EHSH1/Intersectin, a Protein That Contains EH and SH3 Domains and Binds to Dynamin and SNAP-25

A PROTEIN CONNECTION BETWEEN EXOCYTOSIS AND ENDOCYTOSIS*

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In yeast two-hybrid screens for proteins that bind to SNAP-25 and may be involved in exocytosis, we isolated a protein called EHSH1 (for EH domain/SH3 domain-containing protein). Cloning of full-length cDNAs revealed that EHSH1 is composed of an N-terminal region with two EH domains, a central region that is enriched in lysine, leucine, glutamate, arginine, and glutamine (KLERQ domain), and a C-terminal region comprised of five SH3 domains. The third SH3 domain is alternatively spliced. Data bank searches demonstrated that EHSH1 is very similar to Xenopus and human intersectins and to human SH3P17. In addition, we identified expressed sequence tags that encode a second isoform of EHSH1, called EHSH2. EHSH1 is abundantly expressed in brain and at lower levels in all other tissues tested. In binding studies, we found that the central KLERQ domain of EHSH1 binds to recombinant or native brain SNAP-25 and SNAP-23. The C-terminal SH3 domains, by contrast, quantitatively interact with dynamin, a protein involved in endocytosis. Dynamin strongly binds to the alternatively spliced central SH3 domain (SH3C) and the two C-terminal SH3 domains (SH3D and SH3E) but not to the N-terminal SH3 domains (SH3A and SH3B). Immunoprecipitations confirmed that both dynamin and SNAP-25 are complexed to EHSH1 in brain. Our data suggest that EHSH1/intersectin may be a novel adaptor protein that couples endocytic membrane traffic to exocytosis. The ability of multiple SH3 domains in EHSH1 to bind to dynamin suggests that EHSH1 can cluster several dynamin molecules in a manner that is regulated by alternative splicing.

At the plasma membrane of all cells, vesicles are inserted by exocytosis and retrieved by endocytosis (1). A specialized form of exo- and endocytosis operates in nerve terminals (reviewed in Refs. 2–4). In nerve terminals, Ca\textsuperscript{2+} triggers the rapid exocytic fusion of synaptic vesicles with the presynaptic plasma membrane followed by fast endocytosis and recycling. This leads to millisecond trafficking reactions that are precisely timed and targeted. A critical step in synaptic vesicle exocytosis is the formation of the core complex between the vesicle protein synaptobrevin/VAMP and the plasma membrane proteins syntaxin 1 and SNAP-25. Botulinum and tetanus toxins inhibit exocytosis by cleaving the components of the core complex, demonstrating that the core complex is essential for exocytosis (4). Exocytosis outside of synapses probably utilizes mechanisms that are analogous to those of the synapse (5). It is likely that exocytosis in non-neuronal cells also requires the formation of a core complex composed of homologs of SNAP-25 (e.g. SNAP-23), syntaxin 1 (e.g. syntaxins 2, 3, and 4), and synaptobrevin (e.g. cellubrevin). Variations of the model developed for the synapse appear to operate in vesicular membrane traffic at the plasma membrane in all cells and possibly also in vesicular traffic between intracellular compartments. Although much is known about the core complex, its precise function or the mechanism of membrane fusion are unknown. One interesting property of synaptic vesicle exocytosis is the accurate timing of exocytosis. Synaptic vesicle exocytosis is faster and more tightly regulated than any other form of exocytosis. In nerve terminals, Ca\textsuperscript{2+} influx triggers exocytosis by an unknown mechanism that requires the presence of the synaptic vesicle proteins synaptotagmin I or II (6). Because synaptotagmins are Ca\textsuperscript{2+}-binding proteins (reviewed in Ref. 7), they probably represent Ca\textsuperscript{2+} sensors in exocytosis. It is unclear, however, whether Ca\textsuperscript{2+} binding to synaptotagmin regulates fusion and how this relates to core complex formation. After exocytosis, synaptic vesicles are retrieved by endocytosis. The mechanisms involved in endocytosis have been studied in great detail in nerve terminals and fibroblasts (for reviews see Refs. 8–10). The seminal discovery of clathrin coats and clathrin assembly proteins in endocytosis raised the question of how these coats invaginate (reviewed in Ref. 8). Analysis of a Drosophila mutation called shibire revealed that the GTPase dynamin is essential for invagination of clathrin-coated pits (9, 10). Dynamin is thought to drive endocytosis by interacting directly and indirectly with multiple components of the endocytic machinery and with phospholipids (reviewed in Refs. 11–13). Dynamin includes a proline-rich C-terminal sequence that binds to SH3 domains of several proteins, although largely nonstochiometrically (14–19). A large number of proteins with putative functions in endocytosis have been identified in addition to dynamin, clathrin, and the clathrin assembly proteins. This has led to a view whereby endocytosis is driven by a molecular superstructure involving at least 20 different proteins. Among these proteins are phosphoinositide phosphatases called synaptogamins (20–22), two closely related molecules called EPS15 and EPS15r that contain EH domains at their N terminus (23, 24), SH3 domain proteins called amphiphysins...
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Vector Construction and Protein Expression

Plasmids were constructed in pVP16-3, pbTM116, and pLexN for use as yeast two-hybrid prey and bait vectors, respectively; pGEX-KG was for expression as GST fusion proteins (37); and pCMV5 was for expression in COS cells. Most of the vectors used were described previously (33, 34, 38, 39) except for the following plasmids. 1) pGex-KG expression plasmids: pGexEHSH1A, residues 1–855; pGexEHSH1-1, residues 1–440; pGexEHSH1-5, residues 440–682; pGexEHSH1SH3A/B, residues 598–795; pGexEHSH1SH3C, residues 998–1070; pGexEHSH1SH3D/E, residues 1028–1217. 2) pCMV plasmids: pCMV5-EHSH1A, residues 1–1217 with 7 base pairs of 5’ and 200 base pairs of 3’-untranslated regions; pCMV525A-1 and pCMV525B-1, full-length human SNAP-25A and SNAP-25B. 3) pLexN plasmids: pLexN-EHSH1A, residues 14–855; pLexN-EHSH1-5, residues 440–682; pLexN-EHSH1SH3A/B, residues 598–795; pLexN-EHSH1SH3C, residues 998–1070. 4) pVP16-3 constructs: pVP16-3-EHSH1-5, residues 440–682. Proteins were expressed in bacteria or COS cells as described, and GST fusion proteins were purified as reported by Guan and Dixon (37).

Affinity Chromatography and Pull-down Experiments with Immobilized GST Fusion Proteins

Affinity Chromatography—Three frozen rat brains were homogenized in 20 ml of 10 mM HEPES-NaOH, pH 7.4, 0.1 mM NaCl, 1 mM EDTA, and 0.1 g/liter PMSF. Triton X-100 was added to 1% (v/v). The homogenate was extracted by end-over-end rotation for 1 h at 4 °C and centrifuged for 1 h at 100,000 × g to obtain total brain extracts as the supernatant (∼400 mg protein/3 rat brains). Total brain extract was precleared by incubation with glutathione agarose without GST fusion protein for 6 h at 4 °C, followed by centrifugation (800 × g for 2 min). 18 ml of precleared total brain extract was loaded onto the respective GST-protein affinity columns (1 ml of glutathione-agarose resin with 4–5 mg of GST fusion proteins attached) that had been preequilibrated with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM NaCl, 0.25% Triton X-100. Loading was performed by 10 recirculations of the brain extract over the columns under gravity flow at 4 °C. The flow-through from the last cycle was collected, and the columns were sequentially washed with 20 bed volumes of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM NaCl, and 0.5% CHAPS containing 0.1 mM NaCl or 0.5 mM NaCl. After washing, the columns were eluted with the same buffer containing 1 mM NaCl followed by SDS sample buffer. Samples were analyzed by SDS-PAGE and Coomassie staining (20 ml/lane) or immunoblotting (5 ml/lane).

Pull-down Experiments—Approximately 0.1 mg of GST fusion protein immobilized on glutathione agarose for most experiments) or extracts from transfected COS cells (1 mg of protein) in 10 mM HEPES-NaOH, pH 7.4, 0.1 mM NaCl, 0.1 g/liter PMSF, 1 mM EDTA, and 1% Triton X-100 by end-over-end rotation at 4 °C for 12–16 h. The beads were washed six times with 50 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 0.1 g/liter PMSF, 1 mM EDTA, and 1% Triton X-100, and retained proteins were analyzed by SDS-PAGE and Ponceau S staining or immunoblotting. For all experiments, GST alone or irrelevant GST fusion proteins were used as controls.

Immunoprecipitations

Four rat brains were extracted as described above (see affinity chromatography) except that 20 ml of 10 mM HEPES-NaOH, pH 7.4, 0.1 mM NaCl, 1 mM EDTA, 1 g/liter PMSF, 1% Triton X-100, 1 mM MgCl2, and 1 mM CaCl2 were used. Affinity columns (30 mg) of the brain extract were precleared by incubation with protein A- or G-Sepharose for 3 h at 4 °C and then reacted with affinity purified EHSH1 antibodies or preimmune serum (25 mg of protein) on ice for 12–16 h. A 50% slurry of protein A- or G-Sepharose beads (100 ml) were added and incubated with end-over-end rotation for 1 h at 4 °C. Beads were washed five times with the same buffer containing 50 mM Tris-HCl, pH 7.4 and analyzed by SDS-PAGE and immunoblotting.

Antibodies

Three antibodies were raised against GST fusion proteins of EHSH1: S750 was raised against GST-EHSH1–1 (residues 1–440); P229 was raised against GST-EHSH1SH3A/B (residues 598–795); and P227 was raised against GST-EHSH1SH3D/E (residues 1028–1217). Antibodies were affinity purified on immobilized GST fusion proteins as described (40). SNAP-23 antibodies were a gift of Dr. E. Link (Synaptic Systems, Goettingen, Germany). Dynamin antibodies were obtained from Trans-
The first and second SH3 domains (SH3A and SH3B) are available blots (CLONTECH) containing total RNA from rat tissues. Proteins were assayed with a Coomassie Blue-based assay previously (21, 34). Proteins were recovered (38). Proteins were dialyzed against 25,000. All other antibodies were reported previously (12). DAP160 is similar to SH3P18 but is not recognized by the clathrin assembly protein complex (37, 39). The C terminus of DAP160 is more similar to SH3P18 than to EHSH1 and thus may correspond to an EHSH1 isoform. Data bank searches also revealed that the human gene for EHSH1 has been largely sequenced on two adjacent segments on chromosome 21q11.1 (GenBank™ accession numbers AP000049 and AP000050). The gene contains at least 26 exons over 110 kilobases.

**RESULTS**

Identification of EHSH1: Similarities to Xenopus Intersectin, Mammalian SH3P17, and Drosophila DAP160—To find potential new membrane trafficking proteins, we performed yeast two-hybrid screens for proteins interacting with SNAP-25. Clones corresponding to two novel proteins were repeatedly isolated. These include a protein that we named EHSH1 because of its domain structure includes EH and SH3 domains (see below) and a second protein without defined domains that will be described elsewhere.

The yeast two-hybrid prey clones encoding EHSH1 were used to isolate full-length cDNA clones from conventional libraries. A complete amino acid sequence for EHSH1 was assembled from the cDNA sequences and employed in data bank searches. These revealed that the C-terminal third of EHSH1 is identical with SH3P17, a partial cDNA containing multiple SH3 domains (42), except that SH3P17 misses the central SH3 domain present in EHSH1, which is probably alternatively spliced (see below). In addition, after review of this manuscript the Xenopus and human sequences of intersectins were reported and EHSH1. EST data banks searches revealed that the SH3 domains are most similar to human EST clones of EHSH1. This indicates that this SH3 domain is subject to alternative splicing, resulting in proteins that include either four or five SH3 domains. In addition to this alternative splicing, a short sequence in SH3A also appears to be alternatively spliced based on EST data bank searches (underlined in Fig. 1). Both alternatively spliced sequences precisely correspond to exon-intron boundaries in the human gene structure (data not shown).

**Isoforms and Homologs of EHSH1**—EST data bank searches revealed the presence of a second protein closely related to EHSH1. EST sequences from both human and mice encoding this isoform were found (Fig. 2). We have named this protein EHSH2 because it is highly homologous to EHSH1, suggesting that it represents an isoform. Comparison of the EH domains from EHSH1 and EHSH2 with those from EPS15 and 15R shows that they are only distantly related (Fig. 2). DAP160 was initially thought to have only one EH domain (43) but analysis reveals that it also contains two N-terminal EH domains (data not shown). Although DAP160 is similar to EHSH1 in structure, its EH domains are more distantly related to EHSH1 and EHSH2 than they are to each other, suggesting that they are homologs and not orthologs.

**Tissue Distribution of EHSH1 Expression**—To determine which tissues express EHSH1, we performed RNA blotting analyses (Fig. 3). Blots containing equal amounts of poly(A)+-enriched RNA from different rat tissues were hybridized at high stringency with a 32P-labeled probe from the N terminus of EHSH1. mRNAs of approximately 4.2 and 5.5 kb were observed in all tissues. Brain contained the highest levels of the 5.5-kb mRNA and the lowest levels of the 4.2-kb mRNA (Fig. 3). In addition, a large mRNA (approximately 10 kb) was detected only in brain even after prolonged exposure (Fig. 3 and data not shown). Because blotting transfer of large mRNAs is much less efficient than transfer of smaller mRNAs, the relative signals of the 5.5- and 10-kb EHSH1 mRNAs do not necessarily reflect their relative abundance. What is the molecular origin of these distinct mRNAs? Their apparent size differences are too large (approximately 1.5 and 5 kb) to be caused by the two events of alternative splicing identified in the cDNA clones (15 and 213 base pairs). Even the 213-base pair alternatively spliced sequence would not be detectable with the resolution of an RNA blot. Furthermore, RNA blots probed with a probe from the SH3C domain that is alternatively spliced gave results similar to those shown in Fig. 3 (data not shown). mRNAs of different sizes are often caused by differential polyadenylation. However, in this case it seems likely that at least the difference between the two principal mRNAs observed here (5.5 and 10 kb) are due to alternative splicing, because after review of this manuscript, cDNA clones of human intersectin were reported in two variants (31). One of these cDNA variants is very large and encodes several C-terminal domains in addition to the EHSH1/intersectin domains we described above. Because of its size, this variant probably corresponds to the 10-kb message seen on RNA blots.

To determine whether the various mRNAs for EHSH1/intersectin are translated into proteins, we raised antibodies to different parts of EHSH1 and used them for immunoblotting experiments with total proteins from rat tissues. Of the tissues analyzed, all contained a reactive protein of approximately 140 kDa with variable abundance (Fig. 4). Brain expressed by far the highest levels of this protein. The apparent low abundance

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of this protein in muscle is probably due to the high levels of contractile proteins in muscle that dilute other proteins loaded when total proteins are compared between tissues. Direct comparisons of tissue samples with COS cells transfected with a full-length EHSH1 expression vector showed that the 140 kDa protein corresponds in size with full-length EHSH1 (data not shown). In addition to the 140-kDa protein, a 200-kDa EHSH1 protein was observed only in brain. This band was detected with multiple independent antibodies (data not shown). Because of its size and reactivity with multiple EHSH1 antibodies, this protein probably corresponds to the brain-specific splice variant reported for humans (31) and seen in our RNA blots (Fig. 3).

**Fig. 1. Structure of EHSH1.** A, domain model of EHSH1. EHSH1 is composed of two N-terminal EH domains (EH1 = residues 21–85; EH2 = residues 221–286), a central domain rich in lysine, leucine, glutamate, arginine, and glutamine (KLERQ domain, residues 326–702), and five C-terminal SH3 domains labeled A–E (SH3A = residues 738–802; SH3B = residues 911–967; SH3C = residues 994–1056; SH3D = residues 1072–1134; SH3E = residues 1155–1210). Of these, the SH3C domain is alternatively spliced. B, alignment of the rat EHSH1 sequence with that of Xenopus intersectin (GenBank™ accession number AF032118). Identical residues are shaded according to the following color code: EH domains, red; KLERQ domain, green; SH3 domains, blue; linking sequences, yellow. Alternatively spliced sequences in EHSH1 as identified in EST sequences are underlined. Sequences are numbered on the right. Note that the Xenopus intersectin sequence continues beyond the stop codon of the mammalian EHSH1 sequence (dotted line).
tergents or high salt, suggesting that it is a peripheral membrane protein. Immunoblotting analyses of rat brains from animals of different ages revealed that EHSH1 is expressed during embryonic development and throughout adult life (Fig. 3). A significant shift in the ratio of the upper to lower band during development was observed. The upper band increases in abundance after birth; only after postnatal day 18 is a decrease observed. The lower band, by contrast, becomes less prominent after postnatal day 4. The time course of the upper band agrees well with the time course of synaptic development in brain.

EHSH1 Binds to SNAP-25 and SNAP-23—Since we identified EHSH1 in yeast two-hybrid screens for SNAP-25 interacting proteins, the question arises of whether EHSH1 truly binds to SNAP-25. Furthermore, SNAP-25 is present in two splice variants in brain (SNAP-25A and SNAP-25B); both variants are primarily expressed in neurons and are highly homologous to a ubiquitously present protein called SNAP-23 (44, 45). This suggests that SNAP-25 and SNAP-23 perform similar functions in neuronal and non-neuronal cell, respectively, by interacting with the same or similar proteins. To test these questions and suggestions, we prepared GST fusion proteins of the N-terminal half of EHSH1 and of syntaxin 1 and used them together with GST control proteins in pull-down experiments with brain extract and transfected COS cells (Fig. 6). In these experiments, the use of brain extracts allowed us to analyze binding of SNAP-25 and of SNAP-23, whereas transfected COS cells were employed to study binding of SNAP-25A and SNAP-25B; both variants are primarily expressed in neurons and are highly homologous to a ubiquitously present protein called SNAP-23 (44, 45). This suggests that SNAP-25 and SNAP-23 perform similar functions in neuronal and non-neuronal cell, respectively, by interacting with the same or similar proteins. To test these questions and suggestions, we prepared GST fusion proteins of the N-terminal half of EHSH1 and of syntaxin 1 and used them together with GST control proteins in pull-down experiments with brain extract and transfected COS cells (Fig. 6). In these experiments, the use of brain extracts allowed us to analyze binding of SNAP-25 and of SNAP-23, whereas transfected COS cells were employed to study binding of SNAP-25A and SNAP-25B; both variants are primarily expressed in neurons and are highly homologous to a ubiquitously present protein called SNAP-23 (44, 45). This suggests that SNAP-25 and SNAP-23 perform similar functions in neuronal and non-neuronal cell, respectively, by interacting with the same or similar proteins. To test these questions and suggestions, we prepared GST fusion proteins of the N-terminal half of EHSH1 and of syntaxin 1 and used them together with GST control proteins in pull-down experiments with brain extract and transfected COS cells (Fig. 6). In these experiments, the use of brain extracts allowed us to analyze binding of SNAP-25 and of SNAP-23, whereas transfected COS cells were employed to study binding of SNAP-25A and SNAP-25B separately. GST-syntyxin was used as a positive control because syntaxin 1 is the tightest known binding partner for SNAP-25. Proteins bound to the GST columns were analyzed by immunoblotting with antibodies to SNAP-25 and SNAP-23, synaptotagmin, and various control proteins.

The pull-down experiments confirmed that EHSH1 biochemically interacts with SNAP-25 isoforms and with SNAP-23 (Fig. 6). Control immunoblots with multiple synaptic proteins (Rab3a, synaptophysin, synaptotagryin, Rab5, synapsins 1a, Ib, IIa, and IIb, SV2, synaptobrevin, and synaptotagmin) revealed that syntaxin 1 was bound to EHSH1 together with SNAP-25 but that no other synaptic protein tested bound (data not shown). Furthermore, syntaxin 1 expressed in COS cells by transfection was unable to bind in the absence of SNAP-25 (data not shown). Thus the binding of SNAP-25 to EHSH1 is specific for SNAP-25 but can occur with SNAP-25 being simultaneously bound to syntaxin 1. In the immunoblotting analysis of the pull-downs, the strengths of the interactions of SNAP-25 with EHSH1 and syntaxin 1 seemed to be comparable (Fig. 6A).
In the current study we describe a protein called EHSH1 with a fascinating structure composed of multiple copies of three principal regions: two N-terminal EH domains, a central coiled-coil domain that is highly charged and called the KLERQ domain, and five C-terminal SH3 domains. In addition to EHSH1, a second isoform called EHSH2 was identified in EST data banks. Possibly the most interesting property of EHSH1 is its interaction with two proteins involved in different phases of the exocytosis and endocytosis processes.

**DISCUSSION**

To assess this more quantitatively, we measured the interaction of SNAP-25 with EHSH1 and syntaxin 1 in yeast two-hybrid assays using liquid β-galactosidase determinations (Table 1). Although the binding of syntaxin to SNAP-25 was significantly stronger than that of EHSH1, the difference for full-length SNAP-25 was only 2-fold. Both EHSH1 prey clones used (pVPEHSH-5 and -21) interacted equally well. Because pVPEHSH-5 contains only sequences from the KLERQ domain, this domain is sufficient for binding SNAP-25 (Table 1).

We next tested whether all of the sequence of SNAP-25 is required for EHSH1 binding. Botulinum toxins A and E cleave the C terminus of SNAP-25 and inhibit exocytosis without abolishing syntaxin 1 binding (reviewed in Ref. 4). Truncated versions of SNAP-25 that lack the C-terminal residues that are removed by botulinum toxins A and E still bound to both syntaxin 1 and EHSH1 (Table 1). Interestingly, however, syntaxin binding was unchanged, whereas EHSH1 binding was decreased by more than 50%. Taken together, these data suggest that EHSH1 interacts with SNAP-25 nearly as strongly as syntaxin 1 in a reaction that involves the KLERQ domain of EHSH1 and the C terminus of SNAP-25.

**EHSH1 Binds to Dynamin—SH3 domains from amphiphysin, grb2, Crk, and SH3P4, P8, and P13 have been shown to bind to dynamin and/or synaptojanin (14–17, 19). Furthermore, EH domains have been implicated in endocytosis in a number of proteins (46–54). The striking presence of both SH3 and EH domains in EHSH1 posits the question of whether the SH3 domains of EHSH1 also bind to dynamin and/or synaptojanin. To address this question, we used GST fusion proteins of various SH3 domains from EHSH1 to affinity purify proteins from total brain extracts (Fig. 7). Bound proteins were analyzed by Coomassie staining and immunoblotting. Coomassie blue-stained gels of proteins bound to the SH3A/B, SH3C, and SH3D/E domains of EHSH1 revealed that a single major protein of approximately 96 kDa was selectively affinity purified with SH3C and SH3D/E domains. This protein was identified by immunoblots as dynamin (Fig. 7). Binding is specific because GST alone or GST-SH3A/B did not bind strongly, because no other protein was visible on the Coomassie-stained gels, and because immunoblots also showed that the sticky syntaxin 1 protein was not bound (Fig. 7). Dynamin was greatly enriched in the eluate compared with the total homogenate and quantitatively removed from the flow-through. The absence of dynamin from the flow-through indicates that the SH3 domains of EHSH1 bound all of the dynamin molecules present in the brain extract, showing that binding is stoichiometric and of high affinity.

To determine whether other proteins involved in endocytosis are also bound to the SH3 domains and, specifically, whether the first two SH3 domains possibly bind other proline-rich proteins, we analyzed proteins bound to GST fusion proteins of SH3A/B, SH3C, and SH3D/E by immunoblotting with antibodies to synaptojanin and synapsins (Fig. 8). No synapsin binding was observed (data not shown). Low levels of purified synaptojanin were detected only with SH3C and SH3D/E, which contained high levels of dynamin (Fig. 8). No synapsin binding was observed (data not shown). These results indicate that the SH3 domains are relatively specific for dynamin and bind only low levels of other proteins, possibly indirectly via dynamin.

The stoichiometric binding of dynamin to EHSH1 in vitro suggests an in vivo role. To test whether EHSH1 is also complexed to dynamin and SNAP-25 in vivo, we performed immunoprecipitation experiments from brain using two independent EHSH1 antibodies (Fig. 9). The immunoprecipitates were analyzed by immunoblotting for dynamin and SNAP-25. Both proteins were found to co-immunoprecipitate with EHSH1. Dynamin was co-precipitated to a larger extent than SNAP-25, but both were only detected in the immunoprecipitates with EHSH1 antibodies but not with control serum (Fig. 9). These results indicate that EHSH1 exists in a complex with dynamin and SNAP-25 in vivo.
membrane traffic: SNAP-25 and SNAP-23 in exocytosis and dynamin in endocytosis. Although these interactions were characterized primarily in vitro, the following evidence supports a physiological significance of the observed binding reactions: 1) EHS1 is highly enriched in brain but also expressed in other tissues. This distribution parallels the distribution of exo- and endocytic activity that is most prevalent in synapses but also present outside of synapses. 2) A high molecular

![Fig. 7. Binding of dynamin to SH3 domains from EHS1. Affinity chromatography was performed with different GST fusion proteins and rat brain homogenates. GST fusion proteins containing the first two SH3 domains of EHS1 (GST-EHS1SH3A/B), the alternatively spliced central SH3 domain (GST-EHS1SH3C), the last two SH3 domains (GST-EHS1SH3D/E), or GST alone were immobilized on glutathione agarose and packed into a column. Total rat brain homogenate (TH) solubilized in Triton X-100 was loaded onto the column, the flow through (FT) was collected, and the column was extensively washed with the loading buffer (W1 and W2) and sequentially eluted with buffer containing 1 M NaCl (E1) and SDS-PAGE sample buffer (E2). Fractions were analyzed by Coomassie Blue staining (top panels) or immunoblotting for dynamin, syntaxin, and SNAP-25 (bottom panels). Open arrows in the Coomassie-stained panels identify GST fusion proteins. Filled arrows mark dynamin. Numbers on the left indicate positions of molecular mass markers.](image1)

![Fig. 8. Co-purification of synaptojanin with dynamin on immobilized GST fusion proteins of the SH3 domains of EHS1. GST fusion proteins containing the first two SH3 domains, the central SH3 domain, the last two SH3 domains of EHS1 (GST-EHS1SH3A/B, -EHS1SH3C, and -EHS1SH3D/E, respectively), and a GST-only control were immobilized on glutathione agarose and used in pull-down experiments with total rat brain homogenates. Bound proteins were analyzed by SDS-PAGE followed by blotting to nitrocellulose membranes, which were then stained for proteins by Ponceau S (left panels) or analyzed by immunoblotting with antibodies to dynamin (top) or synaptojanin 1 (bottom). Arrows in the Ponceau S-stained panels point to dynamin signals. Numbers on the left indicate positions of molecular mass standards.](image2)

![Fig. 9. Co-immunoprecipitation of dynamin and SNAP-25 with EHS1. Proteins from rat brain homogenates (lanes 4) were immunoprecipitated with two distinct antibodies to EHS1 (lanes 1 and 2) and with preimmune serum for antibody (B) (lane 3). Immunoprecipitates were analyzed by immunoblotting with antibodies to dynamin (top) or SNAP-25 (bottom); signals were visualized by ECL. Numbers on the left indicate positions of molecular mass markers.](image3)
weight neuronal variant of EHSH1 is highly enriched in brain. This agrees well with the specific enrichment of certain dynamin and SNAP-25 isoforms in brain. 3) SNAP-25 binding was confirmed by three independent methods, yeast two-hybrid assays, GST fusion protein pull-downs, and immunoprecipitations. 4) Two different splice variants of SNAP-25 and the ubiquitous homolog SNAP-23 bound to EHSH1 in interactions that were almost as strong as the interactions of the same proteins with syntaxin 1, the strongest currently known binding partner of SNAP-25. 5) A discrete domain in EHSH1, the KLERQ sequence, bound to SNAP-25. 6) Dynamin quantitatively bound to the SH3 domains of EHSH1 as defined in pull-down experiments and immunoprecipitations. Most significantly, the SH3 domains of EHSH1 were capable of completely removing dynamin from the brain extract. No other protein was visible on Coomassie Blue-stained gels. 7) Binding of dynamin to EHSH1 was specific for three of the five C-terminal SH3 domains. Conversely, other endocytic proteins exhibited very little binding.

After the present paper had been reviewed, the sequences of *Xenopus* and human intersectins were published (30, 31). The intersectins likely are orthologs of EHSH1. *Xenopus* intersectin was studied for its binding reactions and was found to bind via its N-terminal domain to a variety of NPF-containing sequences, whereas the C-terminal domain, in agreement with our results, was found to pull down dynamin and synaptotagmin (30). These results provide independent support for our data, which extend these results because the SH3 domains involved in binding are defined, so that we also identified interactions of this protein with SNAP-25 and -23, and because we demonstrated that these interactions are stoichiometric and can be found in complexes in native brain. Although the human intersectins were only studied at the level of DNA sequences without identification of proteins, their sequences provide help in interpreting our protein studies. Two human intersectin variants were identified from cDNAs (31): a short variant corresponding to the protein characterized here and an alternatively spliced variant that likely corresponds to the large mRNA and protein that we observed in brain. Interestingly, the long intersectin/EHSH1 variant contains a C-terminal guanine-nucleotide exchange factor domain, a pleckstrin homology domain, and a C₂ domain. This suggests a special role for EHSH1/intersectins in brain, which involves additional functional domains. In addition to the intersectins, SH3P17 probably is also identical to EHSH1 but contains only the C-terminal part of the protein and is thus incomplete (42). A recently described protein in *Drosophila*, DAP160, is very similar to EHSH1 (43). DAP160 also contains EH and SH3 domains and binds to dynamin. However, DAP160 is more similar to SH3P18 than to EHSH1. Furthermore, the central domain is not well conserved. This suggests that DAP160 may represent an isoform of EHSH1 instead of an ortholog.

What is the functional implication of a protein's binding to both SNAP-25 and dynamin? Individually, both binding activities could potentially fill voids in our current understanding of the functions of these proteins. SNAP-25 has highly conserved domains that do not participate in the formation of the core complex; it seems likely that it binds to other proteins in addition to syntaxin and synaptobrevin. Furthermore, the core complex is probably dissociated by NSF after membrane fusion, and the vesicular and plasma membrane components are presumably segregated (55). This sorting step must involve protein-protein interactions and must be linked to endocytosis because it has to precede endocytosis; it is possible that EHSH1 could function here.

In terms of dynamin binding, EHSH1 contains two interesting aspects that set it apart from other SH3 domain proteins in endocytosis. The first interesting aspect is that EHSH1 contains multiple SH3 domains that bind dynamin. Thus, EHSH1 can bind several molecules of dynamin simultaneously, thereby clustering dynamin molecules. This is quite distinct from the interactions of amphiphysins with dynamin, arguably the best characterized interactions (25). Amphiphysins contain only a single SH3 domain but multimerize, so that an amphiphysin multimer could potentially also cluster dynamins. The very high affinity of the SH3 domains of EHSH1 for dynamin suggest that the interaction is physiological. However, the presence of multiple SH3 domain containing proteins in endocytosis (14–19) is puzzling, and their respective contributions remain to be clarified. The second interesting aspect of EHSH1 with regard to dynamin is its domain structure. Although it is possible that EH domains perform a multitude of functions, their best characterized role is in endocytosis (23, 50–52). Thus EHSH1 is the only vertebrate protein in which EH domains are coupled to SH3 domains, both with a conjectured involvement in endocytosis. Overall, the properties of EHSH1 suggest a function as an adaptor that co-localizes multiple membrane trafficking components.

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REFERENCES

1. Palade, G. E. (1995) Cold Spring Harbor Symp. Quant. Biol. 60, 821–831
2. Bennett, M. K., and Scheller, R. H. (1994) Annu. Rev. Biochem. 63, 63–100
3. Martin, T. P. J. (1997) Trends Cell Biol. 7, 271–276
4. Sudhof, T. C. (1995) Nature 375, 645–653
5. Ferro-Novick, S., and Jahn, R. (1994) Nature 370, 191–193
6. Geppert, M., Geda, Y., Hammer, R. E., Li, C., Rosahl, T. W., Stevens, C. F., and Sudhof, T. C. (1994) Cell 79, 717–727
7. Sudhof, T. C., and Rizo, J. (1997) Neuron 17, 379–388
8. Robinson, M. S. (1994) Curr. Opin. Cell Biol. 6, 539–544
9. Fassler, R., Timpl, R., Birk, A. M., and Colotta, F. (1991) Nature 341, 411–414
10. Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A., Wadsworth, S. C., and Vallee, R. B. (1991) Nature 351, 583–586
11. Urubita, R., Henley, J. R., Cook, T., and McNiven, M. A. (1997) Proc. Natl. Acad. U. S. A. 94, 377–384
12. Zhang, J., Ferguson, S. S., Barak, L. S., Aber, M. J., Giros, B., Lefkowitz, R. J., and Caron, M. G. (1997) Neuron 19, 53–68
13. Schmidt, S. L., McNiven, M. A., and De Camilli, P. (1998) Curr. Opin. Cell Biol. 10, 504–512
14. Wigge, P., Vallis, Y., and McMahon, H. T. (1997) Curr. Biol. 7, 554–560
15. Vial, M., Montiel, L. J., Cussac, D., Corelli, F., Durhame, M., Parker, F., Tocque, B., Roques, B. P., and Garbay, C. (1998) J. Biol. Chem. 273, 5343–5348
16. Sacotte, R. M., and Margolis, R. L. (1997) Cell Signalling 9, 395–401
17. Ringstad, N., Nemoto, Y., and De Camilli, P. (1997) J. Biol. Chem. 272, 19594–19600
18. Nemoto, Y., Arribas, M., Haffner, C., and De Camilli, P. (1997) J. Biol. Chem. 272, 19594–19600
19. Hata, Y., and Sudhof, T. C. (1995) J. Biol. Chem. 270, 28727–28730
20. Wigge, P., and McMahon, H. T. (1998) Trends Neurosci. 21, 339–344
21. Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M., and Kirchhausen, T. (1996) J. Biol. Chem. 271, 751–760
22. Nemoto, Y., Arribas, M., Haffner, C., and De Camilli, P. (1997) J. Biol. Chem. 272, 20817–20821
23. Carbone, R., Fre, S., Iannolo, G., Briasse, F., Mancini, P., Pelicci, P. G., Torrisi, M. R., and Di Fiore P. P. (1997) J. Biol. Chem. 272, 5498–5504
24. Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M., and Kirchhausen, T. (1996) J. Biol. Chem. 271, 751–760
25. Schumacher, C., Kuroda, B. S., Ouchi, T., Di Fiore, P. P., Glassman, R. H., and Hanafusa, H. (1995) J. Biol. Chem. 270, 15341–15347
26. Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takai, K., De Camilli, P., and Brodin, L. (1997) Science 276, 259–263
27. McPherson, P. S., Garcia, E. P., Slepnev, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Nemoto, Y., and De Camilli (1996) Nature 379, 353–357
28. Kurochkin, M., and Sudhof, T. C. (1998) J. Biol. Chem. 273, 23066–23071
29. Nemoto, Y., Arribas, M., Haffner, C., and De Camilli, P. (1997) J. Biol. Chem. 272, 20387–20391
30. Torrisi, M. R., and Di Fiore, P. P. (1997) J. Cell Biol. 138, 15341–15347
31. Okamoto, M., and Sudhof, T. C. (1997) J. Biol. Chem. 272, 31459–31464
32. Rose, M. D., Winston, F., and Hieter, P. (1990) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
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Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 282–287
38. McMahon, H. T., and Sudhof, T. C. (1995) J. Biol. Chem. 270, 2213–2217
39. McMahon, H. T., Missler, M., Li, C., and Sudhof, T. C. (1995) Cell 83, 111–119
40. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
41. Sontag, J.-M., Fykse, E. M., Ushkaryov, Y., Liu, J.-P., Robinson, P. J., and Sudhof, T. C. (1995) J. Biol. Chem. 269, 4547–4554
42. Sparks, A. B., Hoffman, N. G., McConnell, S. J., Fowlkes, D. M., and Kay, B. K. (1996) Nat. Biotech. 14, 741–744
43. Ross, J., and Kelly, R. (1998) J. Biol. Chem. 273, 19108–19119
44. Ravichandran, V., Chawla, A., and Roche, P. A. (1996) J. Biol. Chem. 271, 13300–13303
45. Wang, G., Witkin, J. W., Hao, G., Bankaitis, V. A., Scherer, P. E., and Baldini, G. (1997) J. Cell Sci. 110, 505–513
46. Wendland, B., and Emr, S. D. (1998) J. Cell Biol. 141, 71–84
47. Rizo, J., and Sudhof, T. C. (1998) Nat. Struct. Biol. 5, 839–842