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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 is colocalized 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)[1] 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. Transfection studies

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[1] The abbreviations used are: 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.

suggest that the EH domains may mediate this localization. Intersectin-l 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 epsin (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′-CTGTGCGGATCCAATTTGGACATCTGGGCCATAACG and the reverse primer 5′-CTGTGGAATTCAAGATGGGGGAATATACTCTGGAGG. The PCR product, encoding amino acids 11 to 306 of intersectin, including both EH domains (3), was cloned into the *Bam*HI-*Eco*RI sites of pGEX-2T (Amersham Pharmacia Biotech) and pTrcHisA (Invitrogen). The resulting GST (GST-EHa/b) and His_6 (His_6-EHa/b) fusion proteins were expressed and purified as described (19, 32). Two rabbits (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 His_6-EHa/b, and antibodies were affinity purified using strips of polyvinylidene difluoride membrane containing the same fusion protein (33). For preabsorption experiments, 2173 serum was diluted in phosphate-buffered saline (20 mM NaH_2PO_4, 0.9% NaCl, pH 7.4) containing 1% bovine serum albumin and incubated overnight at 4 °C with either GST or His_6-EHa/b immobilized on polyvinylidene difluoride membranes. A polyclonal antibody against clathrin (34) and monoclonal antibody AC1-M11 against α-adaptin (35) were generous gifts of Dr. Margaret Robinson (Cambridge University). A monoclonal antibody against clathrin was produced from the hybridoma X22 (ATCC) and was also kindly supplied by Dr. Mark McNiven (Mayo Clinic). A monoclonal antibody against dynamin (HUDY-1) was purchased from Upstate Biotechnology Inc. (Albany, NY).

Transfections of COS-7 Cells with Intersectin Constructs—A cDNA construct encoding hemagglutinin-tagged full-length intersectin was prepared by PCR using an intersectin cDNA template with *Pfu* DNA polymerase and the following primers (F stands for forward and R stands for reverse): intersectinFa (5′-GATCCATGGCTCAGTTTGGAACTCCG); intersectinFb (5′-CATGGCTCAGTTTGGAACTCCG); intersectinRa (5′-GATCCCAGTTGTTAAAGCTGTAGGGT); and intersectinRb (5′-CCAGTTGTTAAAGCTGTAGGGT). One PCR reaction was performed with the primer pair intersectinFa with intersectinRb, and a second 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 *Bam*HI cleaved vector. The re-annealed mixtures were ligated into the pCGN-Hygro mammalian expression vector which carries a *Bam*HI site in-frame with the epitope tag. Constructs encoding the EH domains (amino acids 11–306) were prepared in an identical manner using the following primers: EHFa (5′-GATCCAATTTGGACATCTGGGCCATA); EHFb (5′-CAATTTGGACATCTGGGCCATA); EHRa (5′-GATCCAGATGGGGGAATATACTCTGG); and EHRb (5′-CAGATGGGGGAATATACTCTGG). The constructs were verified by sequence analysis and transfected into COS-7 using the calcium phosphate precipitation method (36).

Preparation and Examination of Plasma Membranes—COS-7 cells were platted into tissue culture wells containing 22-mm coverslips previously coated with poly-L-lysine. To coat the coverslips, poly-L-lysine stock (Sigma) was diluted 1:100 in 0.15 M boric acid, pH 8.4, filter sterilized, and incubated over coverslips for 30 min. After incubation, coverslips were washed extensively with sterile water and once with COS-7 cell medium. Plasma membranes were prepared from the cells (either transfected or nontransfected) essentially as described (37). Briefly, 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 (Sonics 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 KCl, 5 mM $MgCl_2$, 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′-CTGTGCGGATCCTCAAGCAAGGCACTGACACTG and the reverse primer 5′-CTGATGAATTCCTCACTAGAGAAGGAAAGGGTT. The resulting PCR product was subcloned into the *Bam*HI-*Eco*RI 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 $MgCl_2$, 0.1 mM $CaCl_2$, 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 $NaPO_4$, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 50 mM Na-pyrophosphate, 50 mM Na-fluoride, 10 μg/ml 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 Domain—cDNA clones encoding Ibp2 (3), 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 Transend™ 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, encoding the amino-terminal 579 amino acids of the clathrin 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, antiserum 2173 yielded a bright, punctate staining pattern that was virtually identical to the pattern seen for clathrin (Fig. 1*A*). 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 antiserum preabsorbed against the EH domains (Fig. 1*B*). Affinity purified antibodies demonstrated an identical immunofluorescence pattern as untreated antiserum (data not shown).

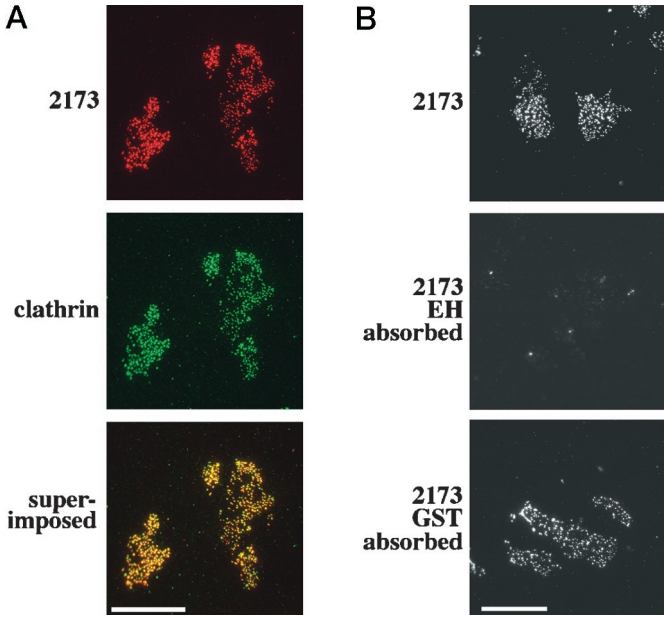

FIG. 1. **Intersectin-s localizes to clathrin-coated pits.** *A*, isolated plasma membranes, prepared by sonication of COS-7 cell monolayers, were stained with an anti-clathrin mouse monoclonal antibody (*clathrin*), revealing a punctate fluorescence (37, 44). Endogenous COS-7 cell intersectin-s displays a similar pattern as revealed with antiserum 2173. Superimposition of the images reveals a high degree of co-localization. *B*, COS-7 cell plasma membranes were stained with antiserum 2173, 2173 serum preabsorbed against the intersectin EH domains, or 2173 serum preabsorbed against GST. All images were captured with a Sony CCD video camera with a time integration of ~3 s. The *scale bar* represents 20 μm.

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. 2*A* (~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. 2*A*), 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 (amino acids 11 to 306) (Fig. 2*A*). By Western blot, both constructs were seen to be highly overexpressed (data not shown). At higher magnification, double-labeling immunofluorescence revealed nearly complete co-localization of both full-length intersectin and its EH domains with clathrin-coated pits on the plasma membrane (Fig. 2*B*). Taken together, the data in Figs. 1 and 2 suggest that intersectin is localized to clathrin-coated pits, possibly through its EH domains, although a role for the SH3 domains in membrane targeting cannot be ruled out.

Expression of Intersectin Isoforms in Mammalian Cells—To further explore the expression of intersectin, we used affinity purified antibody 2173 for Western blots of a crude rat brain extract. Two bands of immunoreactivity were evident (Fig. 3*A*), 190 kDa (intersectin-l) and 145 kDa (intersectin-s) in size. The molecular masses of these bands are in good agreement with the predicted molecular masses of the long (195, 576 Da) and short (137, 711 Da) isoforms of human intersectin, alternatively spliced variants identified through genomic analysis of chromosome 21 (31). To confirm the identity of these bands, we performed *in vitro* binding assays using a fusion protein encoding a region of the intersectin-binding protein, Ibp2, which contains the NPF repeats that interact with the EH domains of intersectin (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. 3*b*). 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. 3*A*). 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. 3*A*, 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 and LVNLD, respectively, which resemble clathrin-binding motifs (18, 46–48). To determine whether the Ibps interact with clathrin, we generated a GST fusion protein encoding the C-terminal 511 amino acids of Ibp2 (GST-Ibp2) that includes the three NPF repeats and the LVNLD sequence (despite repeated attempts, we were unable to generate soluble GST fusion proteins encoding Ibp1). When incubated with rat brain extracts, GST-Ibp2 bound specifically to clathrin (Fig. 6*A*). Consistent with the previous observation that epsin binds to AP2 (5), Ibp2 also bound to AP2, as assessed by Western blots with an antibody to the αa- and αc-isoforms of adaptin (Fig. 6*A*).

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. 6*B*). Further, the negative control protein, luciferase, bound to neither GST fusion protein (Fig. 6*B*). Thus, both Ibp2 and MP90 bind to the terminal domain of clathrin.

To further characterize 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

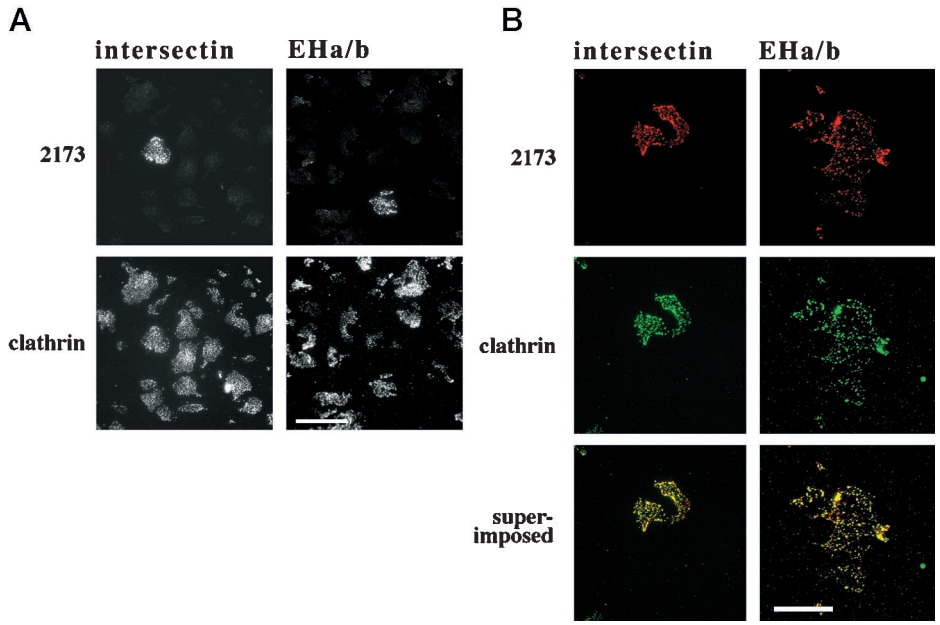

FIG. 2. **Recombinant intersectin and intersectin EH domains target to clathrin-coated pits.** *A*, plasma membranes, isolated from COS-7 cell monolayers overexpressing recombinant intersectin or its two EH domains (*EHa/b*), were processed for immunocytochemistry with rabbit polyclonal antibody 2173 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 40 μm. *B*, at higher magnification, double labeling immunofluorescence of plasma membranes from transfected cells demonstrates a high degree of co-localization between EH domain-containing constructs and clathrin. The *scale bar* represents 20 μm.

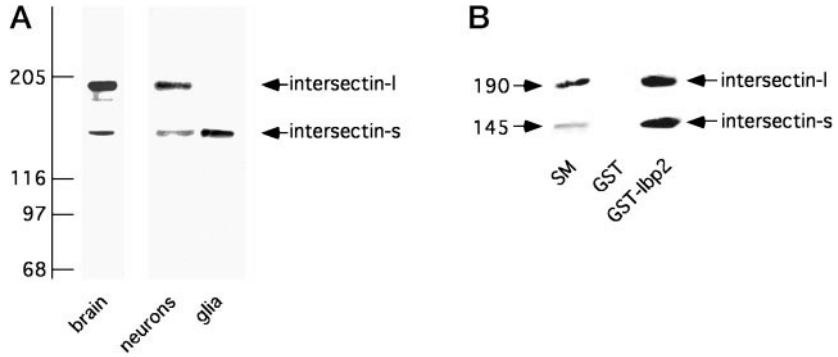

FIG. 3. **Identification of long and short intersectin proteins.** *A*, a rat brain extract, along with extracts from purified glial cultures and enriched neuronal cultures, were processed for Western blots with affinity purified antibody 2173, revealing intersectin-l (~190 kDa) and intersectin-s (~145 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.

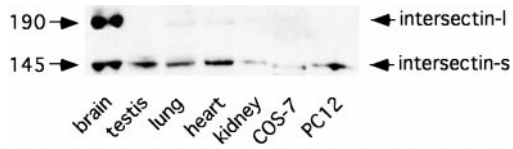

FIG. 4. **Tissue distribution of the intersectin isoforms.** Crude postnuclear supernatants were prepared from a variety of tissues and cell lines and processed for Western blots with antibody 2173.

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).

The presence of EH and SH3 domains in proteins involved in endocytosis (4, 18) provided the first suggestion that intersectin may function in the endocytic process. Support for this idea then came from the observation that frog intersectin and fruit fly Dap160 display SH3 domain-dependent binding to dynamin (3, 30), an enzyme that functions in the formation of clathrin-coated vesicles (24, 25). To begin to study the role of intersectin in mammalian cells, we generated a polyclonal antibody against the EH domains of frog intersectin. Analysis of intact cells in culture revealed a punctate, surface staining for intersectin that was reminiscent of clathrin-coated pits (data not

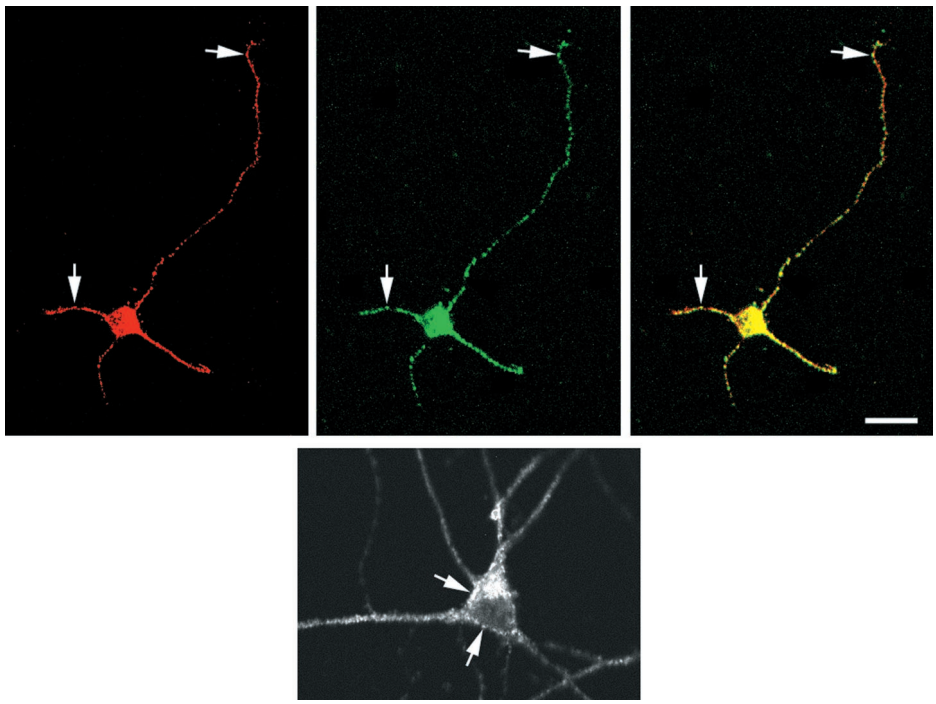

FIG. 5. **Subcellular distribution of intersectin in hippocampal neurons.** A confocal section of intersectin staining in a 1-day old hippocampal neuron is demonstrated in *red*. The distribution of clathrin in the same field as the intersectin staining is shown in *green*. Superimposition of the images (*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.

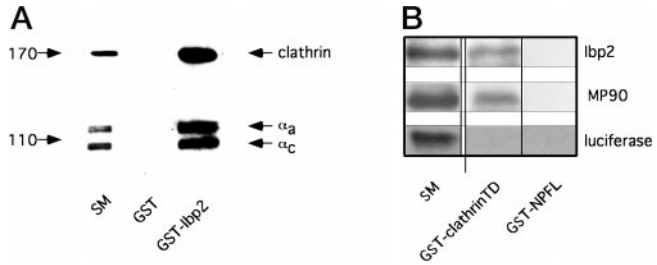

FIG. 6. **Ibp2 binding to clathrin and AP2 *in vitro*.** *A*, a GST fusion protein encoding the C-terminal 511 amino acids of Ibp2 (*GST-Ibp2*), including potential EH- and clathrin-binding domains, and GST alone (*GST*) were incubated with glutathione-Sepharose. The washed beads were then incubated with soluble extracts from rat brain (*SM*), and proteins specifically bound to the beads were resolved by SDS-polyacrylamide gel electrophoresis. Western blots were reacted with an antibody against the clathrin heavy chain or the αa and αc subunits of AP2. *B*, to determine whether the terminal domain of clathrin was involved in Ibp2 binding, biotinylated forms of Ibp2, MP90, and luciferase were prepared *in vitro* by coupled transcription and translation and incubated with agarose beads prebound to two different GST fusion proteins, encoding either the N-terminal 579 amino acids of the clathrin heavy chain (*GST-clathrinTD*) or the peptide NPFL (*GST-NPFL*). Proteins specifically bound to the beads were eluted with SDS-polyacrylamide gel electrophoresis sample buffer and processed for Western blots, along with an aliquot of the biotinylated proteins (*SM*), using streptavidin-alkaline phosphatase.

shown). To explore this further, we prepared plasma membranes from COS-7 cells using a procedure that leads to plasma membranes rich in clathrin-coated pits (37, 43, 44). Immunostaining of these preparations revealed that intersectin is highly co-localized with clathrin. As COS-7 cells only express intersectin-s, protein domains contained within this splice variant (*i.e.* EH and SH3 domains, coiled-coil region) must be sufficient to direct targeting of the molecule to clathrin-coated pits. To begin to address this, we transfected COS-7 cells with full-length recombinant intersectin as well as with a construct encoding just the tandem EH domains. Both constructs localize to clathrin-coated pits, suggesting that the EH domains are sufficient to mediate the subcellular localization of intersectin-s. However, we cannot yet rule out a role for the SH3 domains in this process. For example, SH3 domains can function in directing protein localization (18, 50), and intersectin-s could potentially target to clathrin-coated pits through SH3 domain-dependent interactions with dynamin (3).

Western blot analysis reveals that intersectin-s is ubiquitously expressed, whereas intersectin-l is expressed predominately 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-l, 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-

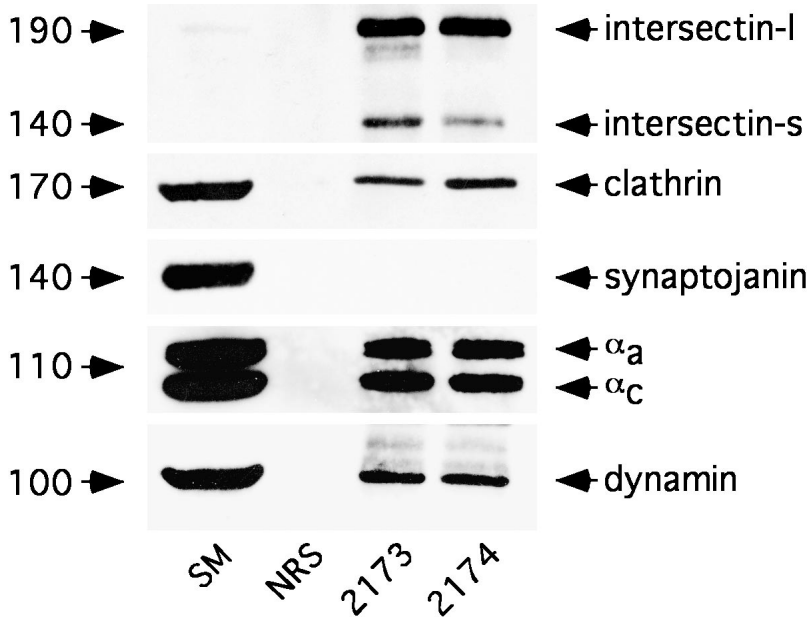

FIG. 7. **Co-immunoprecipitation of clathrin, AP2, and dynamin with intersectin.** Aliquots of antisera 2173 and 2174, as well as pre-immune 2173 antisera (*NRS*), were pre-coupled to protein A-Sepharose beads for 1 h at 4 °C. Pre-coupled beads were washed and incubated overnight at 4 °C with 1 mg of a soluble extract prepared from rat brain synaptosomes (22) and extensively washed the next day. Twenty μg samples of starting material (*SM*) and material bound to the beads were eluted and processed for Western blot analysis with polyclonal antibodies against intersectin (2173) and synaptojanin, and monoclonal antibodies against dynamin, clathrin, and the α_a and α_c subunits of AP2. Immune complexes were detected on separate filter strips by enhanced chemiluminescence (NEN Life Sciences). The antigens and their approximate molecular masses (kDa) are denoted with *arrows* on the *right* and *left sides* of the figure, respectively.

tabolism, and in particular, the metabolism of inositol phospholipids, has been strongly implicated in the endocytosis of synaptic vesicles (58). Further, both PH and C2 domains are found in a wide variety of proteins that function in synaptic vesicle endocytosis including dynamin and synaptotagmin. The exact functional role in neurons of the intersectin-l-specific C terminus remains to be explored.

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 are 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 Ibp2 (epsin 2) has been recently deposited in GenBank™ (accession number AAC79495).[2] The same is true for intersectin, 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.[3] 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 mechanisms 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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Splice Variants of Intersectin Are Components of the Endocytic Machinery in Neurons and Nonneuronal Cells

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