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Minireview

The Eps15 homology (EH) domain

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Abstract The Eps15 homology (EH) domain was originally identified as a motif present in three copies at the NH_2-termini of Eps15 and of the related molecule Eps15R. Both of these molecules are substrates for the tyrosine kinase activity of the epidermal growth factor receptor and hence the name 'Eps15 homology' or EH domain [Wong et al. (1994) Oncogene 9, 1591–1597; Wong et al. (1995) Proc. Natl. Acad. Sci. USA 92, 9530–9534; Fazioli et al. (1993) Mol. Cell. Biol. 13, 5814–5828] was derived. The motif was subsequently found in several proteins from yeast to nematode, thus establishing its evolutionary conservation. Initial studies with filter-binding assays and phage-displayed libraries demonstrated its protein:protein interaction abilities and identified specific ligands. Subsequently, structural analyses established the molecular bases of recognition between EH domains and cognate peptides. To date, several EH-containing and EH-binding proteins have been identified, which establish in the cell a network of protein:protein interactions, defined as the EH network. This network coordinates cellular functions connected with endocytosis, actin remodeling and intracellular transduction of signals.

Key words: EH; Eps15; Endocytosis; Actin remodeling; Signaling

1. The EH domain: definition and binding specificity

The EH domain[1–3] is ~100 amino acids long, and it is present in proteins from Haemosporida Fungi, Plants, Nematodes, Artropoda, Amphibia and Mammalia. We created a profile with a subset of EH domains from different species, by employing domain boundaries according to the nuclear magnetic resonance (NMR) structure of the second EH domain of human Eps15 [4]. This profile was used to search all the public protein databases, with the HMMER software [5]. After elimination of redundancies, we identified 50 proteins containing at least one EH domain. These proteins are listed in Table 1, where they are identified with their EMBL/SwissProt accession numbers.

All non-redundant EH domains were aligned using the Clustalw program. The alignment, shown in Fig. 1, was manually adjusted and drawn using the ESPrit software [6]. Conserved residues are identified according to amino acid physical–chemical properties, and they are colored in red when present at a plurality of >60%. The most conserved (>95%) residues are Leu^{45} and Trp^{49} in the alignment (corresponding to Leu^{165} and to Trp^{169} in the mEps15 protein). They are indicated with a red arrow in Fig. 1.

A variety of approaches, from filter-binding [3] to phage display assays [7], were used to determine the binding abilities of EH domains and identified three classes of binding peptides. Phage display experiments [7] showed that the majority of EH domains bind preferably to peptides containing an NPF (asparagine–proline–phenylalanine) motif (class I peptides). However binding to peptides containing FW (phenylalanine–tryptophan), WW (tryptophan–tryptophan) or SWG (serine–tryptophan–glycine) motifs (class II peptides) was also found. A third class of ligands, containing a H(S/T)F (histidine–serine/threonine–phenylalanine) motif, was found to bind exclusively the EH1 of End3p. Consistent with these findings, the screening of a human fibroblast expression library with the EH domains of Eps15 yielded several clones, representing bona fide EH-binding proteins [8]. Among these were Numb and Numbl [9], Hrb and Hrbl [10] and Epsin 1 [11]. These proteins displayed no sequence homology among themselves except for the presence of one or more copies of the NPF motif, which was in turn demonstrated to be indispensable in vivo for the binding of the identified proteins to the EH domains of Eps15 [8].

2. The EH domain: structure and molecular bases of interaction

The structure of five different EH domains has been determined by NMR, including those of the EH1 of mouse Eps15 [12], the EH2 of human Eps15 [4], the EH3 of human Eps15 [13], the EH of POB1 [14] and the EH of Reps1 [15]. They share the same fold, composed of two closely associated helix–loop–helix motifs, also called EF-hands, connected by a short antiparallel β-sheet (Fig. 2). EF-hands are endowed with Ca^{2+}-binding properties [16]. All EH domains display two EF-hand motif structures but not all of them possess all the residues required for calcium-binding, as defined by the canonical and the pseudo EF-hand consensus sequences [17,18]. Three of the five EH domains bind to calcium through their second canonical EF-hand, formed by helices αC and αD, because their first one does not contain the necessary residues to coordinate the calcium ion. The EH3 of Eps15 binds to Ca^{2+} only through its first EF-hand, because in the second

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Table 1
Accession numbers, domain composition and known interactors of EH-containing proteins

Name	TREMBL/SwissProt accession number	Organism	Domains	EH-binding proteins
EHD-RME-1	Q9N5B7	*C. elegans*	1 EH	
EHD-PAST1	Q94919	*Drosophila melanogaster*	1 EH	
EHD1	Q9UNR3	*Homo sapiens*	COIL, 1 EH	
EHD1-HPAST	O14611	*H. sapiens*	COIL, 1 EH	
EHD2	Q9NZN4	*H. sapiens*	COIL, 1 EH	
EHD3	Q9NZN3	*H. sapiens*	COIL, 1 EH	
EHD4	Q9NZN2	*H. sapiens*	1 EH	
EHD1	Q9WVK4	*Mus musculus*	COIL, 1 EH	
EHD3	Q9QXY6	*M. musculus*	COIL, 1 EH	
EHD4-MPAST2	Q9EQP2	*M. musculus*	1 EH	
Q9W111	Q9W111	*D. melanogaster*	3 EH, COIL	
END3	P39013	*Saccharomyces cerevisiae*	2 EH, COIL	
PAN1	P32521	*S. cerevisiae*	2 EH, COIL	Ent1p, Ent2p, Sla1p, yAP180A, yAP180B
EDE1	P34216	*S. cerevisiae*	3 EH, COIL, UBA	
O94685	O94685	*Schizosaccharomyces pombe*	3 EH, COIL, UBA	
EHS-1	Q9BIF4	*C. elegans*	3 EH, COIL	
EPS15	P42566	*H. sapiens*	3 EH, COIL	Epsin, Hrb, Hrb1, Numb, Numb1, synaptojanin
EPS15	P42567	*M. musculus*	3 EH, COIL	Epsin, Hrb, Hrb1, Numb, Numb1, synaptojanin
EPS15R	Q9UBC2	*H. sapiens*	3 EH, COIL	
EPS15R	Q60902	*M. musculus*	3 EH, COIL	Hrb, Hrb1
GAMMA-SYN	Q9JKC9	*Rattus norvegicus*	COIL, 1 EH	SCAMP1
GAMMA-SYN	Q9UMZ2	*H. sapiens*	COIL, 1 EH	
Intersectin-2	19NYG0	*H. sapiens*	2 EH, COIL, 5 SH3, RhoGEF, PH, C2	
Intersectin-2	19Z0R6	*M. musculus*	2 EH, COIL, 5 SH3, RhoGEF, PH, C2	Epsin
Intersectin	Q9U2T9	*C. elegans*	2 EH, COIL, 4 SH3	Hrb, Epsin
Intersectin-DAP160	Q9VIF7	*D. melanogaster*	2 EH, COIL, 5 SH3	
Intersectin	O95216	*H. sapiens*	2 EH, COIL, 5 SH3, RhoGEF, PH, C2	
Intersectin	Q9Z0R4	*M. musculus*	2 EH, COIL, 5 SH3, RhoGEF, PH, C2	Epsin
Intersectin	Q9WVE9	*R. norvegicus*	2 EH, COIL, 5 SH3	SCAMP1
Intersectin	O42287	*Xenopus laevis*	2 EH, COIL, 5 SH3	Hrb, Epsin
REPS1	Q9BXY9	*H. sapiens*	1 EH, COIL	
REPS1	O54916	*M. musculus*	1 EH, COIL	
POB1	O43428	*H. sapiens*	1 EH, COIL	Eps15, Eps15R, Epsin
SAGA	Q12076	*Aspergillus nidulans*	2 EH, COIL	
Q9LM78	Q9LM78	*Arabidopsis thaliana*	2 EH, COIL	
Q9LTR4	Q9LTR4	*A. thaliana*	1 EH	
Q9XI16	Q9XI16	*A. thaliana*	2 EH, COIL	
Q9NE15	Q9NE15	*C. elegans*	1 EH, COIL	
Q9VKK0	Q9VKK0	*D. melanogaster*	1 EH, COIL	
Q9H223	Q9H223	*H. sapiens*	1 EH	
Q9H4M9	Q9H4M9	*H. sapiens*	1 EH	
Q9NLB8	Q9NLB8	*Plasmodium falciparum*	1 EH	
AAF99472	AAF99472	*Plasmodium vivax*	1 EH	
O88638	O88638	*R. norvegicus*	1 EH	
P36115	P36115	*S. cerevisiae*	1 EH, COIL	
P47030	P47030	*S. cerevisiae*	1 EH	
O14066	O14066	*S. pombe*	1 EH	
Q10172	Q10172	*S. pombe*	2 EH, COIL, WH2	
Q9HGL2	Q9HGL2	*S. pombe*	3 EH, COIL	
Q9USZ7	Q9USZ7	*S. pombe*	2 EH	

motif the residue at position 3 is a lysine instead of an aspartate, a substitution that is predicted to repel a divalent cation. The EH1 of Eps15 presents a pseudo (residues Gln^{25}–Asp^{38}) and a canonical (residues Asp^{61}–Glu^{72}) EF-hand motif. However, both motifs differ in two critical residues with respect to other EF-hand domains [17–19]. In the pseudo EF-hand consensus, the residue at position 11 provides a side chain oxygen ligand for metal-binding; in the EH1 of Eps15 this position is occupied by a leucine (Leu^{35}), which cannot perform this function. In the canonical EF-hand consensus, the residue at position 5 is usually an aspartate/asparagine, which provides a side chain carbonyl for Ca^{2+}-binding. In the Eps15 EH1 domain, this residue is substituted by lysine (Lys^{65}). Accordingly the EH1 of Eps15 does not bind Ca^{2+} at physiological concentrations, as predicted [12].

In all EH domains the binding pocket for the NPF motif is made up by helices αB and αC (blue and yellow in Fig. 2). In the EH2 of Eps15 Leu^{155}, Leu^{165}, and Trp^{169} form the bottom

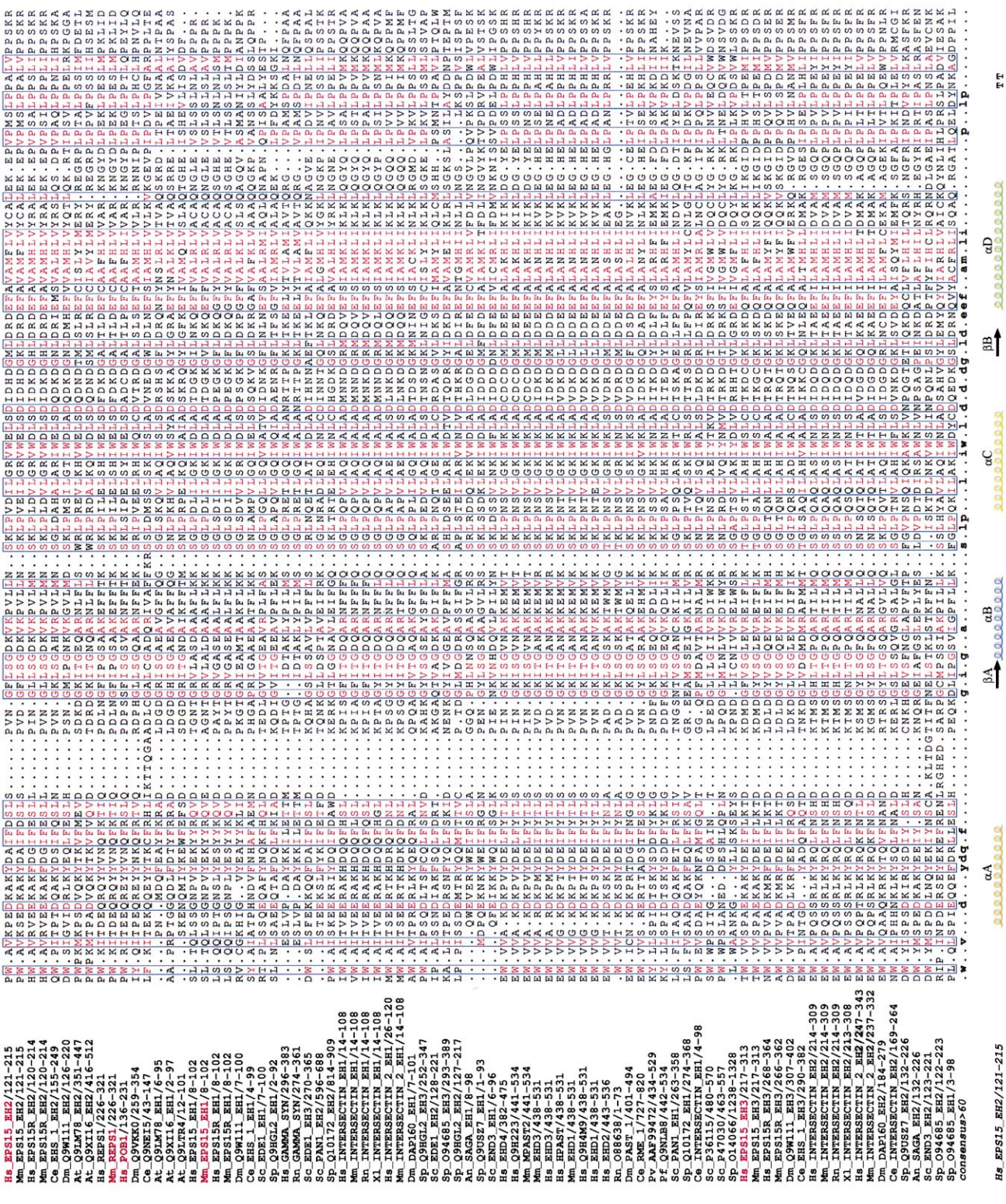

Fig. 1. Multiple alignment of EH domains. Multiple alignments of all EH domains found in public protein databases with the HMMER software. Amino acids are grouped according to physical–chemical properties: H/K/R, polar positive; D/E, polar negative; S/T/N/Q, polar neutral; A/V/L/I/M, non-polar aliphatic; F/Y/W, non-polar aromatic; P/G, small residues; C, cysteine (not groupable with other amino acids). Red color is applied whenever group conservation exceeds 60%. Arrowheads on top of the alignment indicate the residues involved in the binding to the peptide. Red arrows indicate residues whose mutation to Ala completely abolishes the EH:NPF binding. The blue arrow indicates the +3 position, with respect to the conserved Trp, that modulates the alternate binding specificity for NPF- or FW-containing peptides. The secondary structure of the second EH domain of hEps15 is reported at the bottom.

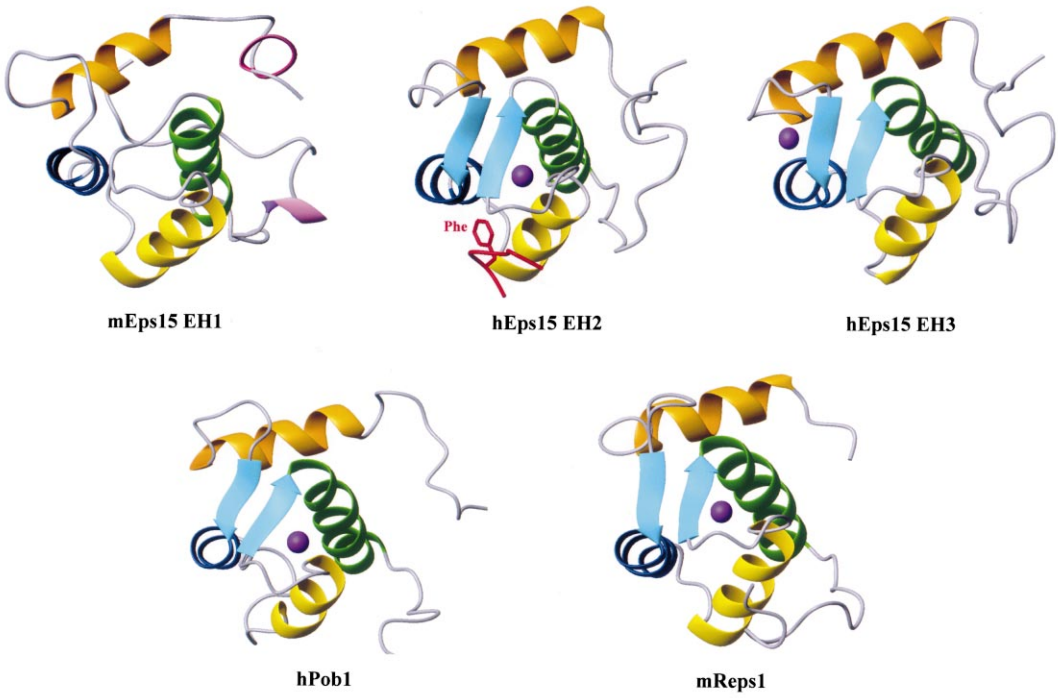

Fig. 2. NMR structures of various EH domains. Known structures of EH domains. The domains represented here are those listed in red in Fig. 1 (mEps15-EH1, PDB code 1qjt; hEps15-EH2 complexed with the NPF peptide of Hrb, in red, PDB code 1ff1; hEps15-EH3, PDB code 1c07; the EH domain of hPOB1, PDB code 1iq3; EH domain of mReps1, PDB code 1fi6). A violet sphere represents the calcium ion. The figures were prepared with the program MOLMOL [53].

of the pocket. The two latter residues are the most conserved among all EH domains, and mutations at these positions are incompatible with binding to NPF-containing peptides [4,7]. The NPF motif (class I peptides) binds to EH domains in a type I Asn–Pro β-turn conformation, and is almost completely buried in the binding pocket formed by helices αB and αC. A type I β-turn conformation is also present in cyclized NPF peptides and they have been shown to be better ligands than the linearized counterparts [20]. The Phe residue in the peptide serves as a central hydrophobic anchor. It is postulated [21] that similar interactions underlie the binding of peptides of other classes. This is supported by findings that FW and NPF motifs bind in the same pocket of the EH3 of Eps15 [13]. Furthermore, all peptides selected by EH domains have at least one aromatic amino acid, which could fit into the binding pocket similarly to the Phe ring in the NPF motif [21].

Four residues in the NPF-containing peptide mediate the interaction with EH domains, i.e. the NPF itself and the residue at position +3 with respect to Asn. This latter residue seems important in determining binding affinity. It contacts several residues on the surface of the domain, and can stabilize the β-turn conformation of the peptide [21]. On the other hand, in the EH domain, the residue at position +3, with respect to the conserved Trp, contributes to the specificity of recognition of the cognate peptide. Domains that prefer NPF have Ala or Ser at this position, while domains that bind FW, WW or SWG display slightly larger amino acids (Cys or Val). It has been shown that mutations (Cys → Ala/Ser) in the third EH domain of Eps15, which binds preferably FW, promote the binding to NPF peptides. Similarly, mutations (Ala → Val/Cys) in the third EH domain of Eps15R, which binds preferably NPF, promote the binding to FW peptides [7]. However it was not possible to switch completely the specificity of recognition from one class of peptides to another [13].

Interestingly the EH3 of Eps15 can bind to a peptide resembling the FW internalization motif of the mannose 6-phosphate receptor (MPR) [22], suggesting the possibility that Eps15 could interact via its EH3 with the MPR in endosomes and clathrin-uncoated vesicles where Eps15 is localized [23]. The EH domain of Reps1 can also bind, albeit with low affinity, to a peptide containing a DPF (aspartate–proline–phenylalanine) motif [15]. This finding supports the experimental evidence that the EH domain of POB1 can bind directly to the C-terminus of Eps15, which contains 12 DPF motifs [24], and that the ability of Eps15 to form tetramers can be attributed to EH domain–DPF interactions [25]. DPF-containing peptides were shown to constitute ligands for the C-terminal appendage domain of α-adaptin [9,26]. The fact that EH domains can also recognize DPF motifs suggests interesting analogies. EH domains recognize the motif NPF with a K_D of approximately 500 μM [4], and the C-terminal subdomain of α-adaptin domain recognizes the DPF motif with similar affinity [26]. Although there are no structural similarities between these domains, the two binding sites possess some common features. Both of them are composed of a hydrophobic pocket of similar size that has a tryptophan at the base, and mutation of this residue to alanine abolishes interaction with their binding counterpart. In both binding sites, residues surrounding the tryptophan can provide specificity for their binding targets [7,21,26].

3. EH domain-containing proteins and their interactors: the EH network

The biological functions of the EH network have recently been reviewed in an extensive fashion [27]. Here we will briefly concentrate on more recent findings that further highlight the involvement of this network in a variety of cellular processes.

When functional information is available, EH-containing and EH-binding proteins are often implicated in the regulation of protein transport/sorting and membrane traffic (see also [27]). For example, in mammals, Eps15 and Eps15R are components of clathrin-coated pits. They also interact with AP2, the major clathrin adaptor complex, and localize to organelles of the endocytic route during receptor internalization [23,28–30]. Accordingly, Eps15 was shown, by interference experiments, to be an essential component of the endocytic machinery [31–33]. Formal genetic evidence for an involvement of the EH network in endocytosis was obtained in yeast [34–38]. More recently, nematode studies provided additional genetic evidence in higher eukaryotes. EHS-1, the *Caenorhabditis elegans* homologue of Eps15, and RME-1, another EH-containing protein, were, in fact, shown to be required for endocytic transport and synaptic vesicle recycling [39,40]. A recent intriguing finding concerns Numb, which is an EH-binding protein associated to Eps15. Numb is a membrane-associated protein that determines cell fate during both nervous and muscle system development in flies and mammals [41–44]. Recently it was shown that mammalian Numb is an endocytic protein [9] that is co-trafficked with internalizing receptors, interacts with AP2, and is involved in clathrin-mediated endocytosis. These results raise the interesting possibility of participation of the endocytic machinery to cell fate determination.

Regulation of actin cytoskeleton is another function linked to EH-containing protein as demonstrated by yeast studies [34,45], and, in mammals, by the interaction of two EH-containing proteins, Reps1 and POB1, with RalBP1, a GTPase activating protein for the CDC42 and Rac GTPases [46,47]. Recent studies of Intersectin further support this contention. The so-called long isoform of Intersectin contains, in addition to EH and SH3 domains, a DH-PH domain, a hallmark of guanine exchange factors (GEFs) for Rho-GTPases. It was shown that Intersectin can actually function as a GEF for CDC42 [48]. In addition, N-WASP, a critical component of the actin remodeling machinery, binds directly to Intersectin, and upregulates its GEF activity. This, in turn, leads to the production of GTP-bound CDC42, which is an activator of N-WASP. Thus Intersectin participates in positive feedback loops, leading to actin re-organization [48].

Evidence is also emerging that links directly EH-containing and EH-binding proteins to intracellular signaling. Intersectin was shown to bind to Sos1, a potent Ras-GEF, via its SH3 domains [49]. Accordingly, the SH3 domains of Intersectin acted as dominant negative mutants on the activation of Ras and mitogen-activated protein kinase (MAPK) [50]. Furthermore, the overexpression of Intersectin activates the Elk-1 transcription factor, in a MAPK-independent manner. This ability resides within the EH domains, as expression of the tandem EH domains is sufficient to activate Elk-1 [51]. Finally a role for endocytic proteins, including members of the EH network, in the nucleus has been proposed. Eps15, Epsin, CALM and AP2 accumulate in the nucleus when nuclear export is inhibited [52]. In the nucleus, Eps15 and CALM acted as positive modulators of transcription in a GAL4-based transactivation assay, suggesting that endocytic proteins participate in the nucleus to transcriptional regulation [52].

4. Outlook

Originally thought to be exclusively involved in the regulation of endocytosis, the network of EH-containing and EH-binding proteins is now being implicated in a variety of other cellular processes. Presently, the EH network extends its ramifications from endocytosis to the control of the actin polymerizing machinery, to neurotransmission, to intracellular signal transduction, to cell fate determination, down to the remote (from the endocytosis' point of view) regions of the nucleus, where a role in transcriptional regulation is suspected. While a role in cancer is already emerging (reviewed in [27]), the increasing connections with basic cellular processes predict further involvement of this protein network in human diseases.

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