with Ral-binding protein 1, a putative effector protein of Ral. *J. Biol. Chem.* **273,** 814–821.

59. Nakashima, S., Morinaka, K., Koyama, S., Ikeda, M., Kishida, M., Okawa, K., Iwamatsu, A., Kishida, S., and Kikuchi, A. (1999). Small G protein ral and its downstream molecules regulate endocytosis of EGF and insulin receptors. *EMBO J.* **18,** 3629–3642.

60. Morinaka, K., Koyama, S., Nakashima, S., Hinoi, T., Okawa, K., Iwamatsu, A., and Kikuchi, A. (1999). Epsin binds to the EH domain of POB1 and regulates receptor-mediated endocytosis. *Oncogene,* in press.

61. Mintz, L., Galperin, E., Pasmanik-Chor, M., Tulzinsky, S., Bromberg, Y., Kozak, C. A., Joyner, A., Fein, A., and Horowitz, M. (1999). EHD1—An EH-domain-containing protein with a specific expression pattern. *Genomics* **59,** 66–76.

62. Page, L. J., Sowerby, P. J., Lui, W. W., and Robinson, M. S. (1999). gamma-Synergin. An eh domain-containing protein that interacts with gamma-adaptin. *J. Cell Biol.* **146,** 993–1004.

63. Wendland, B., McCaffery, J. M., Xiao, Q., and Emr, S. D. (1996). A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. *J. Cell Biol.* **135,** 1485–1500.

64. Raths, S., Rohrer, J., Crausaz, F., and Riezman, H. (1993). end3 and end4: Two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae. J. Cell Biol.* **120,** 55–65.

65. Benedetti, H., Raths, S., Crausaz, F., and Riezman, H. (1994). The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. *Mol. Biol. Cell* **5,** 1023–1037.

66. Kolling, R., and Hollenberg, C. P. (1994). The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquitinated form in endocytosis mutants. *EMBO J.* **13,** 3261–3271.

67. Volland, C., Urban-Grimal, D., Geraud, G., and Haguenauer-Tsapis, R. (1994). Endocytosis and degradation of the yeast uracil permease under adverse conditions. *J. Biol. Chem.* **269,** 9833–9841.

68. Lai, K., Bolognese, C. P., Swift, S., and McGraw, P. (1995). Regulation of inositol transport in *Saccharomyces cerevisiae* involves inositol-induced changes in permease stability and endocytic degradation in the vacuole. *J. Biol. Chem.* **270,** 2525–2534.

69. Tang, H. Y., Munn, A., and Cai, M. (1997). EH domain proteins Pan1p and End3p are components of a complex that plays a dual role in organization of the cortical actin cytoskeleton and endocytosis in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **17,** 4294–4304.

70. Tang, H. Y., and Cai, M. (1996). The EH-domain-containing protein Pan1 is required for normal organization of the actin cytoskeleton in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **16,** 4897–4914.

71. Wendland, B., and Emr, S. D. (1998). Pan1p, yeast eps15, functions as a multivalent adaptor that coordinates protein–protein interactions essential for endocytosis. *J. Cell Biol.* **141,** 71–84.

72. Zolladek, T., Tobiasz, A., Vaduva, G., Boguta, M., Martin, N. C., and Hopper, A. K. (1997). MDP1, a *Saccharomyces cerevisiae* gene involved in mitochondrial/cytoplasmic protein distribution, is identical to the ubiquitin–protein ligase gene RSP5. *Genetics* **145,** 595–603.

73. Tan, P. K., Howard, J. P., and Payne, G. S. (1996). The sequence NPFXD defines a new class of endocytosis signal in *Saccharomyces cerevisiae. J. Cell Biol.* **135,** 1789–1800.

74. Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y., and Jan, Y. N. (1989). numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. *Cell* **58,** 349–360.

75. Rhyu, M. S., Jan, L. Y., and Jan, Y. N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell* **76,** 477–491.

76. Verdi, J. M., Schmandt, R., Bashirullah, A., Jacob, S., Salvino, R., Craig, C. G., Program, A. E., Lipshitz, H. D., and McGlade, C. J. (1996). Mammalian NUMB is an evolutionarily conserved signaling adapter protein that specifies cell fate. *Curr. Biol.* **6,** 1134–1145.

77. Zhong, W., Feder, J. N., Jiang, M. M., Jan, L. Y., and Jan, Y. N. (1996). Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. *Neuron* **17,** 43–53.

78. Zhong, W., Jiang, M. M., Weinmaster, G., Jan, L. Y., and Jan, Y. N. (1997). Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. *Development* **124,** 1887–1897.

79. Ruiz Gomez, M., and Bate, M. (1997). Segregation of myogenic lineages in Drosophila requires numb. *Development* **124,** 4857–4866.

80. Carmena, A., Murugasu-Oei, B., Menon, D., Jimenez, F., and Chia, W. (1998). Inscuteable and numb mediate asymmetric muscle progenitor cell divisions during Drosophila myogenesis. *Genes Dev.* **12,** 304–315. [published erratum appears in *Genes Dev.* **12**(8), 1241, 1998]

81. Knoblich, J. A., Jan, L. Y., and Jan, Y. N. (1995). Asymmetric segregation of Numb and Prospero during cell division. *Nature* **377,** 624–627.

82. Kraut, R., Chia, W., Jan, L. Y., Jan, Y. N., and Knoblich, J. A. (1996). Role of inscuteable in orienting asymmetric cell divisions in Drosophila. *Nature* **383,** 50–55.

83. Lu, B., Rothenberg, M., Jan, L. Y., and Jan, Y. N. (1998). Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in Drosophila neural and muscle progenitors. *Cell* **95,** 225–235.

84. Campos-Ortega, J. A. (1996). Numb diverts notch pathway off the tramtrack. *Neuron* **17,** 1–4.

85. Frise, E., Knoblich, J. A., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (1996). The Drosophila Numb protein inhibits signaling of the Notch receptor during cell–cell interaction in sensory organ lineage. *Proc. Natl. Acad. Sci. USA* **93,** 11925–11932.

86. Guo, M., Jan, L. Y., and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: Interaction of Numb and Notch. *Neuron* **17,** 27–41.

87. Spana, E. P., and Doe, C. Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron* **17,** 21–26.

88. Wakamatsu, Y., Maynard, T. M., Jones, S. U., and Weston, J. A. (1999). NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. *Neuron* **23,** 71–81.

89. Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995). Notch signaling. *Science* **268,** 225–232.

90. Greenwald, I. (1998). LIN-12/Notch signaling: Lessons from worms and flies. *Genes Dev.* **12,** 1751–1762.

91. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999). Notch signaling: Cell fate control and signal integration in development. *Science* **284,** 770–776.

92. Verdi, J. M., Bashirullah, A., Goldhawk, D. E., Kubu, C. J., Jamali, M., Meakin, S. O., and Lipshitz, H. D. (1999). Distinct human NUMB isoforms regulate differentiation vs proliferation in the neuronal lineage. *Proc. Natl. Acad. Sci. USA* **96,** 10472–10476.

93. Bogerd, H. P., Fridell, R. A., Madore, S., and Cullen, B. R. (1995). Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell* **82,** 485–494.

94. Fritz, C. C., Zapp, M. L., and Green, M. R. (1995). A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature* **376,** 530–533.

95. Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82,** 475–483.

96. Fritz, C. C., and Green, M. R. (1996). HIV Rev uses a conserved cellular protein export pathway for the nucleocytoplasmic transport of viral RNAs. *Curr. Biol.* **6,** 848–854.

97. Stutz, F., Izaurralde, E., Mattaj, I. W., and Rosbash, M. (1996). A role for nucleoporin FG repeat domains in export of human immunodeficiency virus type 1 Rev protein and RNA from the nucleus. *Mol. Cell. Biol.* **16,** 7144–7150.

98. Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998). Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. *EMBO J.* **17,** 554–564.

99. Cullen, B. R. (1998). HIV-1 auxiliary proteins: Making connections in a dying cell. *Cell* **93,** 685–692.

100. Stutz, F., Neville, M., and Rosbash, M. (1995). Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell* **82,** 495–506.

101. Neville, M., Stutz, F., Lee, L., Davis, L. I., and Rosbash, M. (1997). The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. *Curr. Biol.* **7,** 767–775.

102. Henderson, B. R., and Percipalle, P. (1997). Interactions between HIV Rev and nuclear import and export factors: The Rev nuclear localisation signal mediates specific binding to human importin-beta. *J. Mol. Biol.* **274,** 693–707.

103. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* **90,** 1041–1050.

104. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90,** 1051–1060.

105. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390,** 308–311.

106. Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997). Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science* **278,** 141–144.

107. Ullman, K. S., Powers, M. A., and Forbes, D. J. (1997). Nuclear export receptors: From importin to exportin. *Cell* **90,** 967–970.

108. Stutz, F., Kantor, J., Zhang, D., McCarthy, T., Neville, M., and Rosbash, M. (1997). The yeast nucleoporin rip1p contributes to multiple export pathways with no essential role for its FG-repeat region. *Genes Dev.* **11,** 2857–2868.

109. Stukenberg, P. T., Lustig, K. D., McGarry, T. J., King, R. W., Kuang, J., and Kirschner, M. W. (1997). Systematic identification of mitotic phosphoproteins. *Curr. Biol.* **7,** 338–348.

110. Kay, B. K., Yamabhai, M., Wendland, B., and Emr, S. D. (1999). Identification of a novel domain shared by putative components of the endocytic and cytoskeletal machinery. *Protein Sci.* **8,** 435–438.

111. McPherson, P. S., de Heuvel, E., Phillie, J., Wang, W., Sengar, A., and Egan, S. (1998). EH domain-dependent interactions between Eps15 and clathrin-coated vesicle protein p95. *Biochem. Biophys. Res. Commun.* **244,** 701–705.

112. Haffner, C., Takei, K., Chen, H., Ringstad, N., Hudson, A., Butler, M. H., Salcini, A. E., Di Fiore, P. P., and De Camilli, P. (1997). Synaptojanin 1: Localization on coated endocytic intermediates in nerve terminals and interaction of its 170 kDa isoform with Eps15. *FEBS Lett.* **419,** 175–180.

113. McPherson, P. S., Garcia, E. P., Slepnev, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1996). A presynaptic inositol-5-phosphatase. *Nature* **379,** 353–357.

114. Nemoto, Y., Arribas, M., Haffner, C., and DeCamilli, P. (1997). Synaptojanin 2, a novel synaptojanin isoform with a distinct targeting domain and expression pattern. *J. Biol. Chem.* **272,** 30817–30821.

115. Khvotchev, M., and Sudhof, T. C. (1998). Developmentally regulated alternative splicing in a novel synaptojanin. *J. Biol. Chem.* **273,** 2306–2311.

116. Ramjaun, A. R., and McPherson, P. S. (1996). Tissue-specific alternative splicing generates two synaptojanin isoforms with differential membrane binding properties. *J. Biol. Chem.* **271,** 24856–24861.

117. Woscholski, R., and Parker, P. J. (1997). Inositol lipid 5-phosphatases—Traffic signals and signal traffic. *Trends Biochem. Sci.* **22,** 427–431.

118. Mitchell, C. A., Brown, S., Campbell, J. K., Munday, A. D., and Speed, C. J. (1996). Regulation of second messengers by the inositol polyphosphate 5-phosphatases. *Biochem. Soc. Trans.* **24,** 994–1000.

119. Sakisaka, T., Itoh, T., Miura, K., and Takenawa, T. (1997). Phosphatidylinositol 4,5-bisphosphate phosphatase regulates the rearrangement of actin filaments. *Mol. Cell. Biol.* **17,** 3841–3849.

120. McPherson, P. S., Takei, K., Schmid, S. L., and De Camilli, P. (1994). p145, a major Grb2-binding protein in brain, is co-localized with dynamin in nerve terminals where it undergoes activity-dependent dephosphorylation. *J. Biol. Chem.* **269,** 30132–30139.

121. Ramjaun, A. R., Micheva, K. D., Bouchelet, I., and McPherson, P. S. (1997). Identification and characterization of a nerve terminal-enriched amphiphysin isoform. *J. Biol. Chem.* **272,** 16700–16706.

122. Ringstad, N., Nemoto, Y., and De Camilli, P. (1997). The SH3p4/Sh3p8/SH3p13 protein family: Binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. *Proc. Natl. Acad. Sci. USA* **94,** 8569–8574.

123. de Heuvel, E., Bell, A. W., Ramjaun, A. R., Wong, K., Sossin, W. S., and McPherson, P. S. (1997). Identification of the major synaptojanin-binding proteins in brain. *J. Biol. Chem.* **272,** 8710–8716.

124. Qualmann, B., Roos, J., DiGregorio, P. J., and Kelly, R. B. (1999). Syndapin I, a synaptic dynamin-binding protein that associates with the neural Wiskott–Aldrich syndrome protein. *Mol. Biol. Cell* **10,** 501–513.

125. Srinivasan, S., Seaman, M., Nemoto, Y., Daniell, L., Suchy, S. F., Emr, S., De Camilli, P., and Nussbaum, R. (1997). Disruption of three phosphatidylinositol-polyphosphate 5-phosphatase genes from *Saccharomyces cerevisiae* results in pleiotropic abnormalities of vacuole morphology, cell shape, and osmohomeostasis. *Eur. J. Cell Biol.* **74,** 350–360.

126. Stolz, L. E., Huynh, C. V., Thorner, J., and York, J. D. (1998). Identification and characterization of an essential family of inositol polyphosphate 5-phosphatases (INP51, INP52 and INP53 gene products) in the yeast *Saccharomyces cerevisiae. Genetics* **148,** 1715–1729.

127. Singer-Kruger, B., Nemoto, Y., Daniell, L., Ferro-Novick, S., and De Camilli, P. (1998). Synaptojanin family members are implicated in endocytic membrane traffic in yeast. *J. Cell Sci.* **111,** 3347–3356.

128. Ahle, S., and Ungewickell, E. (1986). Purification and properties of a new clathrin assembly protein. *EMBO J.* **5,** 3143–3149.

129. Keen, J. H. (1987). Clathrin assembly proteins: Affinity purification and a model for coat assembly. *J. Cell Biol.* **105,** 1989–1998.

130. Kohtz, D. S., and Puszkin, S. (1988). A neuronal protein (NP185) associated with clathrin-coated vesicles. Characterization of NP185 with monoclonal antibodies. *J. Biol. Chem.* **263,** 7418–7425.

131. Zhou, S., Sousa, R., Tannery, N. H., and Lafer, E. M. (1992). Characterization of a novel synapse-specific protein. II. cDNA cloning and sequence analysis of the F1-20 protein. *J. Neurosci.* **12,** 2144–2155.

132. Zhang, B., Koh, Y. H., Beckstead, R. B., Budnik, V., Ganetzky, B., and Bellen, H. J. (1998). Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron* **21,** 1465–1475.

133. Perry, D. G., Hanson, V., Benuck, M. L., and Puszkin, S. (1991). Neuronal protein NP185 in avian and murine cerebellum: Expression during development and evidence for its presence in nerve endings. *J. Histochem. Cytochem.* **39,** 1461–1470.

134. Sousa, R., Tannery, N. H., Zhou, S., and Lafer, E. M. (1992). Characterization of a novel synapse-specific protein. I. Developmental expression and cellular localization of the F1-20 protein and mRNA. *J. Neurosci.* **12,** 2130–2143.

135. Morris, S. A., Schroder, S., Plessmann, U., Weber, K., and Ungewickell, E. (1993). Clathrin assembly protein AP180: Primary structure, domain organization and identification of a clathrin binding site. *EMBO J.* **12,** 667–675.

136. Ye, W., and Lafer, E. M. (1995). Clathrin binding and assembly activities of expressed domains of the synapse-specific clathrin assembly protein AP-3. *J. Biol. Chem.* **270,** 10933–10939.

137. Ye, W., Ali, N., Bembenek, M. E., Shears, S. B., and Lafer, E. M. (1995). Inhibition of clathrin assembly by high affinity binding of specific inositol polyphosphates to the synapse-specific clathrin assembly protein AP-3. *J. Biol. Chem.* **270,** 1564–1568.

138. Hao, W., Tan, Z., Prasad, K., Reddy, K. K., Chen, J., Prestwich, G. D., Falck, J. R., Shears, S. B., and Lafer, E. M. (1997). Regulation of AP-3 function by inositides. Identification of phosphatidylinositol 3,4,5-trisphosphate as a potent ligand. *J. Biol. Chem.* **272,** 6393–6398.

139. Dreyling, M. H., Martinez-Climent, J. A., Zheng, M., Mao, J., Rowley, J. D., and Bohlander, S. K. (1996). The t(10;11) (p13; q14) in the U937 cell line results in the fusion of the AF10 gene and CALM, encoding a new member of the AP-3 clathrin assembly protein family. *Proc. Natl. Acad. Sci. USA* **93,** 4804–4809.

140. Tebar, F., Bohlander, S. K., and Sorkin, A. (1999). Clathrin assembly lymphoid myeloid leukemia (CALM) protein: Localization in endocytic-coated pits, interactions with clathrin, and the impact of overexpression on clathrin-mediated traffic. *Mol. Biol. Cell* **10,** 2687–2702.

141. Lohi, O., and Lehto, V. P. (1998). EAST, a novel EGF receptor substrate, associates with focal adhesions and actin fibers. *FEBS Lett.* **436,** 419–423.

142. Ponting, C. P., Schultz, J., Milpetz, F., and Bork, P. (1999). SMART: Identification and annotation of domains from signaling and extracellular protein sequences. *Nucleic Acids Res.* **27,** 229–232.

143. Lohi, O., Poussu, A., Merilainen, J., Kellokumpu, S., Wasenius, V. M., and Lehto, V. P. (1998). EAST, an epidermal growth factor receptor- and Eps15-associated protein with Src homology 3 and tyrosine-based activation motif domains. *J. Biol. Chem.* **273,** 21408–21415.

144. Piper, R. C., Cooper, A. A., Yang, H., and Stevens, T. H. (1995). VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae. J. Cell Biol.* **131,** 603–617.

145. Komada, M., and Kitamura, N. (1995). Growth factor-induced tyrosine phosphorylation of Hrs, a novel 115-kilodalton protein with a structurally conserved putative zinc finger domain. *Mol. Cell. Biol.* **15,** 6213–6221.

146. Takeshita, T., Arita, T., Higuchi, M., Asao, H., Endo, K., Kuroda, H., Tanaka, N., Murata, K., Ishii, N., and Sugamura, K. (1997). STAM, signal transducing adaptor molecule, is associated with Janus kinases and involved in signaling for cell growth and c-myc induction. *Immunity* **6,** 449–457.

147. Asao, H., Sasaki, Y., Arita, T., Tanaka, N., Endo, K., Kasai, H., Takeshita, T., Endo, Y., Fujita, T., and Sugamura, K. (1997). Hrs is associated with STAM, a signal-transducing adaptor molecule. Its suppressive effect on cytokine-induced cell growth. *J. Biol. Chem.* **272,** 32785–32791.

148. Patki, V., Lawe, D. C., Corvera, S., Virbasius, J. V., and Chawla, A. (1998). A functional PtdIns(3)P-binding motif. *Nature* **394,** 433–434.

149. Burd, C. G., and Emr, S. D. (1998). Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol. Cell* **2,** 157–162.

150. Gaullier, J. M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R. (1998). FYVE fingers bind PtdIns(3)P. *Nature* **394,** 432–433.

151. Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M., and Stenmark, H. (1998). EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* **394,** 494–498.

152. Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999). Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J. Cell Sci.* **112,** 1303–1311.

153. Chang, C. P., Lazar, C. S., Walsh, B. J., Komuro, M., Collawn, J. F., Kuhn, L. A., Tainer, J. A., Trowbridge, I. S., Farquhar, M. G., Rosenfeld, M. G., *et al.* (1993). Ligand-induced internalization of the epidermal growth factor receptor is mediated by multiple endocytic codes analogous to the tyrosine motif found in constitutively internalized receptors. *J. Biol. Chem.* **268,** 19312–19320.

154. Nesterov, A., Kurten, R. C., and Gill, G. N. (1995). Association of epidermal growth factor receptors with coated pit adaptins via a tyrosine phosphorylation-regulated mechanism. *J. Biol. Chem.* **270,** 6320–6327.

155. Mellman, I. (1996). Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* **12,** 575–625.

156. Glickman, J. N., Conibear, E., and Pearse, B. M. (1989). Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO J.* **8,** 1041–1047.

157. Nesterov, A., Wiley, H. S., and Gill, G. N. (1995). Ligand-induced endocytosis of epidermal growth factor receptors that are defective in binding adaptor proteins. *Proc. Natl. Acad. Sci. USA* **92,** 8719–8723.

158. Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L., and Beguinot, L. (1996). Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr974-containing internalization motif. *J. Biol. Chem.* **271,** 13377–13384.

159. Nesterov, A., Carter, R. E., Sorkina, T., Gill, G. N., and Sorkin, A. (1999). Inhibition of the receptor-binding function of clathrin adaptor protein AP-2 by dominant-negative mutant mu2 subunit and its effects on endocytosis. *EMBO J.* **18,** 2489–2499.

160. Dickson, R. B., Hanover, J. A., Willingham, M. C., and Pastan, I. (1983). Prelysosomal divergence of transferrin and epidermal growth factor during receptor-mediated endocytosis. *Biochemistry* **22,** 5667–5674.

161. Hanover, J. A., Beguinot, L., Willingham, M. C., and Pastan, I. H. (1985). Transit of receptors for epidermal growth factor and transferrin through clathrin-coated pits. Analysis of the kinetics of receptor entry. *J. Biol. Chem.* **260,** 15938–15945.

162. Wiley, H. S. (1988). Anomalous binding of epidermal growth factor to A431 cells is due to the effect of high receptor densities and a saturable endocytic system. *J. Cell Biol.* **107,** 801–810.

163. Wiley, H. S., Herbst, J. J., Walsh, B. J., Lauffenburger, D. A., Rosenfeld, M. G., and Gill, G. N. (1991). The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. *J. Biol. Chem.* **266,** 11083–11094.

164. Lund, K. A., Opresko, L. K., Starbuck, C., Walsh, B. J., and Wiley, H. S. (1990). Quantitative analysis of the endocytic system involved in hormone-induced receptor internalization. *J. Biol. Chem.* **265,** 15713–15723.

165. Lamaze, C., and Schmid, S. L. (1995). Recruitment of epidermal growth factor receptors into coated pits requires their activated tyrosine kinase. *J. Cell Biol.* **129,** 47–54.

166. Marsh, M., and Helenius, A. (1989). Virus entry into animal cells. *Adv. Virus Res.* **36,** 107–151.

167. Wendland, B., Emr, S. D., and Riezman, H. (1998). Protein traffic in the yeast endocytic and vacuolar protein sorting pathways. *COCB* **10,** 513–522.

168. Kubler, E., and Riezman, H. (1993). Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO J.* **12,** 2855–2862.

169. Lappalainen, P., and Drubin, D. G. (1997). Cofilin promotes rapid actin filament turnover *in vivo. Nature* **388,** 78–82.

170. Yang, S., Cope, M. J., and Drubin, D. G. (1999). Sla2p is associated with the yeast cortical actin cytoskeleton via redundant localization signals. *Mol. Biol. Cell* **10,** 2265–2283.

171. Lamaze, C., Fujimoto, L. M., Yin, H. L., and Schmid, S. L. (1997). The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J. Biol. Chem.* **272,** 20332–20335.

172. Gaidarov, I., Santini, F., Warren, R., and Keen, J. (1999). Spatial control of coated-pit dynamics in living cells. *NCB* **1,** 1–7.

173. Witke, W., Podtelejnikov, A. V., Di Nardo, A., Sutherland, J. D., Gurniak, C. B., Dotti, C., and Mann, M. (1998). In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly. *EMBO J.* **17,** 967–976.

174. She, H. Y., Rockow, S., Tang, J., Nishimura, R., Skolnik, E. Y., Chen, M., Margolis, B., and Li, W. (1997). Wiskott–Aldrich syndrome protein is associated with the adapter protein Grb2 and the epidermal growth factor receptor in living cells. *Mol. Biol. Cell* **8,** 1709–1721.

175. Simpson, F., Hussain, N., Qualmann, B., Kelly, R., Kay, B., McPherson, P., and Schmid, S. (1999). SH3-domain-contain-

ing proteins function at distinct steps in clathrin-coated vesicle formation. *NCB* **1,** 119–124.

176. Wendland, B., Steece, K. E., and Emr, S. D. (1999). Yeast Epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis. *EMBO J.* **18,** 4383–4393.
177. Merilainen, J., Lehto, V. P., and Wasenius, V. M. (1997). FAP52, a novel, SH3 domain-containing focal adhesion protein. *J. Biol. Chem.* **272,** 23278–23284.
178. Floyd, S., and De Camilli, P. (1998). Endocytosis proteins and cancer: A potential link? *Trends Cell Biol.* **8,** 299–301.
179. Di Fiore, P. P., and Gill, G. N. (1999). Endocytosis and mitogenic signaling. *Curr. Opin. Cell Biol.* **11,** 483–488.
180. Rowley, J. D. (1998). The critical role of chromosome translocations in human leukemias. *Annu. Rev. Genet.* **32,** 495–519.
181. Schichman, S. A., Caligiuri, M. A., Gu, Y., Strout, M. P., Canaani, E., Bloomfield, C. D., and Croce, C. M. (1994). ALL-1 partial duplication in acute leukemia. *Proc. Natl. Acad. Sci. USA* **91,** 6236–6239.
182. Corral, J., Lavenir, I., Impey, H., Warren, A. J., Forster, A., Larson, T. A., Bell, S., McKenzie, A. N., King, G., and Rabbitts, T. H. (1996). An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: A method to create fusion oncogenes. *Cell* **85,** 853–861.
183. Djabali, M., Selleri, L., Parry, P., Bower, M., Young, B. D., and Evans, G. A. (1992). A trithorax-like gene is interrupted by chromosome 11q23 translocations in acute leukaemias. *Nat. Genet.* **2,** 113–118. [published erratum appears in *Nat. Genet.* **4**(4), 431, 1993]
184. Iida, S., Seto, M., Yamamoto, K., Komatsu, H., Tojo, A., Asano, S., Kamada, N., Ariyoshi, Y., Takahashi, T., and Ueda, R. (1993). MLLT3 gene on 9p22 involved in t(9;11) leukemia encodes a serine/proline rich protein homologous to MLLT1 on 19p13. *Oncogene* **8,** 3085–3092.
185. Dreyling, M. H., Schrader, K., Fonatsch, C., Schlegelberger, B., Haase, D., Schoch, C., Ludwig, W., Loffler, H., Buchner, T., Wormann, B., Hiddemann, W., and Bohlander, S. K. (1998). MLL and CALM are fused to AF10 in morphologically distinct subsets of acute leukemia with translocation t(10;11): Both rearrangements are associated with a poor prognosis. *Blood* **91,** 4662–4667.
186. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* **12,** 3663–3674.
187. Ohno, M., Fornerod, M., and Mattaj, I. W. (1998). Nucleocytoplasmic transport: The last 200 nanometers. *Cell* **92,** 327–336.
188. Freedman, D. A., and Levine, A. J. (1998). Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. *Mol. Cell. Biol.* **18,** 7288–7293.
189. Juven-Gershon, T., Shifman, O., Unger, T., Elkeles, A., Haupt, Y., and Oren, M. (1998). The Mdm2 oncoprotein interacts with the cell fate regulator Numb. *Mol. Cell. Biol.* **18,** 3974–3982.
190. Prives, C. (1998). Signaling to p53: Breaking the MDM2-p53 circuit. *Cell* **95,** 5–8.
191. Juven-Gershon, T., and Oren, M. (1999). Mdm2: The ups and downs. *Mol. Med.* **5,** 71–83.
192. Freedman, D. A., Wu, L., and Levine, A. J. (1999). Functions of the MDM2 oncoprotein. *Cell. Mol. Life Sci.* **55,** 96–107.
193. ter Haar, E., Musacchio, A., Harrison, S. C., and Kirchhausen, T. (1998). Atomic structure of clathrin: A beta propeller terminal domain joins an alpha zigzag linker. *Cell* **95,** 563–573.
194. Musacchio, A., Smith, C. J., Roseman, A. M., Harrison, S. C., Kirchhausen, T., and Pearse, B. M. (1999). Functional organization of clathrin in coats: Combining electron cryomicroscopy and X-ray crystallography. *Mol. Cell* **3,** 761–770.
195. Marsh, M., and McMahon, H. T. (1999). The structural era of endocytosis. *Science* **285,** 215–220.

Received September 15, 1999