Cell Biochem Biophys
DOI 10.1007/s12013-010-9137-5

REVIEW PAPER

The Structure and Function of Endophilin Proteins

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Abstract Members of the BAR domain protein superfamily are essential elements of cellular traffic. Endophilins are among the best studied BAR domain proteins. They have a prominent function in synaptic vesicle endocytosis (SVE), receptor trafficking and apoptosis, and in other processes that require remodeling of the membrane structure. Here, we discuss the role of endophilins in these processes and summarize novel insights into the molecular aspects of endophilin function. Also, we discuss phosphorylation of endophilins and how this and other mechanisms may contribute to disease.

Keywords Endocytosis · Synaptic vesicles · Clathrin · Dynamin · Tyrosine kinase receptors · Apoptosis · Cancer · Neurodegeneration

Introduction

A hallmark of living cells is the busy traffic of proteins and other molecules between specialized membrane compartments, including the plasma membrane. Protein traffic is tightly interwoven with membrane traffic, which is primarily mediated by a large number of transport vesicles. The Bin/Amphiphysin/Rvs (BAR) domain proteins constitute a heterogeneous superfamily of cytoplasmic proteins with a strong commitment to membrane traffic (www.bar-superfamily.org) [1]. Among the best characterized BAR domain proteins are the endophilins, which in addition to the BAR domain contain a variable region and an SH3 domain. Among the many intriguing processes that involve endophilin activity are synaptic vesicle retrieval, receptor tyrosine kinase signaling, mitochondrial network dynamics, apoptosis and autophagy. Dysregulation of endophilin function has been linked to both cancer and neurodegenerative diseases.

This review is motivated by a number of recent developments in endophilin research. In earlier reviews, much attention was given to the lysophosphatidic acid acyl transferase (LPAAT) activity attributed to endophilin at the time of writing, which candidated as a key aspect of endophilin function [2–4]. However, although still cited occasionally, the LPAAT activity of endophilins has been shown to represent an experimental artifact [5, 6], which enforces a shift of focus towards other functional mechanisms. Fortunately, this setback is compensated for by the fact that the structure of the endophilin BAR domain is now very well characterized and its interaction with the lipid bilayer is understood in considerable detail, based on studies both at the biophysical level and in living systems. In addition, there has been exciting recent progress in the identification of the role played by the endophilin B subfamily. Also, we are now able to produce an overview of the post-translational modification of the endophilins, and discuss how this and other modifications of endophilin expression or function might lead to disease.

Discovery

The endophilins were found 13 years ago, by screening an embryonic mouse cDNA expression library with a

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Published online: 25 December 2010

proline-rich peptide ligand for SH3 domains [7]. Three positive clones named SH3p4, SH3p8, and SH3p13 were identified, corresponding to the three endophilin A genes endophilin A1, A2, and A3, respectively. Renaming to endophilin was motivated by the affinity of SH3p4 to endocytic proteins [8]. An independent PCR-based screen of cDNA from normal human brain and brain tumor tissue also identified the three isoforms of endophilin A, named in that study SH3GL for SH3-containing Grb2-like protein [9]. Endophilin A is also known as EEN (extra eleven nineteen) protein, identified as a partner in a fusion protein created by a translocation between chromosomes 11 and 19 in a leukemia patient [10].

The existence of a second endophilin subfamily, endophilins B, was noted in 2000 [2]. Using the proapoptotic protein Bax as bait, two groups pulled out endophilin B1 in yeast two-hybrid screens, naming it SH3GLB1 or Bif-1 (Bax-interacting factor-1) [11, 12]. Using SH3GLB1 itself as bait, a second protein, SH3GLB2 (endophilin B2), was identified as an interacting partner [12].

The use of several naming conventions for the same proteins, with independent numbering of isoforms, is the unavoidable consequence of the multiple paths to their discovery. However, this situation has now become a nuisance and may inhibit the dissemination of valuable information across research fields such as those concerned with synaptic endocytosis and cancer. We therefore urge investigators of this protein family to use "endophilin" in future reports, as also recommended by the UniProt consortium. If another name is preferred, we suggest to always also enter "endophilin" as an alternative designation in the title.

To address the evolution of endophilins, we performed searches based on primary structural alignments. We searched for proteins containing both a BAR and an SH3 domain, and with significant similarity to human endophilin. By this criterion, endophilins are found in other unikonts than animals, e.g., fungi, amoebozoa and choanoflagellates (A. Jung, unpublished observations). Endophilins are also present in some archeaplastida (green plants). By contrast, they seem to be absent in chromalveolates (e.g., ciliates, water molds) and excavates (e.g., trypanosomes, trichomonades).

Below, reference will be made to the *Drosophila melanogaster* (fruit fly) system. In *Drosophila*, there is only one isoform of the endophilin A protein, encoded by the *endoA* gene [13, 14]. Similarly, there is only one gene encoding endophilin B, *endoB* [13].

Localization

Endophilin A1 is essentially brain-specific, while endophilin A2 is expressed in all tissues. Expression of endophilin A3 is high in brain and testes [9, 15, 16] but may not be limited to these tissues [9]. Endophilin B1 and B2 are present in most organs, including brain [5, 12, 17]. Endophilin splice variants have been documented, in particular for endophilin A3 and the B forms. They differ in localization and time of expression. For example, both brain- and testes-specific variants of endophilin B1 have been described [16–18]. In *Drosophila*, endophilin A is strongly enriched in the CNS and eyes, while endophilin B is enriched in the adult and larval CNS, eyes, and ovaries [13, 14, 19].

At the subcellular level, endophilins A are concentrated at presynaptic terminals (primarily A1 and A3 in mammals) where they localize to synaptic vesicle membranes and the cytosol, and to a lesser extent to the membrane at resting synapses [13–15, 20–23]. In response to synaptic stimulation, endophilins A are recruited to the presynaptic membrane. Both in neurons and other cell types, they also accumulate at the extra-synaptic plasma membrane. Moreover, the cytosol typically exhibits a strong endophilin A immunosignal both in punctate and diffuse patterns, concentrated in the perinuclear space [15, 24–28]. Subcellular fractionation studies indicate that endophilins A associate with the light membrane fraction (enriched in ER/Golgi, endosomes and lysosomes) and mitochondria, and also appears in solution in the cytosol [21, 27, 29, 30]. In germ cells from testis, endophilin A3 is found in the acrosome region [31].

Endophilin B1 staining has been described as both diffuse and punctate/reticular, located in the cytosol, and with perinuclear enrichment [12, 30]. Endophilin B1 is recruited to mitochondria in response to pro-apoptotic stimuli (see below). An interesting but unexplored finding is that endophilin B1, like endophilins A, is associated with synaptic vesicles [5, 30]. The subcellular distribution of endophilin B2 is not well known.

The results cited above concern the subcellular localization of endogenous endophilins. Roughly congruent findings have been made when analyzing endophilins encoded by exogenous genes, introduced by cellular transfection [5, 11, 12, 17, 25, 27, 30, 32–35]. A putative nuclear localization signal is found in both endophilins A and B (Fig. 1a). Accordingly, endophilins have sometimes been detected in the nucleus. However, this seems to occur rarely and mostly in transfected cells. The functional significance remains unclear. Nuclear translocation of endophilin can be a robust phenomenon under abnormal circumstances, in which endophilin is sequestered by another protein residing in the nucleus (see below) [26].

Overall Structure

All endophilins consist of an N-terminal BAR domain, a variable middle region and a C-terminal SH3 domain

(Fig. 1). BAR domains are dimerization domains that are able to induce and stabilize membrane curvature and to "sense" (i.e., bind selectively to) already curved membrane. The endophilin BAR domain (henceforth the endoBAR) forms a crescent-shaped dimer, with each monomer made up of three kinked, antiparallel alpha-helices (Fig. 1b) [36–38]. The endoBAR belongs to the N-BAR class, implying that an amphipathic helix, Helix 0 (H0), is apposed to the N-terminus of the actual BAR domain (Fig. 1a). The endoBAR also has an insert in Helix 1, the most N-terminal of the three alpha helices. The Helix 1 insert (H1I, residues ~60 to 88 in mammalian endophilin A1) protrudes from the concave endoBAR surface and establishes a second amphipathic helix in addition to Helix 0. Here, we name this helix the central amphipathic helix (CAH, residues ~63–75; Fig. 1a, b).

The higher-order structure of the central variable region is unknown. However, it is known that the variable region is important in determining whether endophilin promotes or inhibits receptor-mediated endocytosis [35]. It contains several phosphoresidues, implying that it has a role in the post-translational regulation of endophilin activity. It also harbors a calcium channel binding site (Fig. 1a) [39]. These aspects are treated in more detail below.

SH3 domains are common protein-recognition modules (reviewed in [40]). The structure of the rat endophilin A2 SH3 domain has been solved by X-ray crystallography [41]. It adopts the β-barrel core typical of SH3 domains, with a hydrophobic groove that accommodates proline-rich peptide sequences present in binding partners (Fig. 1c). It is highly similar to the NMR structure of the human endophilin A1 and A3 SH3 [42]. Despite this high degree of structural similarity, the SH3 domains of the three paralogs can exhibit specificity in their interaction with binding partners (see, for example [43]).

Comparing the dimerization constant of the BAR domain (K_D, 10 μM) with a rough estimate of the synaptic concentration of endophilin A (~1 μM), Gallop et al. noted that the monomer might be the predominant form in the cytosol. However, more precise estimates of the free endophilin concentration in situ are needed to establish the cytosolic endophilin conformation with certainty. Electron paramagnetic resonance (EPR) studies have clearly indicated that the dimer is the predominant form on the membranes of small artificial vesicles [36, 44].

The Endophilin A Subfamily

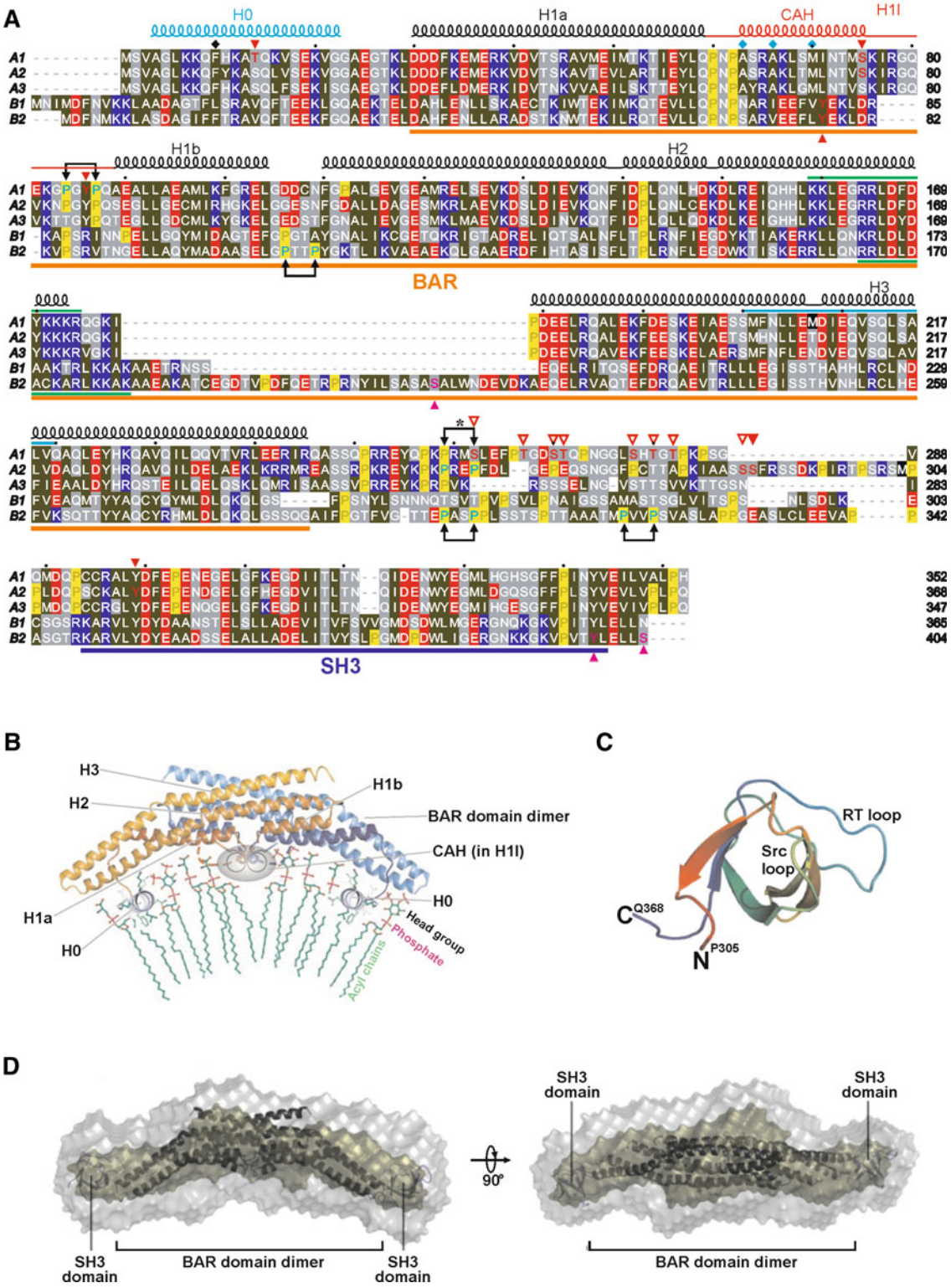
Synaptic Vesicle Endocytosis

Soon after their discovery, endophilins A were linked to synaptic vesicle endocytosis (SVE) [15, 29, 45]. In biochemical studies, they were found to bind synaptojanin and dynamin I, both major players in clathrin-dependent SVE [15, 29, 46]. Dynamin is a large GTPase involved in scission of the newly formed vesicles, whereas synaptojanin is a poly-phosphoinositide phosphatase involved in vesicle uncoating (reviewed in [47, 48]). Endophilin A is concentrated in presynaptic nerve terminals, and strongly overlaps in distribution with dynamin I, synaptojanin and amphiphysin I [15]. Amphiphysin is a brain-enriched protein with structural and functional resemblance to endophilin (reviewed in [49]) and involved in SVE in mammals but not flies [50–52].

The importance of endophilin A in SVE was first verified by ultrastructural analysis in the lamprey. Microinjection of endophilin antibodies into giant axons caused a stimulus-dependent depletion of synaptic vesicles and produced large infoldings of the plasma membrane—hallmarks of a severe block in SVE [53]. A defect in SVE was also demonstrated in *Drosophila endoA* mutants by electrophysiology and the endocytic marker FM1-43 [13, 14; see also 22].

Observations in both the lamprey and *Drosophila* models suggest that endophilin acts at multiple steps of the endocytic process. In the first microinjection study with endoSH3-directed antibodies, numerous shallow clathrin-coated pits accumulated around the active zones [53]. Such shallow clathrin-coated pits were recently observed also after microinjection of whole antibodies or Fab fragments targeting the endophilin BAR domain [54]. Moreover, shallow coated pits were detected in boutons of *Drosophila* mutants with partial loss-of-function mutations in *endoA* [13, 14; see also 22]. Although these observations are consistent with a role of endophilin in promoting the invagination of coated pits, it remains unclear whether this function may involve membrane bending, or reorganization of the clathrin coat or the actin cytoskeleton. In relation to the role of endophilin early in SVE suggested by these findings, it may be noted that also dynamin has been implicated at early stages of endocytosis, in addition to its established role in fission [48].

In another fly study, based on complete loss-of-function alleles, shallow coated pits were also observed, but a more prominent finding was accumulation of late-stage, deeply invaginated coated pits in boutons severely depleted of vesicles [14]. Late-stage coated pits also accumulated in lamprey axons microinjected with a peptide (PP-19) that blocks the binding of endophilin to dynamin (and with synaptojanin, see below) [46]. Detailed analysis of these coated pits failed to reveal any dynamin rings similar to those seen in *Drosophila shibire* (dynamin) mutants. This suggests that endophilin may work upstream of dynamin to mediate its recruitment to fission at the neck region. This possibility recently gained support from studies in non-neuronal cells [55]. In mouse fibroblasts lacking all



dynamin isoforms, clathrin-coated pits were shown to exhibit long narrow necks covered with endophilin. These and other findings suggest a model in which the BAR domain of endophilin (together with other BAR proteins and actin) promotes narrowing of the neck region prior to the recruitment of dynamin [55].

Studies in different systems have also implicated endophilin in the recruitment of synaptojanin, the protein implicated in regulation of vesicle uncoating. Microinjection in the lamprey giant synapse of PP-19 led to the appearance of numerous free coated vesicles, in addition to the deeply invaginated coated pits discussed above [46].

◀ **Fig. 1** Endophilin structure. **a** Alignment of human endophilin A1 (accession EAW58661.1), A2 (EAW69229.1), A3 (EAW62422.1), B1 (EAW73181.1), and B2 (CAI12365.1). Hydrophobic (*olive green*), positively charged (*dark blue*), negatively charged (*red*), and proline residues (*yellow*) are indicated. The three helices, H1a/b, H2 and H3, which constitute the BAR domain backbone are indicated (*black spirals*); extended connections between the spirals correspond to helical kinks. The amphipathic Helix 0 (H0, *light blue spirals*) that precedes the BAR domain, and the Helix 1 insert (H1I) with the central amphipathic helix (CAH)(*red horizontal line with spiral*) are indicated. *Red triangles/residue lettering* indicates phosphorylation sites in human endophilins, with high and low confidence sites marked by closed and open triangles, respectively. *Pink triangles/lettering* indicate conserved residues identified as phosphosites in mouse orthologues. The *green bars* above and below the alignment indicate putative nuclear localization domains in endophilins A and B, respectively (NLD consensus: two basic residues, 10 spacer residues, and another basic region consisting of at least three basic residues out of five residues). This region also harbors a positive patch (171-173KKK in endophilins A) at each tip of the BAR crescent, thought to promote the interaction with the negatively charged membrane. The *light blue bar* marks a region critical for efficient binding of endophilins A to Ca^{2+}-channels [39]. Each pair of *connected arrows* indicate a minimal proline-rich domain (PRD; P-X-X-P). The *asterisk* marks the PRD proposed to interact with the endophilin SH3 domain in a Ca^{2+}-dependent manner [39]. Mutations of 10F (*black diamond*) or 63A/66A/70 M (*light blue diamonds*) perturb the amphipathic helices, and thereby the membrane interactions of endophilin (see text). **b** Binding of the endophilin N-BAR dimer to the cytoplasmic leaflet of the phospholipid bilayer. Modified from Gallop et al. [36]. Reprinted by permission from Macmillan Publishers Ltd: The EMBO Journal, copyright 1996. H0 is disordered in solution but adopts an α-helical fold upon membrane binding. The CAH is partially preformed in solution but becomes much more ordered upon membrane binding. H0 and the CAH both tend to be oriented orthogonally to the long axis of the BAR crescent, as shown. With this orientation, the curvature induced by their wedge effect matches the curvature of the BAR concavity. A detailed description of the CAH interaction with the membrane can be found in Jao et al. [44]. **c** The crystal structure of the endophilin A2 SH3 domain. Reproduced from Loll et al. [41], with permission (http://journals.iucr.org/). **d** The SAXS-based shape reconstruction of mouse endophilin A2 in solution. The crystal structures of the N-BAR and SH3 domains have been manually docked into the SAXS envelope. Reproduced from Wang et al. [59], with permission from Elsevier, copyright 2008. (Color figure online)

Strong evidence of a functional interaction between endophilin and synaptojanin was also obtained in *Drosophila*. Firstly, synaptojanin gene (*synj*) null mutants exhibited defects strikingly similar to those of *endoA* nulls, both at the electrophysiological and ultrastructural levels. Also, the phenotype of *synj*; *endoA* double mutants did not differ substantially from that of the individual *synj* or *endoA* mutants. Moreover, *endoA* overexpression could partially rescue partial loss-of-function *synj* mutants. Finally, in *endoA* null mutants, synaptojanin protein was mislocalized at the synapse, and found to be destabilized by immunoblotting. Similar results were obtained in *C. elegans*, including the demonstration that while GFP-synaptojanin is mislocalized in endophilin mutants, GFP-endophilin is targeted correctly in synaptojanin mutants [22].

To summarize, endophilin A is thought to act upstream of dynamin in the final stage of endocytosis and to assist synaptojanin in the uncoating of the retrieved vesicle. Also, endophilin probably has a function early in SVE that remains to be clarified.

Intramolecular Interactions?

It has been proposed that the endophilin SH3 domain can bind to a minimal proline-rich motif, located in the variable region of endophilin itself [39]. This interaction, unusual by being Ca^{2+} dependent, was suggested to negatively regulate binding of the convex surface of the endoBAR Helix 3 to Ca^{2+} channels (Fig. 1a) [39]. Another auto-interaction model is inspired by studies of syndapins, also known as pacsins (reviewed in [56]). Syndapins are F-BAR and SH3 domain-containing proteins that, like endophilin, are involved in synaptic vesicle recycling and receptor-mediated endocytosis. The isolated F-BAR domain of syndapin is able to tubulate membranes, but full-length syndapin is not [57, 58]. Crystal structures and other evidence indicate that this difference arises because the SH3 domain autoinhibits the tubulation activity of the F-BAR domain. Specifically, the SH3 domain forms a molecular clamp by contacting the positively charged concave face of the F-BAR domain. Whether a similar mechanism regulates the membrane deformation activity of endophilin is not known. However, endophilin differs from syndapin in that not only the isolated endoBAR domain but also full-length endophilin can tubulate membranes in vitro. Hence, at least in the absence of other proteins, endophilin does not appear to be autoinhibited. Moreover, the endophilin structure in solution has been determined by small angle X-ray scattering (SAXS; Fig. 1d) and found to be rigid [59]. Finally, both the SAXS and the membrane-bound structures are similar to the crystal structure [36–38, 59, 60]. Taken together, these findings suggest that endophilin function does not require extensive conformational changes.

Structure/Function Relationships Involving the Endophilin A Bar Domain

Purified endophilin can bind and evaginate lipid bilayers into narrow tubules [5, 37, 60, 61]. When increasing the protein concentration, endophilin can also produce small vesicles (see also [1]). This membrane bending ability relates to SVE, which includes the formation of a tubular vesicle neck that is normally kept short by the action of dynamin [55]. Several other proteins involved in clathrin-mediated endocytosis (CME) can also induce membrane curvature. The same is true for proteins engaged in the formation of tubular membrane invaginations, such as T-tubules or the subsynaptic reticulum at the *Drosophila* neuromuscular junction (NMJ).

The tubulation/vesiculation activity of endophilin resides in the N-terminus, and a fragment consisting of the first 125 residues is sufficient to exert it [5]. This fragment includes only half of the N-BAR domain and thus tubulation does not require the intact BAR crescent. Instead, it likely draws heavily on the membrane insertion and wedging effect of Helix 0 and the CAH, both included in the N-terminal fragment [62]. Mutating a conserved hydrophobic residue in Helix 0 to a negatively charged residue (phenylalanine to glutamate, *F10E*) abolishes or severely reduces the lipid binding and tubulation activity [5, 36]. Moreover, lipid-facing hydrophobic residues are found at positions 63, 66, and 70 in the CAH of mammalian endophilins. Introduction of a membrane-repulsive negative charge at one of these positions (*A66D*) completely disrupts the tubulation activity [37, see also 63]. Introduction of hydrophilic residues (*A63S/A66S/M70Q* or *"SSQ"*) at all three positions reduces the number of tubules. Interestingly, the tubular diameter is also increased, suggesting that the CAH might be a target for adjusting the membrane bending activity of endophilin. The *A66D* and *SSQ* mutations in CAH do not affect lipid binding, while some other CAH mutations reduce it [36].

A striking effect is observed when replacing Alanin-66 in the CAH with Tryptophan (*A66W*). Tryptophane is also a hydrophobic residue but takes up more space than alanine. The *A66W* mutation shifts the balance from tubulation to extensive vesiculation, and thus can be seen as a gain-of-function mutation in vitro [37, 63].

In addition to curvature induction, BAR domains are well known for their ability to recognize curved membranes [1]. To which extent curvature sensing is relevant to physiological endophilin function remains unknown. What is becoming clear, however, is that it is primarily Helix 0 rather than the BAR scaffold that mediates curvature sensing. Among other findings, this conclusion is based on a comparison of the binding of both motifs to single liposomes of varying diameters [64]. Strikingly, the curvature sensing ability of the isolated Helix 0 peptide matches that of the entire BAR domain. Recently, isolated amphipathic peptides located in other (including non-BAR) proteins have also been demonstrated to display strong curvature sensitivity [65]. Thus, the idea that BAR domains are exceptional in their ability to sense membrane curvature is loosing ground.

Effects of Targeted N-Bar Mutations in a Living System

The in vitro studies of membrane curvature induction have been very helpful for understanding the structure/function relationship of endophilin, especially by pinning down the dominant roles played by the amphipathic helices both in lipid binding, curvature induction and sensing. However, the readout from the tubulation assays is hard to translate into physiological conditions. For example, the *A66W* mutation enhances vesiculation at the expense of tubulation, but does this represent a gain or a loss of endophilin function at real synapses? To address such issues, we tested the ability of mutated *endoA* transgenes to rescue the viability and synaptic function of *endoA* null mutants in *Drosophila*. We found that rescue was totally prevented by *I10E*, the *Drosophila* mutation homologous to *F10E* that targets Helix 0. Milder effects were observed after mutating the CAH. For example, *A66D* did not impede rescue, and while *SSQ* diminished the viability and motor activity of adult flies, the larval viability and synaptic function were not affected. Replacing the entire H1I, which includes the CAH, with an alpha-helical stretch [37], substantially impeded endophilin function but did not abolish it. Thus, in accordance with the tubulation assays, our findings generally indicated that the integrity of Helix 0 is essential for basic endophilin activity, whereas the CAH may primarily serve a regulatory function and/or be critical under high SVE demand.

In the flies, expression of the $endoA^{A66W}$ mutant transgene led to a dramatic disruption of eye development, in addition to other developmental effects [66]. The mechanism(s) is not yet known. However, preliminary evidence suggests that $endoA^{A66W}$ expression impedes EGFR-mediated signaling, which controls many aspects of *Drosophila* eye development (A. Jung, unpublised observations). For example, the eye phenotype can be reverted by enhancing EGFR signaling in the *A66W* mutants. By working as a gain-of-function mutation, *A66W* might enhance the endophilin-regulated removal of EGFR from the cell surface, thereby inhibiting EGFR signaling (see below). If this scenario holds true, it would identify alteration of the protein-membrane interaction of endophilin as a central mechanism in its functional regulation.

Are endophilins A always, and only, Involved in Clathrin-Mediated Endocytosis?

In the first genetic study of synaptic endophilin A function, performed in the NMJ of *Drosophila endoA* null mutants, a severe reduction in the ability of this synapse to sustain neurotransmitter release during intense stimulation was observed [14] (see above). With endophilin being involved in CME, this indicates that CME is normally the major mechanism underlying retrieval of synaptic vesicles. It was also observed that some steady-state release persisted in the *endoA* nulls (10–20% of the initial level). The authors found no positive evidence of remaining clathrin-dependent endocytosis, and suggested that the kiss-and-run mode of vesicle recycling is the basis for the residual release. In kiss-and-run, the synaptic vesicles release transmitter through a small pore, without collapsing into the plasma membrane. Hence, there is no need for vesicle reformation

by clathrin-dependent endocytosis (reviewed in [67, 68]). The assertion that kiss-and-run underlies the residual release in *endoA* nulls was later challenged [69]. However, those involved in this dispute all seemed to agree that endophilin serves CME, not kiss-and-run.

Taking the opposite viewpoint, McMahon and colleagues posited in 2006 that endophilin might possibly be involved in kiss-and-run. Also, with reference to CME, they stated that "…the observed accumulation of clathrin-coated intermediates after endophilin depletion could equally be due to a compensatory upregulation in this pathway as opposed to its inhibition." [36]. In the case of SVE, we find this alternative scenario hard to reconcile with the available data. For example, it seems unlikely that compensatory upregulation rather than a block in CME should lead to massive accumulation of coated intermediates at specific stages, as observed when perturbing endophilin. Also, a large body of genetic, cell biological, and biochemical evidence links the function of endophilin closely together with that of synaptojanin, a major player in CME but never implicated in kiss-and-run [8, 22, 29, 46, 70]. We thus believe that the viewpoint expressed by McMahon and colleagues is untenable when it comes to SVE. However, it is important to emphasize that an involvement of endophilin in any form of CME should not be taken for granted without positive evidence.

At present, little is known about the possible role of endophilin in the several other forms of endocytosis that exist besides CME (reviewed in [71]).

The Endophilin B Subfamily

Structure and Localization in Comparison to Endophilin A

Higher-order structural information about endophilins B is not yet available. However, based on the solved structure of endophilin A domains and the use of alignments and secondary structure prediction tools, some reasonable conclusions can be drawn [37, 72]. The core structure of the BAR dimer is conserved in endophilin B, as well as the presence of a Helix 0 and a H1I with a CAH. Helix 0 in endophilin B might be slightly longer (one or two turns) than in endophilin A. Perhaps the most striking difference between endophilin A and B is that the loop region between Helix 2 and 3, located at the tips of the endoBAR crescent, is generally longer in B than in A (Fig. 1a). The role of this extended loop in endophilin B is unknown. However, a couple of observations suggest that it might serve important functions. Firstly, the loop is subject to post-translational modification (see below). Secondly, a somatic mutation in the loop of endophilin B1 was detected in the DNA from a neuroendocrine carcinoma [73].

Whereas endophilins A are involved in plasma membrane internalization, endophilins B are largely associated with intracellular organelles, where they are implicated in the control of membrane dynamics. What is the molecular basis for this differential distribution? Mochizuki and colleagues transformed COS-7 cells with wildtype or chimeric forms of the endoBAR, and obtained qualitative data suggesting that the distribution of endoBAR depends on the origin of the H1I [37]. For example, wildtype endoBAR-A1 was most exclusively localized to the plasma membrane, whereas wildtype endoBAR-B1 localized to the perinuclear cytoplasm. A chimeric mutant of endoBAR-B1 in which the original B1 H1I was replaced with the H1I from A1 was both found in cytoplasm and at the plasma membrane. This suggests that the endoA1-H1I tends to bias the endophilin distribution towards the plasma membrane. However, protein regions outside the H1I must also influence the subcellular localization, as a mutant lacking the H1I still localized to the plasma membrane [37]. Accordingly, endophilin lacking the H1I does not nullify endophilin A function in *Drosophila*, as would be expected if plasma membrane targeting was completely abrogated [66]. Another COS-7 cell study demonstrated a strong accumulation of endophilin A1 (and A3) in a perinuclear, cytoplasmic tubulo-vesicular network thought to largely represent the ER. Distinct plasma membrane labeling was also observed, but only in some of the cells shown [27]. Although these findings in cell cultures are valuable, we feel that more studies, including rigorous data quantification and also involving cells in situ, are needed to fully establish the roles of HI1 and other endophilin domains in the subcellular distribution of endophilin. Moreover, nothing is known about the targeting mechanisms at the level of molecular interactions.

Endophilin B1: Mitochondria, Apoptosis and Autophagy

Bax (B-cell lymphoma-2-associated X protein) is the bait used in two-hybrid screens to identify endophilin B [11, 12]. It is a multidomain pro-apoptotic protein located in the cytosol in healthy cells. Upon reception of death signals, Bax translocates to the outer mitochondrial membrane (OMM). It also undergoes a conformational change needed for its activation. Together with a similar protein (BAK), activated Bax permeabilizes the OMM through a mechanism that is not fully clarified (reviewed in [74]). By allowing the release of cytochrome *c* from the mitochondria, OMM permeabilization triggers the caspase cascade leading to cell death. In HeLa cells, RNAi-mediated knockdown of endophilin B1 expression abolishes the conformational change of Bax, as well as the release of cytochrome *c* and caspase activation [75]. Conversely,

over-expression of endophilin B1 has opposite, pro-apoptotic, effects [11]. In healthy cells, endophilin B1 is mostly distributed in the cytosol. However, a fraction of the protein also resides on intracellular membraneous compartments, including the Golgi complex and the mitochondria [5, 11, 32]. Further work has led to a model in which endophilin B1 translocates massively from the cytosol to the OMM in response to apoptotic stimulation [11, 32, 75]. At the OMM, endophilin B1 transiently interacts with the similarly translocated Bax and stimulates its conformational change, thus acting to support apoptosis. An interesting aspect of the interaction between Bax and endophilin B1 is that it constitutes a target for oncogenic Src kinase, as discussed below.

Yeast two-hybrid analysis has demonstrated that an endophilin B1 deletion mutant consisting of residues 1–84 is able to bind Bax. By contrast, binding to Bax is abrogated both by truncating the eight N-terminal residues of endophilin B1 and by a point mutation (*Val5Met*) also at the N terminus [12]. Hence, the extreme N terminus of endophilin B1 is critical for the Bax interaction. By occurring immediately next to Helix 0, the binding of Bax to endophilin B1 would be expected to strongly affect the dynamics of endophilin B1, although it is unclear exactly how.

Recently, the interaction between endophilin B1, Bax and the OMM was modeled in vitro by incubation of purified proteins together with large vesicles having an OMM-like membrane lipid composition [76]. It was found that the pro-apoptotic conformational change of Bax takes place on the OMM, not in the cytoplasmic solution. Also, the change can be induced by the endophilin B1 N-BAR domain alone; full-length endophilin is not required. Interestingly, the mitochondrion-specific membrane lipid cardiolipin is needed for the BAX conformational change to take place. The reason for this requirement is unknown. Finally, although the endoBAR is able to deform OMM-like liposomes, this ability is distinct from its ability to activate BAX [76].

In healthy cells not undergoing apoptosis, endophilin B1 stabilizes the normal morphology of the mitochondrial network [32]. Knockdown of endophilin B1 in HeLa cells induced formation of misshaped mitochondria with a highly variable diameter and unusual branching patterns. Knockdown of dynamin related protein-1 (Drp-1) produced a different phenotype, characterized by formation of a highly connected mitochondrial network. Simultaneous knockdown of endophilin B1 and Drp-1 produced the same phenotype as Drp-1 knockdown alone, leading to the conclusion that Drp-1 acts upstream of endophilin B1 in the control of the morphological dynamics of mitochondria [32]. Note that this is opposite to the belief that endophilins A act upstream of dynamin at the plasma membrane (see above) [55].

Recently, endophilin B1 has also been implicated in autophagy, a tightly regulated process that degrades the cell's own components. Autophagy serves several adaptive and homeostatic functions, and is also implicated in cell death and disease. A key component is the autophagosome, a double-membrane vesicle, in which organelles and proteins are sequestered and later degraded by fusion of the autophagosome with lysosomal compartments (reviewed in [77]). Formation of autophagosomes from precursor organelles depends on the early recruitment of class III phosphoinositide 3-kinase (PI3KC3) and Beclin-1/autophagy-related gene (ATG) 6, but also involves several other ATGs. In cell lines, Wang and colleagues observed that endophilin B1 translocates to early-stage autophagosomes during starvation, and that the number of autophagosomes is reduced in cells derived from endophilin B1 knockout mice [78]. This suggests that endophilin B1 aids in autophagosome formation. Subsequent studies demonstrated that endophilin B1 indirectly binds to Beclin-1 to stimulate PI3KC3 activity, although it probably also promotes autophagosome formation through other means [78]. Time-lapse microscopy further indicated that small vesicles with membrane-associated endophilin B1 expand by recruiting and fusing with vesicles carrying the membrane-integrated autophagy-related protein ATG9, a co-activator of PI3KC3 [79]. Again, this suggests that endophilin B1 may have an important role in the construction of autophagosomes.

It is well established that apoptosis is a negative regulator of tumorigenesis [80]. Increasing evidence suggests that this is also the case for autophagy. Since endophilin B1 is involved in both apoptosis and autophagy, this protein would therefore be predicted to act as a tumor suppressor. Accordingly, although endophilin B1 knockout mice develop normally, they display a significantly higher tumor incidence than controls [78]. This aspect of endophilin B1 function is treated more fully below.

Receptor Trafficking

Endophilins A

Endophilins participate in the regulation of receptor tyrosine kinase (RTK) trafficking and signaling. In particular, endophilin involvement in the internalization of the epidermal growth factor receptor (EGFR, or Erb-1) has been studied in detail [81]. Upon binding of EGF, the EGFR is autophosphorylated, which recruits and activates the E3 ubiquitin ligase Casitas B-lineage lymphoma (Cbl). Cbl ubiquitylates the EGFR, thereby triggering EGFR endocytosis. Adaptors such as Eps15 have ubiquitin-binding domains that enable them to recruit the ubiquitylated EGFR to clathrin-coated pits. In parallel, Eps15 promotes

the formation of AP2 complexes that stimulate the formation of the pits. Besides serving as a ubiquitin ligase, Cbl also recruits endophilin A1 via the adaptor Cbl-interacting protein of 85 kDa (CIN85), allowing endophilin A1 to assist in CME [81]. The Cbl–CIN85–endophilin A3 complex was similarly shown to strongly promote the downregulation of the hepatocyte growth factor (HGF) receptor Met, another RTK [82]. Transfection of COS7 cells with the isolated endophilin A3 SH3 domain prevented HGF bound to Met from being internalized, indicative of a dominant negative effect. Met receptor downregulation was also impaired in the endoSH3-transfected cells. Moreover, following HGF stimulation, the receptors remained in the phosphorylated (activated) state for longer than mock-transfected controls [82].

Thus, these data indicated that endophilin A1 and A3 promote RTK endocytosis, and that the signaling mediated by the receptors is inhibited as a result. However, the notion that endocytosis always serves to inhibit TRK signaling may be too simplistic (reviewed in [83]). Indeed, a case involving the third endophilin A paralog, endophilin A2, illustrates that a different relationship can exist between the two processes [84]. In this study, overexpression of endophilin A2 in 293T cells increased EGF-induced endocytosis of the EGFR, but it also increased the phosphorylation of ERK1/2. This suggests that the EGFR/Ras/MAPK cascade was activated rather than inhibited (although it should be kept in mind that increased EGFR phosphorylation was not demonstrated directly in this study).

To further complicate matters and going back to endophilin A3, this paralog has also been reported to *inhibit* receptor-mediated endocytosis, although this effect involved other receptor types than TRK [35]. In this study, transferrin uptake was analyzed in COS-7 cells. It was found that whereas transfection with endophilin A1 did not affect uptake efficiency, transfection with full-length endophilin A3 strongly reduced it. Moreover, transfection with an endophilin A1 chimera carrying the A3 instead of the original A1 variable region inhibited transferrin uptake with a similar potency as full-length endophilin A3. This strongly suggests that the variable region has a central role in regulating receptor-mediated endocytosis. Furthermore, secondary structure analysis indicated that the endophilin A1 (and A2) variable regions carry short α-helices in their N-terminal region. By contrast, this region contains a β-turn in endophilin A3. Subsequent mutational analyses revealed that this difference underlies much of the differential effects of endophilin A1 and A3.

This study also showed that transfection of full-length endophilin A3 inhibits agonist-induced endocytosis of the dopamine D2 receptor (D2R), a G protein-coupled receptor. By contrast, endophilin A1 enhanced basal D2R endocytosis, even before the application of dopamine. Thus, opposite effects of the two endophilin paralogs were demonstrated in the same system. Somewhat surprisingly, expression of the isolated SH3 domain of endophilin A3 also inhibited endocytosis, albeit less efficiently than full-length endophilin A3. This effect might be due to a dominant negative effect on the pro-endocytotic endophilin A1, as the SH3 domains of A1 and A3 are similar in structure [41]. In any case, these results demonstrate that both the SH3 domain and the variable region are key players in endophilin's regulation of receptor-coupled endocytosis, and that the variable region determines the direction of this influence (see also [31]).

Endophilins A have also been implicated in the trafficking of other types of receptors and membrane proteins, including the β1-adrenergic receptor and the vesicular glutamate transporter [20, 23].

Endophilins B and TRK Traffic

Endophilin B1 has recently been found to participate in the trafficking of TrkA, the TRK that serves as a high-affinity receptor for nerve growth factor (NGF). In PC12 cells, endophilin B1 partially colocalized with the early endosome antigene (EEA) and to a lesser extent with the lysosome marker LAMP2 [30]. Biochemical and immunocytochemical analysis further suggested that stimulation with NGF induces endocytosis of NGF-bound TrkA and accumulation of a NGF-TrkA-endophilin B1 complex in early endosomes. The complex then translocates via late endosomes to lysosomes. Along the way, endophilin B1 presumably detaches and returns to the early endosomes. In cells subjected to endophilin B1 knockdown, the TrkA migration from early endosomes to lysosomes was intensified, leading to increased TrkA degradation and reduced signaling downstream of TrkA activation. Another interesting observation was that endophilin B1 knockdown resulted in enlarged EEA-positive compartments. The mechanism is not fully clarified but evidence was provided in support of the idea that recycling from early endosomes back to the plasma membrane is compromised in endophilin B1-depleted cells [30].

Of note, the regulatory influence of endophilin B1 on the Trk receptor family appears to be rather specific. Firstly, endophilin B1 does not interact with the transferrin receptor. Also, downregulation of the transferrin receptor is not affected by endophilin B1 knockdown. Secondly, there seems to be no interaction between endophilin B1 and the EGFR [30]. This is in contrast to the well-documented involvement of endophilins A in EGFR endocytosis (see above).

Post-Translational Modification

Several of the presynaptic proteins that are essential for SVE are phosphoproteins, including dynamin I, amphiphysin I and II, synaptojanin, AP180, epsin, and Eps15. These proteins are denoted dephosphins by some investigators, because they are dephosphorylated by calcineurin in response to stimulation of the nerve terminals. Dephosphorylation stimulates SVE, and may be particularly important for triggering activity-dependent bulk endocytosis, a SV retrieval pathway that supplements CME during intense synaptic activity (reviewed in [85]). Phosphorylation by cyclin-dependent kinase 5 (Cdk5) inhibits the inositol 5-phosphatase activity of synaptojanin 1, while dephosphorylation by calcineurin stimulates it. Interestingly, binding of endophilin to synaptojanin also stimulates the phosphatase activity of synaptojanin [86]. One phosphorylation target of Cdk5 in synaptojanin is Serine-1144. A phosphomimetic mutation at this site reduces binding of endophilin to synaptojanin [86; see also 87]. Thus, dephosphorylation induced by synaptic activity assists both endophilin's recruitment and its stimulation of synaptojanin's enzymatic activity [86].

The phosphorylation status of endophilin itself at synapses is currently unclear. However, it is now evident that endophilins are in general subject to phosphorylation (Fig. 1a). In PC12 and other cell lines, human endophilin A1 is phosphorylated by Rho-kinase at Thr-14 [88]. Thr-14 phosphorylation inhibits the binding between endophilin and CIN85, and overexpression of endophilin A1 with the phosphomimetic mutation *T14D* inhibits EGFR endocytosis. This suggests that Thr-14 phosphorylation serves to regulate EGFR signaling. Thr-14 is located in H0, which has a critical role in membrane binding and deformation (see above). In addition to inhibiting endophilin binding to CIN85, Thr-14 phosphorylation might well abrogate endophilin-mediated membrane binding/curvature induction by perturbing Helix 0. Another example of Helix 0 phosphorylation comes from large-scale proteomics efforts applied to a *Drosophila* embryonic cell line, where Thr-20 in endophilin B has been identified as a phosphosite [89].

The CAH is also targeted by post-translational modification. Phosphoproteomics on HeLa cells has identified Ser-75, located at the C-terminal root of the CAH, as a phosphosite in human endophilin A1 [90]. Similarly, Ser-78 is a phosphosite in *Drosophila* endophilin A [89], Tyr-80 in human endophilin B1 [91], and Tyr-77 in endophilin B2 [92, 93]. In mammals, CAH phoshosites are implicated in cancer development (see below). To recapitulate, both amphipathic helices in the N-BAR domain can be phosphorylated, in accordance with their prominent functional role. Moreover, amphipathic helix phosphorylation is conserved, occurring in both mammals and flies.

The central variable domain is another preferred target of phosphorylation. Phosphoproteomics on *Drosophila* Schneider 2 cells identified a series of serines as phosphosites with high likelihood (Ser-285, Ser-289, Ser-293 and Ser-297; [90, 94]. Similarly, seven serine/threonine residues in the variable region of human endophilin A1 are potential phosphosites, although their true number may be lower due to some ambiguity associated with the technique used (Fig. 1a) [90].

The expanded loop in endophilin B (see above) has also been found to be phosphorylated in mammalian endophilin B2. Finally, in the endophilin B2 SH3 domain, both the terminal serine and the (−6) threonine at the C-terminus have consistently been identified as phosphosites (Fig. 1a) [90].

In conclusion, several phosphosites are found both in endophilin A and B. Although other explanations are possible, co-existence of lightly and heavily phosphorylated forms in the same tissues might help to explain the observation that endophilin A often migrates as a doublet on Western blots, both in *Drosophila* and mammals [66, 88, 95, 34, 82].

Endophilin Phosphorylation In Tumorigenesis

Endophilin phosphorylation has been linked to the development of neoplasms in several studies. Firstly, the Thr-14 mutation directed against Helix 0 and interfering with endophilin A1/CIN85 mutual binding (see the previous section), would be expected to promote cell transformation by deregulation of EGFR signaling [88]. Secondly, Tyr-86, located at the root of the endophilin A1 H1I, was found to be phosphorylated in a large-scale survey of phosphotyrosine sites in lysates from lung cancer cell lines and tumors [96]. Thirdly, in transformed fibroblasts, a complex of focal adhesion kinase (FAK) and Src kinase phosphorylates Tyr-315, located in the SH3 domain of endophilin A2 [97]. Tyr-315 phosphorylation inhibits the binding of endophilin to dynamin. Accordingly, this modification inhibits endocytosis of matrix metalloproteinases (MMPs), enzymes that attack the extracellular matrix. The resulting increase in the cell-surface presentation of the MMPs would tend to aggravate invasive tumor growth [97].

In addition to endophilin A2, Src kinase also interferes with an anoikis-related function of endophilin B1, thus targeting members of both endophilin subfamilies [91]. Anoikis is a Bax-dependent form of apoptosis triggered by cell detachment, which serves to impede metastasis. Binding between Bax and endophilin B1 promotes anoikis and thus counteracts the spreading of tumor cells. However, the protective interaction between the two proteins is suppressed by Src-dependent phosphorylation at Tyr-80, located in the CAH of endophilin B1 [91]. In addition to perturbing Bax/

endophilin B1 binding, Tyr-80 phosphorylation would be expected to diminish the curvature-inducing activity of endophilin B1, which is likely to depend partially on the CAH as observed in endophilin A.

With a few exceptions, such as the Src and Rho kinases, the kinases responsible for phosphorylating the endophilin residues discussed above remains to be identified.

Endophilins and Disease

No clinical condition is known to be caused specifically by a dysfunction of endophilins A or B. While endophilins have been linked sporadically to other pathological processes such as virus production [98] and development of scoliosis [99], their most intimate relationship with disease clearly appears to be with cancer. In addition, endophilins interact with a number of proteins whose mutated forms are implicated in neurodegenerative disorders.

Cancer

Mechanisms whereby endophilin dysfunction might promote the development of malignant processes have been discussed above. Here, we expand on this theme, giving more reference to the clinic. Regarding endophilin A1, a series of recent studies found reduced expression in neoplasms of various origins, such as breast carcinoma (alterations in the endophilin A1 gene detected in one third of a tumor sample; [100], laryngeal carcinoma [101], pituitary adenomas [102] and other tumors [103]). Known causes of reduced endophilin A1 expression include deletion, promotor methylation and, more rarely, point mutations [100, 104; see also 105]. Notably, in the breast carcinoma study, alterations of the endophilin A1 gene were associated with poor prognosis [100]. Moreover, endophilin A1 expression was strongly reduced in metastatic tumors, compared to those that did not develop metastasis [104].

The consistent downregulation of endophilin A1 in neoplasms points towards a role of this isoform as a tumor suppressor. This is in accordance with the ability of the CIN85-endophilin A1 complex to assist downregulation of EGFR and other growth factor receptors, so that loss of endophilin expression or function (e.g., by phosphorylation) would promote cell growth and proliferation through receptor over-activation. The importance of the CIN85-linked regulatory function of endophilin A1 in relation to clinical cancer remains to be fully clarified. However, in a study looking for predictive markers of chemotherapy resistance in ovarian carcinomas, losses in one or both of the two loci encoding CIN85 and endophilin A1 was detected in 70% of the resistant tumors, but only in 15% of the treatment-sensitive tumors [106].

The endophilin A2 paralog interacts with the cytoplasmic protein endophilin binding protein (EBP). EBP is an intersectin-like protein that binds endophilin A2 simultaneously with Sos, a guanine nucleotide exchange factor (GEF) for the Ras GTPase. Interestingly, the cytoplasmic EBP-endophilin A2 complex suppresses Ras signaling and Ras-induced cellular transformation. This suggests that, similarly to endophilin A1, endophilin A2 could act as a tumor suppressor [43]. On the other hand, over-expression of endophilin A2 has been shown to increase, rather than decrease, ERK1/2 activation (see above, [84]). Moreover, upregulation of endophilin A2 can be oncogenic, as nude mice injected with NIH3T3 cells transfected with endophilin A2 develop tumors [107].

Endophilin A2 is also involved in an oncogenic fusion with the MLL protein, providing the historical basis for the endophilin A2 alias EEN (see "Discovery" section). The *MLL* (myeloid–lymphoid leukemia) gene encodes a histone methyl transferase that regulates HOX genes and embryonic development. Chromosomal rearrangements involving *MLL* are associated with infant leukemias of poor prognosis. Translocations of *MLL* leads to production of chimeric proteins in which the MLL N-terminus is fused with the C-terminus of the fusion partner. Several fusion partners have been found, one of which is Endophilin A2 (EEN). How the MLL-endophilin A2 fusion protein promotes leukemia is still unclear, but some possible mechanisms have emerged. First, while wildtype endophilin normally localizes to the cytoplasm, MLL-endophilin A2 localizes to the nucleus. When both proteins are expressed in the same cell, endophilin A2 is translocated to the nucleus as a result of heterodimer formation with MLL-endophilin A2 [26]. The resulting reduction of the endophilin A2 level in the cytoplasm was proposed to attenuate the endocytosis-based downregulation of growth factor receptors, causing over-activation of their associated signaling cascades. Nuclear sequestration of endophilin A2 might also reduce the ability of the EBP-endophilin A2 complex to counteract Ras-induced cell transformation. This effect would be made even worse by the fact that MLL-endophilin A2 also partially sequesters EBP [43]. The assumption underlying these suggested mechanisms is that endophilin A2 acts as a tumor suppressor. However, as noted above, endophilin A2 can also promote tumorigenesis.

Expression of the MLL-endophilin A2 fusion protein inhibits the development and function (including endocytosis) of dendritic cells in a leukemia mouse model. Dendritic cells (DC) are thought to play a central role in the induction of anti-tumor immunity. The MLL-endophilin A2 has been suggested to shift the cell developmental program away from that leading to proper DC development [108]. More work is required to clarify to what extent this

and other mechanisms contribute to the oncogenic effect of the MLL-endophilin A2 fusion. In conclusion, the contribution of endophilin A2 to clinical cancer is poorly understood and is likely to be complex.

Endophilin A3 expression is lower in oral squamous cell carcinoma tumors with lymph node metastasis than in those without metastasis [109]. Moreover, endophilin A3 interacts directly with metastasis-associated protein 1 (Mta1), so named because the expression level of the corresponding gene correlates with the metastatic tendency of cancer cell lines and tissues [24]. Unfortunately, the implication of the endophilin A3-Mta1 interaction is unknown.

Turning towards the other endophilin subfamily, endophilin B1 mRNA expression was found by gene profiling to be downregulated in lung carcinomas [110] and by immunohistochemistry to be reduced in malignant epithelial cells in the stomach, compared to normal mucosal cells [111]. Subsequently, Wang and colleagues reported that endophilin B1 expression is also reduced during the malignant transition from normal colorectal mucosa to colorectal adenocarcinoma, both at the mRNA and protein levels [112]. In accordance with these results showing endophilin B1 downregulation, the chromosomal region harboring the endophilin B1 gene suffers from loss of heterozygosity in a wide variety of tumor types. ([113]; see also references provided in [112]). Recently, Lim and colleagues quantified protein expression in four different cell lines with increasing metastatic potential in a mouse model [114]. About 200 of the more than 1,000 proteins analyzed displayed altered expression when comparing non-metastatic cells with cells of increased metastatic propensity. One of these proteins was endophilin B1, which also in this case showed a reduction in expression. By immunohistochemistry on human breast tumor tissue, endophilin B1 expression was also observed to be lower in invasive carcinoma than in metastatic carcinoma obtained from lymph nodes [114].

These studies thus suggest that endophilin B1 expression is invariably reduced in cancer. However, other reports undermine this simple picture, although they are fewer in number. Firstly, in addition to colorectal cancer [112], Wang and colleagues also evaluated endophilin B1 expression in prostate cancer [115]. This time their data clearly indicated an *increase* in endophilin B1 expression, occurring at the transition from benign prostatic hyperplasia (a physiological cell proliferation) to high grade prostatic intraepithelial neoplasia (a putative premalignant lesion of adenocarcinoma). Secondly, high-intensity diffuse cytoplasmic endophilin B1 expression was described in Merkel cell carcinoma of the skin [116]. Finally, in a study aiming to identify somatic mutations of the endophilin B1 gene in leukemias and carcinomas of various types, only one mutation was detected among 284 cancer tissues (located in the extended H2/H3 loop as already mentioned above). This led the authors to conclude that endophilin B1 mutations are rare in cancers, so that endophilin B1 deregulation must be explained by other mechanisms [73].

Interestingly, the typical finding concerning endophilin B2, the other endophilin B paralog, has also been that of increased expression. Endophilin B2 expression was both elevated in primary tumors in a transgenic mouse model of prostate cancer [117] and in malignant tumors derived from tissues of developing teeth [118]. Moreover, Brooks, Pollack and their colleagues used gene expression profiling and cluster analysis to define three classes of prostate cancer based on distinct expression patterns. Endophilin B2 was most highly expressed in lymph node metastases of the most aggressive class [119; see also 120].

Thus, to summarize the endophilin expression changes found to be associated with cancer development: the endophilin A isoforms are downregulated, endophilin B1 is most often downregulated but may be upregulated, and B2 is upregulated. There is no obvious explanation for the mixed results obtained for endophilin B1, nor for the general endophilin B1 downregulation/B2 upregulation discrepancy. However, speculations can be made based on the involvement of these proteins in apoptosis. Firstly, upregulation of pro-apoptotic factors such as endophilin B1 is the expected reaction to molecular defects that threaten to allow a cell to liberate itself from proliferative control. However, for those cells that manage to escape apoptosis and continue to accumulate genetic alterations, the selection pressure would favor disruption of the pro-apoptotic machinery, including endophilin B1. The affected cells would then be either in an early state in which pro-apoptotic factors are upregulated or in an advanced—and genetically more deranged—state, in which endophilin B1 and other pro-apoptotic proteins are downregulated or eliminated. The relative duration and distribution of these states in the tumor cell population would determine the main direction taken by the endophilin B1 expression at any given moment.

The generally opposite changes in expression observed for the endophilin B1 and B2 paralogs can be explained by assuming that B2 is pro-apoptotic and, similarly to B1, strives to eliminate the cell in the critical phase before control over it is lost. However, endophilin B2 might for some reason be a less sensitive target for downregulation than B1, and therefore persist in an upregulated state. Alternatively, endophilin B2 is anti-apoptotic, so that cells with upregulated endophilin B2 are positively selected during tumor development. The observation that the highest endophilin B2 expression is seen in the most aggressive tumors [119] might be taken as support for the latter of the two possibilities.

Neurodegenerative Diseases

Endophilins A interact with a variety of proteins responsible for neurodegenerative disease. Wanker and colleagues reported that endophilin A3 antibodies co-immunoprecipitated huntingtin in brain extract from a patient with Huntingtons disease (HD); [34]. They also found that endophilin A3 binds huntingtin exon 1 protein (HDex1p). HDex1p is an N-terminal fragment of huntingtin that contains the polyglutamine (poly-Q) tract whose degree of expansion correlates with the gravity of the disease (reviewed in [121]). The binding of endophilin A3 to HDex1p was enhanced by poly-Q expansion. Moreover, transfected HDex1p carrying an expanded poly-Q tract formed insoluble aggregates in COS-1 cells, and the amount of these aggregates was doubled by co-expression of endophilin A3. How endophilin exerts this effect is not known with certainty. However, there is evidence that the poly-Q tract is stabilized by a proline-rich domain (PRD) placed next to it, so that the risk of aggregation is reduced [122]. The PRD also mediates the interaction with endophilin, by binding to its SH3 domain. We speculate that the binding of endophilin might interfere negatively with the stabilizing effect of the PRD. In any case, the implications of these findings for endophilin's role in HD pathogenesis is unclear, as the toxicity of the aggregates is debated [121]. In addition to endophilin A3, mutant huntingtin perturbs several other synaptic proteins, including those involved in SVE. This may well contribute to the severe synaptic dysfunction seen in HD [123].

Another polyglutamine disorder, spinocerebellar ataxia type 2 (SCA2), is caused by an expanded poly-Q tract in ataxin-2. The cerebellar Purkinje cells are particularly vulnerable, but other neuronal types are also affected (reviewed in [124]). In COS-7 cells and cultured hippocampal neurons, endophilin A1 (and A3) colocalize with ataxin-2, in particular at the ER in the perinuclear zone [95]. Moreover, ataxin-2 binds to endophilins A1 and A3 in yeast and mammalian cells, including neurons. A role of the endophilin A/ataxin-2 interaction at the plasma membrane is indicated by the observation that ataxin-2 also binds Cbl, CIN85, and Src kinase [95]. Together with endophilin, these proteins form a complex involved in downregulation of RTK such as EGFR and Met (see above). In contrast to huntingtin, the interaction between ataxin-2 and endophilin was not affected by poly-Q expansion in ataxin-2. Interestingly, EGFR endocytosis was diminished in fibroblasts carrying a homozygous deletion in the gene encoding ataxin-2. This suggests that ataxin is a positive regulator of EGFR signaling [95].

Recently, an upregulation of endophilin A1 expression has been detected in the temporal cortex of patients suffering from Alzheimer's disease (AD) [125]. Endophilin A upregulation was also observed in an AD mouse model in the same study, and shown to result from the binding of the amyloid-β protein to amyloid-β binding alcohol dehydrogenase (ABAD). The amyloid-β/ABAD interaction is known to increase neuronal stress and to impair learning and memory. It remains unclear how the amyloid-β/ABAD interaction promotes endophilin expression. In transfected cortical neurons, endophilin enhanced the ability of germinal center kinase-like kinase (GCK) to stimulate another kinase, c-Jun N-terminal kinase (JNK) [125]. Such endophilin-dependent boosting of the JNK-stimulating activity of GCK had already been demonstrated earlier in the HEK293 cell line [126]. JNK is thought to contribute to AD pathogenesis by a number of mechanisms, including a triggering of neuronal apoptosis (reviewed in [127]). To summarize, endophilin is upregulated in AD and may contribute to cell death at least partly by activating JNK-dependent apoptosis.

Conclusion and Future Perspectives

Efforts of many research groups have amply documented the involvement of the endophilins in a wide variety of biological processes, both in health and disease. This functional diversity may appear remarkable at first sight, considering the few protein domains contained in these proteins. On the other hand, the ability of the BAR domain to actively interact with the ubiquitous cell membrane is useful in many contexts. Also, the SH3 domain is a versatile adaptor module. In this light, the diverse nature of the endophilin engagements may seem less surprising.

What more do we need to know? As regards endophilins A, we now have a much improved understanding of their interaction with the membrane. However, we still lack a precise description of their molecular actions, and of the timing and exact consequences of their dynamic interactions with binding partners such as dynamin and synaptojanin in the course of CME. Similarly, the manner in which endophlin B1 induces conformational changes in Bax, and why a particular OMM lipid is needed for this action, must be resolved. The dissociation between the BAX-regulating action of endophilin B1 and its membrane deformation activity may constitute a general warning against overemphasizing the latter activity of the endophilins.

The role of endophilin B2 is even more obscure than that of B1, as we do not know where and how B2 modifies the action of B1 and other binding partners. However, we should be able to obtain this information fairly quickly through genetic manipulations, combined with the methods already used to characterize the other endophilins. Moreover, it is still unresolved which factors that trigger endophilin recruitment to (and dismissal from) the membrane.

Could it be small GTPases, perhaps binding to the concave BAR surface—or would the presence of the H1I prevent such an interaction? Answers to such questions would greatly aid our understanding of the function also of the many other existing BAR domain proteins. The consequences of endophilin phosphorylation is another largely unresolved issue, in particular in relation to synaptic function. Luckily, the greatly advanced charting of endophilin phosphosites opens up for a systematic analysis of the importance of post-translational modification in synaptic and other processes, for example based on phoshomimetic or –blocking mutations. The identification of the endophilin-phosphorylating kinases is probably done most efficiently using mass spectrometry in combination with RNAi-based knockdown of genes encoding the various kinases, or similar large-scale strategies. Finally, concerning the role of endophilin in cancer, there is a lot of clinical evidence to suggest an involvement, but most of it has the nature of guilt by association. Moreover, the changes in the endophilin expression level that accompanies tumor development are not always consistent. It should be possible to design interventional experiments in animal models, in which more controlled changes in endophilin expression and function can be used to gauge the importance of endophilin dysfunction in tumorigenesis.

Clearly, the endophilinists will have a lot to do in the future!

Note added in proof In a recent paper (Bai et al. 2010. Cell 143: 430–441) it is demonstrated that in C. elegans endophilin null mutants, synaptic vesicle endocytosis can be rescued efficiently by endophilin protein lacking the SH3 domain, but not by protein containing a mutant N-BAR domain deficient in membrane tubulation. Another paper (Sundborger et al. J. Cell Sci., in press) provides evidence that in the lamprey synapse, endophilin recruits dynamin to a restricted part of the neck of clathrin-coated pits, forming a complex that promotes budding of new synaptic vesicles.

Acknowledgements The authors thank Viktor Lund for critical reading of the manuscript. Supported by the Dagmar Marshall Foundation and Fonden for Lægevidenskabens Fremme (O.K, A.J.) and the Swedish Research Council/DBRM (L.B.).

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