Splice Variants of Intersectin Are Components of the Endocytic Machinery in Neurons and Nonneuronal Cells*

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We recently identified and cloned intersectin, a protein containing two Eps15 homology (EH) domains and five Src homology 3 (SH3) domains. Using a newly developed intersectin antibody, we demonstrate that endogenous COS-7 cell intersectin localizes to clathrin-coated pits, and transfection studies suggest that the EH domains may direct this localization. Through alternative splicing in a stop codon, a long form of intersectin is generated with a C-terminal extension containing Dbl homology (DH), pleckstrin homology (PH), and C2 domains. Western blots reveal that the long form of intersectin is expressed specifically in neurons, whereas the short isoform is expressed at lower levels in glia and other nonneuronal cells. Immunofluorescence analysis of cultured hippocampal neurons reveals that intersectin is found at the plasma membrane where it co-localized with clathrin. Ibp2, a protein identified based on its interactions with the EH domains of intersectin, binds to clathrin through the N terminus of the heavy chain, suggesting a mechanism for the localization of intersectin at clathrin-coated pits. Ibp2 also binds to the clathrin adaptor AP2, and antibodies against intersectin co-immunoprecipitate clathrin, AP2, and dynamin from brain extracts. These data suggest that the long and short forms of intersectin are components of the endocytic machinery in neurons and nonneuronal cells.

The Eps15 homology (EH) domain is an important protein-protein interaction module functioning in endocytosis. The core of the EH domain-binding motif is composed of the amino acids asparagine-proline-phenylalanine (NPF) (1–3). This sequence is often found at the C terminus of EH domain-binding proteins where the free carboxylate can contribute to binding (3). The EH domain was originally identified in the epidermal growth factor receptor phosphorylation substrate Eps15 (4). Through its EH domains, Eps15 binds to epsin, a recently identified protein implicated in endocytosis (5). Eps15 is localized to the rim of clathrin-coated pits (6), likely through its interactions with AP2 (7–10) and/or with epsin (5). EH domains are also found in the yeast proteins Pan1p and End3p, which are required for endocytosis and normal organization of the actin cytoskeleton (11–15).

The Src homology 3 (SH3) domain, a 50–70-amino acid motif that binds to proline-rich ligands (16, 17) has also been implicated in endocytosis (18). For example, amphiphysins I and II are nerve terminal-enriched proteins that demonstrate SH3 domain-dependent binding to proline-rich sequences in dynamin and synaptojanin (19–23), enzymes which function in the endocytosis of clathrin-coated vesicles (20, 24, 25). In fact, overexpression of the SH3 domains of amphiphysins I and II leads to a functional block in endocytosis in a number of different systems (26–29).

A link between EH and SH3 domain-mediated protein-protein interactions has been revealed with the identification and cloning of *Xenopus laevis* intersectin, a protein containing two N-terminal EH domains, a central helix forming region that has a high probability of forming coiled-coil interactions, and five C-terminal SH3 domains (3). Intersectin is homologous to Dap160, a *Drosophila* protein with two EH domains and four SH3 domains that was identified based on its affinity for dynamin (30). Through this unique combination of protein-protein interaction modules, intersectin has the potential to form macromolecular complexes between EH domain- and SH3 domain-binding proteins. A human form of intersectin has also been recently identified through genomic analysis of chromosome 21 (31). Interestingly, those authors determined that intersectin undergoes alternative splicing in the stop codon leading to a short form (intersectin-s), which has the same domain structure as the *Xenopus* protein, and a long form (intersectin-l) that contains a C-terminal extension with Dbl Homology (DH), Pleckstrin Homology (PH), and C2 domains (31).

In this manuscript, we have generated an antibody against the EH domains of frog intersectin which we have used to characterize the expression and localization of the antigenically related mammalian protein. Western blots reveal that intersectin-s is expressed in a wide variety of tissues and cell lines, including in COS-7 cells, where it localizes to clathrin-coated pits on the plasma membrane.

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* Abbreviations used: EH, Eps15 homology; DH, Dbl homology; Dap, dynamin-associated protein; GST, glutathione S-transferase; Ibp, intersectin-binding protein; MP90, mitotic phosphoprotein of 90 kDa; PH, pleckstrin homology; SH3, Src homology 3; SM, starting material; PCR, polymerase chain reaction.

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suggest that the EH domains may mediate this localization. Intersectin-I is expressed predominately in neurons where it is also co-localized with clathrin. We previously identified two related proteins that bind to the EH domains of intersectin: intersectin-binding protein (Ibp)1, the mouse homologue of epibrassin (5), and Ibp2 (3). Here, we demonstrate that Ibp2 binds to clathrin and AP2, suggesting that it may be involved in the localization of intersectin at clathrin-coated pits. In fact, clathrin, AP2, and dynamin were found to co-immunoprecipitate with intersectin. Taken together, these data suggest that both the short and long alternatively spliced forms of intersectin are components of the molecular machinery for endocytosis in non-neuronal cells and neurons.

**EXPERIMENTAL PROCEDURES**

**Antibodies—**To generate an intersectin antibody, a full-length intersectin cDNA clone from X. laevis (3) was used as a template in PCR reactions with Pfu DNA polymerase (Stratagene) with the forward primer 5'-CTGTGGCCGGATCCAAATTTGGACATCTGGGGCCATACCG and the reverse primer 5'-CTTTGGAATCCGAGGGAATATACCTGGAGG. The PCR product, encoding amino acids 11 to 366 of intersectin, including both EH domains (3), was cloned into the BamHI-EcoRI sites of pGEX-2T (Amersham Pharmacia Biotech). The resulting GST (GST-EHa/b) and His6 (His6-EHa/b) fusion proteins were expressed and purified as described (19, 32). Two rabbit antisera (2173, 2174) were injected with approximately 50 μg of GST-EHa/b using Titermax adjuvant (CytRx Corp.) with standard protocols. Antibody production was monitored by Western blots against His6-EHa/b, using Titermax adjuvant (CytRx Corp.) with standard protocols. Antigen detection was performed with the primer pair intersectinFa with intersectinRb, and a reverse primer 5'-GATCCCAGTTGTTAAAGCTGTAGGGT); and intersectinRa (5'-CCAGTTGTTAAAGCTGTAGGGT). One PCR reaction was performed with this primer pair intersectinFa with intersectinRb, and a reverse reaction used intersectinFb with intersectinRa. The two reaction products were mixed, and the double-stranded DNA was denatured and allowed to re-anneal; one-fourth of the re-annealed molecules contain sticky ends that are compatible for ligation to a BamHI cleaved vector. The re-annealed mixtures were ligated into the pCGN-Hygro mammalian expression vector which carries a BamHI site in-frame with the epitope tag. Constructs encoding the EH domains (amino acids 11–366) were prepared in an identical manner using the following primers: F (stands for forward and R stands for reverse): intersectinFa (5'-GATCCATGGCTGACATTTTGGAACCTCG); intersectinRh (5'-CATGGCTGACTTTGAACCTCG); intersectinRa (5'-GATCCATGGCTGACATTTTGGAATATACCTGGAGG); and intersectinRb (5'-GACATGGCTGACATTTTGGAATATACCTGGAGG). The constructs were verified by sequence analysis and transfection into COS-7 cells using the calcium phosphate precipitation method (36).

**Preparation and Examination of Plasma Membranes—**COS-7 cells were plated into tissue culture wells containing 22-mm coverslips. The cells were maintained for 1 h at 4 °C and were then washed and sonicated in 12 ml of buffer A (25 mM HEPES, pH 7.0, containing 25 mM KCl, 2.5 mM magnesium acetate, and 0.2 mM dithiothreitol) for 2 s using a 1/2-inch tapered horn 1 cm above the coverslip at setting 5.0 (Sonic Materials Vibra Cell). The cells were then washed three times in buffer A and fixed in buffer B (20 mM HEPES, pH 6.8, 100 mM KC1, 5 mM MgCl2, 3 mM EGTA, and 3% paraformaldehyde). Following fixation, coverslips were washed in phosphate-buffered saline before being processed for immunofluorescence analysis.

**Analysis of Tissue and Subcellular Distribution—**Postnuclear supernatants from different tissues and cell lines were prepared and processed for Western blots as described (22). Rat medial septal neurons were prepared as described (38) as were glial cells from rat hippocampus (39). To examine the subcellular distribution of intersectin in neurons, dissociated cell cultures were prepared from the CA3 and dentate regions of hippocampi from P1 rat pups as described (40). Cells were maintained in culture from 1–7 days before processing for immunofluorescence analysis. Images were captured with a Zeiss scanning laser confocal microscope 410.

**Binding Assays—**A GST fusion protein, encoding the C-terminal 511 amino acids of Ibp2 (GST-Ibp2), was prepared by PCR from an Ibp2 cDNA template (3) using Pfu DNA polymerase (Stratagene) with the forward primer 5'-CTGTGGGCATCCTCAAGAAGCCACTGCACCTG and the reverse primer 5'-CTGTGGACATTTGGACATCTGGGGCCAAGGAAAGGGGTT. The resulting PCR product was subcloned into the BamHI-EcoRI sites of pGEX-2T (Amersham Pharmacia Biotech). GST-Ibp2 was expressed and purified as described previously (32), except that the plasmid was transformed into BL21 Escherichia coli, and the cells were grown and induced at 30 °C. GST-Ibp2 was used in binding assays with a Triton X-100 soluble rat brain extract as described (22). For immunoprecipitation analysis, rat brain synaptosomes were resuspended to a protein concentration of approximately 10 mg/ml in buffer C (20 mM HEPES, pH 7.4, 128 mM NaCl, 3 mM KCl, 1.2 mM MgCl2, 0.1 mM CaCl2, 11 mM glucose) and incubated for 1 h at 37 °C. Synaptosomal membranes were then pelleted, resuspended in buffer D (20 mM HEPES, pH 7.4, 50 mM NaPO4, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 50 mM Na2-Ascorbate, 50 mM Na-fluoride, 10 μM PMSF), and disrupted by sonication. The samples were spun in a microcentrifuge at maximal velocity, and the supernatants were incubated with anti-intersectin antisera (2173, 2174), pre-coupled to protein A-Sepharose. Following an overnight incubation at 4 °C, the beads were washed in buffer D, and proteins were recovered with SDS gel sample buffer.

**Binding of Biotinylated Ibp2 and MP90 to Clathrin Terminal Domains—**COS-7 cDNA clones encoding Ibp2 (3) and MP90 (Ref. 41; generous gift of Dr. Todd Stukenberg, Harvard Medical School), and Luciferase (Promega Co.) were in vitro transcribed and translated in the presence of biotinylated lysine tRNA using the Transient® nonradioactive translation detection system (Promega Co.) according to the manufacturer instructions. The biotinylated proteins were diluted in phosphate-buffered saline and incubated overnight at 4 °C with GST fusion proteins, essentially as described (37). The amino-terminal 19 amino acids of the heavy chain (Ref. 42; generous gift of James Keen, Kimmel Cancer Institute) or the amino acids NPFL (3), pre-bound to glutathione-Sepharose. The beads were subsequently pelleted by microcentrifugation and washed three times in 1 ml of phosphate-buffered saline, and the bound proteins were eluted with SDS gel sample buffer and prepared for Western blot analysis using streptavidin conjugated to alkaline phosphatase.

**RESULTS**

**Intersectin Localizes to Clathrin-coated Pits—**We have recently identified and cloned X. laevis intersectin, a novel protein composed of multiple EH and SH3 domains, protein modules implicated in endocytosis (3). To explore the subcellular localization of mammalian intersectin, we raised two rabbit polyclonal antisera (2173, 2174) against the tandem EH domains of the frog protein because the primary structures of this region are highly related (i.e. 87% identical). We then used the 2173 antibody for immunofluorescence analysis of plasma membranes prepared from COS-7 cells using a procedure that leads to membranes rich in clathrin-coated pits (37, 43, 44). In double-labeling experiments, antisera 2173 yielded a bright, punctate staining pattern that was virtually identical to the pattern seen for clathrin (Fig. 1A). The specificity of the staining was confirmed by pre-absorption experiments. Both untreated and GST preabsorbed antisera yielded bright, punctate staining, whereas staining was virtually undetectable using antisera preabsorbed against the EH domains (Fig. 1B). Affinity purified antibodies demonstrated an identical immunofluorescence pattern as untreated antisera (data not shown).
To begin to explore the mechanism of intersectin targeting, we performed immunofluorescence analysis on plasma membranes prepared from COS-7 cells transfected with recombinant full-length intersectin. At the level of exposure used for Fig. 2A (~300 msec integration using a Sony CCD video camera), membranes from transfected cells, which represent approximately 10% of the clathrin-positive membranes, were strongly positive with antibody 2173. Endogenous intersectin, which is weakly detectable in the nontransfected cells at this level of exposure (Fig. 2A), is readily detectable upon longer exposures (~3 s integration; see Fig. 1). Interestingly, a similar staining pattern was observed in COS-7 cells transfected with a construct encoding the two EH domains of intersectin (31). Both the 190 and the 145 kDa species bind to the fusion protein, suggesting that they are intersectin-l and intersectin-s, respectively (Fig. 3b). This result is further strengthened by the observation that both species also bind specifically to a GST fusion protein encoding the peptide sequence NPFL (data not shown). Western blot analysis of extracts from a wide variety of tissues and cell lines demonstrates that intersectin-l is expressed predominately in the brain, whereas intersectin-s is ubiquitously expressed (Fig. 4). Interestingly, intersectin-l is expressed in neurons and is not detected in glia (Fig. 3A). Intersectin-s is expressed in glia, and the low levels of intersectin-s seen in neuronal cultures is likely because of glia contamination of the neurons (Fig. 3A, Ref. 38). Also of interest, the pheochromocytoma cell line, PC12, expresses intersectin-s (Fig. 4), whereas intersectin-l was only weakly detectable, even after NGF-induced differentiation (data not shown).

**Localization of Intersectin in Neurons**—To explore the subcellular localization of intersectin, we performed immunofluorescence analysis of hippocampal neurons in culture using confocal microscopy (45). Intersectin immunoreactivity appeared as small spots, 0.5–1.0 μm in diameter, which were located within cell bodies in Golgi-like structures, at the plasma membrane and throughout the length of axons and dendrites (Fig. 5, upper left and bottom panels). These intersectin punctae corresponded to regions enriched in clathrin (Fig. 5), although not all intersectin positive punctae were positive for clathrin (note the red staining in the top right panel).

**Intersectin Interacts Indirectly with Components of Clathrin-coated Pits**—Through its EH domains, intersectin binds through the NPF tripeptide with Ibp1 and Ibp2 (3). Interestingly, Ibp1 and Ibp2 also contain the peptide sequences LVDLD through the NPF tripeptide with Ibp1 and Ibp2 (3). Interestingly, Ibp1 and Ibp2 also contain the peptide sequences LVDLD (3). Both the 190 and the 145 kDa species bind to the fusion protein, suggesting that they are intersectin-l and intersectin-s, respectively (Fig. 3b). This result is further strengthened by the observation that both species also bind specifically to a GST fusion protein encoding the peptide sequence NPFL (data not shown). Western blot analysis of extracts from a wide variety of tissues and cell lines demonstrates that intersectin-l is expressed predominately in the brain, whereas intersectin-s is ubiquitously expressed (Fig. 4). Interestingly, intersectin-l is expressed in neurons and is not detected in glia (Fig. 3A). Intersectin-s is expressed in glia, and the low levels of intersectin-s seen in neuronal cultures is likely because of glia contamination of the neurons (Fig. 3A, Ref. 38). Also of interest, the pheochromocytoma cell line, PC12, expresses intersectin-s (Fig. 4), whereas intersectin-l was only weakly detectable, even after NGF-induced differentiation (data not shown).

Several clathrin-binding proteins, including arrestin3 (42), interact with clathrin through the N terminus of its heavy chain, a region known as the terminal domain (49). To determine whether the terminal domain is responsible for the binding of the Ibps, we tested the binding of Ibp2 and MP90, a related mitotic phosphoprotein (41), prepared in vitro by coupled transcription and translation, to a GST fusion protein encoding the amino-terminal 579 amino acids of the clathrin heavy chain. Both Ibp2 and MP90 bound to the N-terminal fusion protein, whereas neither protein bound to a control GST fusion to the peptide NPFL (Fig. 6B). Further, the negative control protein, luciferase, bound to neither GST fusion protein (Fig. 6B). Thus, both Ibp2 and MP90 bind to the terminal domain of clathrin.

We further characterized the interaction of intersectin with clathrin-coated pit components, we performed immunoprecipitation analysis from rat brain synaptosomes using antibodies against intersectin. Anti-intersectin antisera from two different rabbits (2173 and 2174) both immunoprecipitated intersectin-l and intersectin-s (Fig. 7). Both clathrin and AP2 were also
co-immunoprecipitated with intersectin as assessed with specific antibodies (Fig. 7). Further, dynamin was also observed to co-immunoprecipitate with the intersectin antibodies, but synaptojanin did not under these conditions (Fig. 7). Thus, intersectin interacts in vivo with distinct components of the endocytic machinery.

**DISCUSSION**

We have recently identified and cloned *X. laevis* intersectin, a protein containing two EH and five SH3 domains (3). Intersectin is related to a *Drosophila* protein, Dap160 (30), and is highly similar (81% identical) to human intersectin that was cloned based on genomic analysis of chromosome 21 (31). Of interest, human intersectin undergoes alternative splicing in the stop codon, leading to a short form with the same domain structure as the frog protein and a long form with a C-terminal extension containing DH, PH, and C2 domains (31).

**Fig. 2.** Recombinant intersectin and intersectin EH domains target to clathrin-coated pits. A, plasma membranes, isolated from COS-7 cell monolayers overexpressing recombinant intersectin or its two EH domains (EHab), were processed for immunocytochemistry with rabbit polyclonal antibody 2173 and a mouse monoclonal antibody against clathrin. Both full-length intersectin and the intersectin EH domains demonstrate a similar staining pattern. Only a fraction of the membranes that are positive for endogenous clathrin are positive for transfected full-length intersectin or its EH domains. The images were captured with a Sony CCD video camera with a time integration of ~300 ms. The scale bar represents 20 μm.

**Fig. 3.** Identification of long and short intersectin isoforms. A, a rat brain extract, along with extracts from purified glial cultures and enriched neuronal cultures, were processed for Western blots with affinity purified antibody 2173, revealing intersectin-l (~190 kDa) and intersectin-s (~145 kDa). The weak band of ~180 kDa seen in the brain extracts is presumed to be a proteolytic fragment of intersectin-l. B, a GST fusion protein encoding the C-terminal 511 amino acids of Ibp2 (GST-Ibp2), encoding the EH domain-binding sites, and GST alone (GST), were incubated with glutathione-Sepharose. The washed beads were then incubated with soluble extracts from rat brain (SM), and proteins specifically bound to the beads were processed for Western blots with antibody 2173.

**Fig. 4.** Tissue distribution of the intersectin isoforms. Crude postnuclear supernatants were prepared from a variety of tissues and cell lines and processed for Western blots with antibody 2173.
A confocal section of intersectin staining in a 1-day old hippocampal neuron is demonstrated in red. The distribution of clathrin in the same field as the intersectin staining is shown in green. Superimposition of the images (upper right panel) demonstrates a high degree of co-localization (yellow) of intersectin with clathrin (two examples are indicated by the arrows). The distribution of intersectin immunoreactivity in a CA3 pyramidal neuron cultured for 7 days reveals intersectin in Golgi-like structures as well as at the plasma membrane (arrows, bottom panel). Scale bar equals 10 μm.

**Fig. 5. Subcellular distribution of intersectin in hippocampal neurons.**

Western blot analysis reveals that intersectin-s is ubiquitously expressed, whereas intersectin-l is expressed predominantly in brain. This agrees with Northern blots demonstrating two transcripts for human intersectin, a 5.3-kilobase ubiquitously expressed transcript and an approximately 15-kilobase brain-specific transcript (31). Although we do not currently have an intersectin-l specific antibody, Western blots suggest that intersectin-l is the major variant expressed in neurons. Using confocal microscopy, we determined that intersectin is expressed at the plasma membrane of rat hippocampal neurons in culture where it is co-localized with clathrin. Thus, like intersectin-s, intersectin-l is likely a component of clathrin-coated pits. Intersectin was found over the entire surface of the neuron including the dendrites and axons. Exo-endocytic recycling of synaptotagmin-positive synaptic vesicles occurs throughout all processes of the neuron at this stage in culture (51). These results raise the interesting possibility that intersectin-l and intersectin-s may function in the endocytosis of synaptic vesicles and general endocytosis, respectively. Many other proteins involved in endocytosis appear to have a similar specialization including synaptojanin, which has a 145-kDa isoform that is highly expressed in neurons, and a 170-kDa isoform, produced by alternative splicing of an exon encoding a stop codon, which is widely distributed (52, 53). In fact, AP2, clathrin, dynamin, and amphiphysin I and II are all expressed at higher levels in neuronal versus nonneuronal cells, and many of these proteins have neuron-specific isoforms.

The nature of the specialized function of intersectin-l in synaptic vesicle endocytosis is difficult to predict. Intersectin-l contains DH, PH, and C2 domains. DH domains promote guanine-nucleotide exchange on Rho and, as is the case in intersectin-s, the DH domain is followed by a PH domain in all guanine-nucleotide exchange factors (54). Thus, although not tested, it is possible that intersectin-l has guanine-nucleotide exchange factor activity that may control Rho-dependent processes within neurons (55). PH domains mediate interactions with inositol phospholipids (56) and C2 domains can mediate Ca\(^{2+}\)-dependent phospholipid binding (57). Phospholipid me-
interceptin As a Component of Endocytosis

The identification of intersectin-s and intersectin-l as components of clathrin-coated pits is supported by the biochemical characterization of intersectin protein interactions. We have previously demonstrated that through its EH domains, intersectin interacts with mouse Ibp1 and Ibp2 (3). We now demonstrate that Ibp2 binds to clathrin and AP2 in vitro. The importance of the interaction of Ibp2 with clathrin/AP2 and intersectin is underscored by the observation that both clathrin and AP2 co-immunoprecipitate with intersectin from rat brain synaptosomes. As the isolated EH domains of intersectin appear to be sufficient to target the protein to clathrin-coated pits, it is tempting to speculate that Ibp2, and possibly Ibp1, mediate this subcellular localization. Indeed, Chen et al. (5) have suggested that epsin, the rat homologue of mouse Ibp1, may function as a linker between the EH domains of Eps15 and clathrin-coated pit components. The potential targeting role of the Ibps/epsins is reminiscent of the functional role of the amphiphysins in synaptic vesicle endocytosis as these proteins, through interactions with both AP2 (19, 59) and clathrin (22, 48, 60), appear to target synaptojanin and dynamin to endocytic sites (61). The Ibps/epsins appear to represent a growing protein family as the sequence of the rat homologue of mouse Ibp1, as two genes has been observed for mouse (62).

Taken together, the data presented in this manuscript suggest a role for intersectin in endocytosis. The protein is localized to clathrin-coated pits in the different cell types we have examined, where through SH3 domains, it could regulate the function of dynamin. Owen et al. (29) have recently demonstrated that the SH3 domain of amphiphysin II prevents dynamin self-assembly into rings, thereby blocking dynamin function. In our own hands, the SH3 domains of intersectin inhibit transferrin receptor endocytosis in a cell-permeabilized assay. Thus, it is possible that intersectin functions by binding to dynamin at clathrin-coated pits and inhibiting its access to constricted vesicular necks, its assembly into rings, or its enzymatic activity. In neurons, intersectin-l may have additional roles through promotion of guanine-nucleotide exchange on Rho. Regardless of the precise mechanism of intersectin function, the data reported here implicate the intersectin isoforms in clathrin-mediated endocytosis, both in neurons and in non-neuronal cells.

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