View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

 FEBS Letters 478 (2000) 43–51

Intersectin 2, a new multimodular protein involved in clathrin-mediated endocytosis[1]

Carles Pucharcos, Xavier Estivill, Susana de la Luna*

Down Syndrome Research Group, Medical and Molecular Genetics Center, IRO, Hospital Duran i Reynals, Avia. de Castelldefels Km 2.7, L'Hospitalet de Llobregat, 08907 Barcelona, Spain

Received 2 May 2000; received in revised form 16 June 2000

Edited by Felix Wieland

Abstract Intersectin 1 (*ITSN1*) is a binding partner of dynamin that has been shown to participate in clathrin-mediated endocytosis. Here we report the characterization of a new human gene, *ITSN2*, highly similar to *ITSN1*. Alternative splicing of *ITSN2* generates a short isoform with two EH domains, a coiled-coil region and five SH3 domains, and a longer isoform containing extra carboxy domains (DH, PH and C2 domains), suggesting that it could act as a guanine nucleotide exchange factor for Rho-like GTPases. *ITSN2* expression analysis indicates that it is widely expressed in human tissues. Intersectin 2 isoforms show a subcellular distribution similar to other components of the endocytic machinery and co-localize with Eps15. Moreover, their overexpression, as well as the corresponding *ITSN1* protein forms, inhibits transferrin internalization. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Intersectin; Endocytosis; Clathrin; Alternative splicing

1. Introduction

The clathrin-mediated endocytosis pathway occurring at the plasma membrane of eukaryotic cells plays a crucial role in synaptic vesicle recycling in neurons, as well as in the internalization of receptor–ligand complexes and the maintenance of membrane identity in all cells. The uptake process mediated by clathrin-coated vesicles (CCVs) seems to require the coordinated interaction of many proteins to form large macromolecular complexes, and even though a growing list of proteins involved in CCV formation has emerged in recent years, the precise mechanism of this process remains unknown (reviewed in [1–3]).

The main structural component of CCVs is the 190-kDa protein clathrin heavy chain, which when associated with a 25-kDa clathrin light chain forms complexes called triskelions. The triskelions can organize themselves into a caged lattice forming the scaffold of the nascent CCVs [4]. Clathrin is recruited to the plasma membrane by the adapter protein AP-2, a heterotetramer that recognizes the signals present at the cytoplasmic tails of membrane proteins [5]. AP-2 also binds to Eps15 [6,7], epsin [8], amphiphysin heterodimers [9,10], auxilin [11] and AP180 [12]. The interaction occurs through the AP-2 α-adaptin appendage domain and a conserved DPF/W (aspartate-proline-phenylalanine/tryptophan) motif present in the ligands [11]. All these molecules participate not only in the rearrangement of the clathrin coat, but also act as antennae to attract other proteins to the growing CCVs. The fission of the clathrin-coated invaginated pit into free vesicles is thought to be mediated by the GTPase dynamin, which oligomerizes itself forming a helical collar around the neck of the endocytic pits [13,14]. The carboxy-end of dynamin contains a proline/arginine-rich domain able to interact with several SH3 (Src3 homology) domain-containing proteins including amphiphysin I and II, endophilin, syndapin and intersectin (reviewed in [3,15]).

Human intersectin is a multimodular protein containing two EH (Eps15 homology) domains, a central coiled-coil region and five consecutive SH3 domains. It exists also as a longer isoform, mainly expressed in brain, with an extended carboxy-end region composed of a DH (Dbl homology), a PH (pleckstrin homology) and a C2 domain [16,17]. Intersectin orthologues have been identified in rat (named EHSH1 [18]), mouse (named Ese1 [19]), *Xenopus laevis* [20] and *Drosophila melanogaster* (named Dap160 [21]). The short isoform of intersectin binds to several molecules involved in clathrin-mediated endocytosis through its different domains. Thus, the EH domains bind to epsins [19,20], the coiled-coil region interacts with the central region of Eps15 and Eps15R [19], and the SH3 domains interact with dynamin [18–20] and synaptojanin [18,20]. Intersectin has also been proposed to be a connection between endocytosis and exocytosis since its coiled-coil region binds to SNAP25 and SNAP23 [18]. The ability of intersectin to interact with multiple components of the endocytic and exocytic machinery leads to the suggestion that it acts as a scaffolding protein necessary for the formation of CCVs.

We have isolated a new human gene encoding for intersectin 2 (*ITSN2*), a putative paralogue gene of intersectin (which we herein will name intersectin 1, *ITSN1*). *ITSN2* is expressed as two main forms likely resulting from alternative splicing events. The intersectin 2 multimodular composition, the high degree of similarity with intersectin 1 and its subcellular localization suggest a role in clathrin-mediated endocytosis, a fact that we have confirmed by showing that its overexpression inhibits transferrin uptake.

*Corresponding author. Fax: (34)-93-2607776.
E-mail: sluna@iro.es

[1] The sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers AF182198 and AF182199.

0014-5793/00/$20.00 © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0014-5793(00)01793-2

2. Materials and methods

2.1. cDNA cloning and sequencing

In silico searches were performed using BLAST2 programs [22] at the National Center for Biotechnology Information (NCBI) against dbest with default parameters (http://www.ncbi.nlm.nih.gov). The expressed sequence tags (ESTs) were assembled into clusters using the Sequencher software (Gene Codes Corporation). cDNA was synthesized by reverse transcription of 3 μg of total human fetal brain and 0.5 μg of human fetal liver poly(A)$^+$ RNA (Clontech, Palo Alto, CA, USA) using the Ready-to-Go kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and random hexamers as primers, according to the manufacturer's recommendations. 1 μl of the cDNA was used as a template in three independent PCR reactions with primers designed on the in silico DNA: IT2-1f (238–266) and IT2-2r (1959–1939), IT2-3f (1840–1860) and IT2-4r (3291–3271), and IT2-16f (3236–3255) and IT2-17r (4915–4895). The positions of the primers listed in this section are given in brackets according to sequence in Fig. 1A; the f or r included in the name indicates the sense in the nucleotide sequence (f: forward; r: reverse). Rapid amplification of 5′-ends (5′-RACE) was carried out using the Marathon Ready cDNA kit (Clontech) from human fetal and adult brain with primer IT2-9r (1192–1173) as the gene-specific primer (GSP), and primer IT2-21r (630–609) as the nested specific primer (NSP). Two different 3′-RACE reactions were performed with the same kit and the following pairs of primers: IT2-16f (3236–3255) as GSP and IT2-20f (4801–4822) as NSP, and IT2-16f as GSP and IT2-18f (3715–3734) as NSP, respectively. Reactions were carried out as described in the manufacturer's protocol. RT-PCR and RACE products were cloned in pGEM-T Easy (Promega, Madison, WI, USA) and their DNA sequence determined with an ABI dye terminator cycle sequencing-ready reaction kit (Perkin Elmer, Foster City, CA, USA) and an automated DNA sequence analyzer ABI-Prism 377 XL Upgrade (Perkin Elmer).

Protein analysis for domain and pattern recognition was carried out using the following public programs: Profilescan (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html), SMART ([23]; http://smart.EMBL-Heidelberg.de) and COILS2 (http://www.isrec.isb-sib.ch/software/COILS_form.html).

2.2. Northern blotting

Northern blots containing poly(A)$^+$ RNA from human adult tissues (Cat. No. 7760-1), human fetal tissues (Cat. No. 7756-1) and different human brain sections (Cat. No. 7769-1) were purchased from Clontech. Northern blots were hybridized following the manufacturer's recommendations with the probes indicated in Section 3 labelled by random priming. Blots were washed sequentially in 2×SSC/0.1% SDS, 0.2×SSC/0.1% SDS and 0.1×SSC/0.1% SDS, at 65°C for 20 min each and exposed to X-ray film for 2–5 days at −70°C.

2.3. Vector construction

The different *ITSN2* open reading frames were subcloned as N-terminal HA-tagged fusions in the pCDNA-HA1 expression vector [24]. The N-terminus of the long isoform was generated by PCR with the forward primer IT2*Bam*HI/ATG (5′-GGATCCGCTCAGTTTCCCACAGCT-3′) and the reverse primer IT2-21r, then digested with *Bam*HI/*Sal*I and cloned into pCDNA3-HA1. The rest of the sequence was extended by ligating a *Hin*dIII-*Hin*dIII fragment and a *Hin*dIII-*Bst*XI fragment obtained from the cDNA clones described in Section 3.

To generate the short isoform, the 3′-end of *ITSN2-L* from nucleotide 3279 to end, in pCDNA3-HA1, was replaced by a *Bst*EII-*Xba*I fragment containing the specific 3′-end of *ITSN2-S*, generated by PCR with the forward primer IT2-16f and the reverse primer IT2*Xba*I/Stop (5′-GCTCTAGAACCATCTCTCATGAGA-3′).

Plasmids containing the open reading frames of *ITSN1-S* and *ITSN-L* were generated subcloning the corresponding cDNA fragments [17] into pCDNA3-HA1 with the 5′-end of *ITSN1* generated by PRC with primers IT1*Bam*HI/ATG (5′-CGCGGATCCATGGCTCAGTTTCCA-3′) and 76-26 (5′-ATTCCGTTCCCACTCAAGT-3′).

2.4. Protein expression analysis

COS-7 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and antibiotics. Transfections were carried out with Lipofectin (Life Technologies, Rockville, MA, USA) following the manufacturer's instructions. Cells were harvested and processed at a final time of 48 h post-transfection.

Protein expression was checked by Western blotting using total cell extracts. Briefly, transfected cells were harvested in phosphate-buffered saline (PBS) and cell pellets resuspended in Laemmli–SDS buffer. Cell extracts were run on 7.5% SDS–PAGE gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Filters were blocked in 10% dry milk powder/0.05% Tween-20 in PBS, and HA-tagged proteins detected with the anti-HA monoclonal antibody HA.11 (BabCo, Richmond, CA, USA) and enhanced chemiluminescence (WestPico Chemiluminescent Substrate, Pierce, Rockford, IL, USA).

2.5. Immunofluorescence and transferrin internalization assay

For immunofluorescence experiments, approximately 3×10^5 COS-7 cells were plated on 60-mm dishes containing 12-mm coverslips. Cells were transfected using 10 μg of Lipofectin (Life Technologies) together with 5 μg of recombinant plasmids in serum-free DMEM, and 16 h after transfection cells were washed with DMEM and incubated for another 30 h in DMEM with 5% of FBS.

Cells on coverslips were washed in PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized in 0.1% Triton X-100 for 10 min and blocked in PBS–10% FBS for 30 min. Cells were incubated with the primary antibodies in PBS–1% FBS for 1 h, washed intensively in PBS–1% FBS, then incubated with the secondary antibodies in PBS–10% FBS for 45 min and washed again with PBS–1% FBS. Coverslips were mounted using Citifluor (Citifluor Ltd, Cambridge, UK) and analyzed on an Olympus microscope BX60. As primary antibodies we used the anti-HA monoclonal antibody HA.11 (1 μg/ml) and a rabbit polyclonal anti-Eps15 (1 μg/ml; #C20, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies used were a goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody and a goat anti-rabbit Cy3-conjugated antibody (1:250; Amersham Pharmacia Biotech).

For the transferrin uptake assay, transfected cells were washed three times with serum-free DMEM, incubated for 1 h in serum-free DMEM, and then incubated for 25 min in DMEM containing 75 nM tetramethylrhodamine isothiocyanate (TRITC)-conjugated transferrin (Molecular Probes, Eugene, OR, USA) and 1 mg/ml bovine serum albumin. After washing thoroughly with PBS to reduce non-specific staining, cells were fixed, permeabilized and incubated with the anti-HA antibody as described above, in order to identify the transfected cells.

3. Results and discussion

3.1. Cloning of the human intersectin 2 cDNA

The use of *ITSN1* [17] as a template in BLAST searches against the human EST database identified several ESTs highly similar but not identical, suggesting the existence of a novel human gene. The ESTs were grouped in four independent clusters along the coding region of the *ITSN1* long isoform, allowing us to design primers in each one of the clusters to produce overlapping cDNA fragments. RT-PCR reactions using the combination of primers indicated in Section 2 on RNA from two different human tissues, brain and liver, rendered DNA fragments of the expected sizes and several clones were sequenced for each case. To isolate the full-length cDNA, we carried out two nested RACE reactions with primers IT2-9r and IT2-21r (5′-RACE) and IT2-16f and IT2-20f (3′-RACE). The assembly of the RT-PCR and RACE clones gave rise to a continuous 6091 bp long cDNA with an open reading frame of 1696 amino acids (Fig. 1A). Since this cDNA is highly similar all along its sequence to *ITSN1*, we propose to name this new gene *ITSN2* (accepted by the Human Gene Nomenclature Committee). There are two putative consecutive translation initiation codons preceded by an in-frame stop codon, but we favor the second one at nucleotide 241 because it presents a better consensus sequence for eukaryotic initia-

A

```
GGCTGAGGCGGCGCGGCGGAGCCCTGAGCGGCTGTGACAGGCTACGCAACAGGTTCGCGGGCGGCGGCCTGACGACCAAGCCAGCTGCAGTGGCGGCGACGGCGGCAGAGCAGGGTCTCC  120
CCGCGCCTGCCCGCGCCCAGGCTGCCGGTGCTGAGGGACGCGGAGTCGCGCTGTGACGAGCGGGAGGCGCGGCGAGGGCGCCAGATGGCTGAGAGCTAGCAAGGAAAACTCAGGACCATG  240
                                                                                                    *
              IT2-1f
ATGGCTCAGTTTCCCACAGCTATGAATGGAGGGCCAAACATGTGGGCTATTACCTCTGAAGAACGTACTAAGCATGACAGGCAGTTTGATAACCTCAAACCTTCAGGAGGTTACATAACA  360
 M  A  Q  F  P  T  A  M  N  G  G  P  N  M  W  A  I  T  S  E  E  R  T  K  H  D  R  Q  F  D  N  L  K  P  S  G  G  Y  I  T   40

GGTGATCAAGCACGTAATTTTTTCCTACAATCAGGTCTGCCGGCCCCTGTTTTAGCTGAAATATGGGCTTTATCAGACCTAAACAAGGATGGGAAGATGGATCAGCAAGAGTTCTCCATA  480
 G  D  Q  A  R  N  F  F  L  Q  S  G  L  P  A  P  V  L  A  E  I  W  A  L  S  D  L  N  K  D  G  K  M  D  Q  Q  E  F  S  I   80

GCTATGAAACTCATCAAACTGAAGCTTCAAGGCCAACAGTTGCCTGTGGTTCTCCCTCCTATTATGAAGCAACCCCCTATGTTTTCTCCATTAATTTCTGCTCGTTTTGGAATGGGAAGC  600
 A  M  K  L  I  K  L  K  L  Q  G  Q  Q  L  P  V  V  L  P  P  I  M  K  Q  P  P  M  F  S  P  L  I  S  A  R  F  G  M  G  S  120
                IT2-21r
ATGCCCAATCTGTCCATTCCTCAGCCATTGCCTCCAGCTGCACCTATAACATCATTGTCTTCTGCGACTTCAGGGACCAACCTTCCTCCCTTAATGATGCCCACTCCCCTAGTGCCTTCT  720
 M  P  N  L  S  I  P  Q  P  L  P  P  A  A  P  I  T  S  L  S  S  A  T  S  G  T  N  L  P  P  L  M  M  P  T  P  L  V  P  S  160

GTTAGCACATCATCATTACCAAATGGAACCGCCAGTCTCATTCAGCCTTTACCCATTCCTTATTCTTCTTCAACATTGCCTCATGGGTCATCTTATAGTCTGATGATGGGAGGATTTGGA  840
 V  S  T  S  S  L  P  N  G  T  A  S  L  I  Q  P  L  P  I  P  Y  S  S  S  T  L  P  H  G  S  S  Y  S  L  M  M  G  G  F  G  200

GGTGCTAGTATACAGAAAGCGCAGTCTCTGATTGATTTAGGATCTAGTAGCTCAACTTCCTCGACTGCTTCACTCTCAGGGAACTCACCCAAGACTGGGACCTCAGAGTGGGCAGTTCCT  960
 G  A  S  I  Q  K  A  Q  S  L  I  D  L  G  S  S  S  S  T  S  S  T  A  S  L  S  G  N  S  P  K  T  G  T  S  E  W  A  V  P  240

CAGCCTACAAGATTAAAATATCGGCAAAAATTTAATACTCTTGACAAAAGTATGAGTGGATATCTCTCAGGTTTTCAAGCTAGAAATGCCCTTCTTCAGTCAAATCTTTCTCAAACTCAG  1080
 Q  P  T  R  L  K  Y  R  Q  K  F  N  T  L  D  K  S  M  S  G  Y  L  S  G  F  Q  A  R  N  A  L  L  Q  S  N  L  S  Q  T  Q  280
                                                                                                        IT2-9r
CTGGCTACTATTTGGACTCTGGCTGACGTTGATGGTGATGGACAGCTAAAAGCAGAAGAGTTTATTCTTGCAATGCACCTTACTGACATGGCCAAAGCTGGACAGCCATTACCACTGACT  1200
 L  A  T  I  W  T  L  A  D  V  D  G  D  G  Q  L  K  A  E  E  F  I  L  A  M  H  L  T  D  M  A  K  A  G  Q  P  L  P  L  T  320

TTACCTCCTGAGCTTGTTCCTCCATCTTTCAGAGGAGGAAAGCAAATTGATTCCATTAATGGAACTCTGCCTTCATATCAGAAAATGCAAGAAGAGGAGCCTCAGAAGAAATTACCAGTT  1320
 L  P  P  E  L  V  P  P  S  F  R  G  G  K  Q  I  D  S  I  N  G  T  L  P  S  Y  Q  K  M  Q  E  E  E  P  Q  K  K  L  P  V  360

ACTTTTGAGGACAAACGGAAAGCCAACTATGAGCGAGGGAACATGGAGCTGGAAAAGCGACGCCAAGCCTTGATGGAGCAGCAACAAAGGGAGGCAGAACGTAAAGCCCAGAAAGAAAAG  1440
 T  F  E  D  K  R  K  A  N  Y  E  R  G  N  M  E  L  E  K  R  R  Q  A  L  M  E  Q  Q  Q  R  E  A  E  R  K  A  Q  K  E  K  400

GAAGAGTGGGAACGAAAACAGAGAGAATTACAAGAACAAGAATGGAAGAAACAACTTGAATTAGAAAAACGCTTAGAGAAGCAACGGGAATTGGAGAGACAACGAGAGGAAGAAAGGAGA  1560
 E  E  W  E  R  K  Q  R  E  L  Q  E  Q  E  W  K  K  Q  L  E  L  E  K  R  L  E  K  Q  R  E  L  E  R  Q  R  E  E  E  R  R  440

AAAGACATAGAAAGACGAGAGGCAGCAAAACAGGAACTTGAACGACAACGTCGCTTAGAATGGGAGAGAATTCGGCGACAGGAGCTTCTCAATCAAAAGAATAGAGAACAAGAAGAAATT  1680
 K  D  I  E  R  R  E  A  A  K  Q  E  L  E  R  Q  R  R  L  E  W  E  R  I  R  R  Q  E  L  L  N  Q  K  N  R  E  Q  E  E  I  480

GTCAGGTTAAACTCTAAAAAGAAGAATCTTCATCTTGAGTTGGAAGCACTGAATGGCAAACATCAGCAGATCTCAGGCAGACTTCAGGATGTCCGACTCAAAAAGCAAACTCAAAAGACT  1800
 V  R  L  N  S  K  K  K  N  L  H  L  E  L  E  A  L  N  G  K  H  Q  Q  I  S  G  R  L  Q  D  V  R  L  K  K  Q  T  Q  K  T  520
                                              IT2-3f
GAGCTGGAAGTTCTGGATAAGCAGTGTGACTTGGAAATTATGGAAATCAAGCAACTTCAACAGGAACTTCAGGAATATCAGAATAAGCTTATCTATCTGGTACCTGAGAAGCAATTATTA  1920
 E  L  E  V  L  D  K  Q  C  D  L  E  I  M  E  I  K  Q  L  Q  Q  E  L  Q  E  Y  Q  N  K  L  I  Y  L  V  P  E  K  Q  L  L  560
                          IT2-2r
AATGAAAGAATTAAAAACATGCAGTTCAGTAACACACCTGATTCAGGGGTCAGTTTACTTCATAAAAAATCATTAGAAAAGGAAGAATTATGCCAAAGACTTAAAGAACAGTTAGATGCT  2040
 N  E  R  I  K  N  M  Q  F  S  N  T  P  D  S  G  V  S  L  L  H  K  K  S  L  E  K  E  E  L  C  Q  R  L  K  E  Q  L  D  A  600

CTTGAAAAAGAAACTGCATCTAAGCTGTCAGAAATGGATTCTTTTAACAATCAACTAAAGTGTGGGAATATGGATGACTCTGTTCTTCAGTGCCTTTTGTCTCTGCTAAGCTGTCTCAAC  2160
 L  E  K  E  T  A  S  K  L  S  E  M  D  S  F  N  N  Q  L  K  C  G  N  M  D  D  S  V  L  Q  C  L  L  S  L  L  S  C  L  N  640

AACCTCTTCCTCTTACTTAAGGAACTGAGAGAAACCTACAACACACAGCAGTTAGCCCTTGAACAGCTTTATAAGATCAAACGTGACAAGTTGAAGGAAATTGAAAGGAAAAGATTAGAA  2280
 N  L  F  L  L  L  K  E  L  R  E  T  Y  N  T  Q  Q  L  A  L  E  Q  L  Y  K  I  K  R  D  K  L  K  E  I  E  R  K  R  L  E  680

CTAATGCAGAAAAAGAAACTAGAAGATGAGGCTGCAAGGAAAGCAAAGCAAGGAAAAGAAAACTTATGGAAAGAAAATCTTAGAAAGGAGGAAGAAGAAAAACAAAAGCGACTCCAGGAA  2400
 L  M  Q  K  K  K  L  E  D  E  A  A  R  K  A  K  Q  G  K  E  N  L  W  K  E  N  L  R  K  E  E  E  E  E  K  Q  K  R  L  Q  E  720

GAAAAAACACAAGAAAAAATTCAAGAAGAGGAACGGAAAGCTGAGGAGAAACAACGTAAGGATAAGGATACTTTGAAAGCTGAGGAGAAAAAACGTGAGACAGCTAGTGTTTTGGTGAAT  2520
 E  K  T  Q  E  K  I  Q  E  E  E  R  K  A  E  E  K  Q  R  K  D  K  D  T  L  K  A  E  E  K  K  R  E  T  A  S  V  L  V  N  760

TATAGAGCATTATACCCCTTTGAAGCAAGGAACCATGATGAGATGAGTTTTAATTCTGGAGATATAATTCAGGTTGATGAAAAAACCGTAGGAGAACCTGGTTGGCTTTATGGTAGTTTT  2640
 Y  R  A  L  Y  P  F  E  A  R  N  H  D  E  M  S  F  N  S  G  D  I  I  Q  V  D  E  K  T  V  G  E  P  G  W  L  Y  G  S  F  800

CAAGGAAATTTTGGCTGGTTTCCATGCAATTATGTAGAAAAAATGCCATCAAGTGAAAATGAAAAAGCTGTATCTCCAAAGAAGGCCTTACTTCCTCCTACAGTTTCTTTATCTGCTACC  2760
 Q  G  N  F  G  W  F  P  C  N  Y  V  E  K  M  P  S  S  E  N  E  K  A  V  S  P  K  K  A  L  L  P  P  T  V  S  L  S  A  T  840

TCAACTTCCTCTGAACCACTTTCTTCAAATCAACCAGCATCAGTGACTGATTATCAAAATGTATCTTTTTCAAACCTAACTGTAAATACATCATGGCAGAAAAAATCAGCCTTCACTCGA  2880
 S  T  S  S  E  P  L  S  S  N  Q  P  A  S  V  T  D  Y  Q  N  V  S  F  S  N  L  T  V  N  T  S  W  Q  K  K  S  A  F  T  R  880

ACTGTGTCCCCTGGATCTGTATCACCTATTCATGGACAGGGACAAGTGGTAGAAAACTTAAAAGCACAGGCCCTTTGTTCCTGGACTGCAAAGAAAGATAACCACTTGAACTTCTCAAAA  3000
 T  V  S  P  G  S  V  S  P  I  H  G  Q  G  Q  V  V  E  N  L  K  A  Q  A  L  C  S  W  T  A  K  K  D  N  H  L  N  F  S  K  920

CATGACATTATTACTGTCTTGGAGCAGCAAGAAAATTGGTGGTTTGGGGAGGTGCATGGAGGAAGAGGATGGTTTCCCAAATCTTATGTCAAGATCATTCCTGGGAGTGAAGTAAAACGG  3120
 H  D  I  I  T  V  L  E  Q  Q  E  N  W  W  F  G  E  V  H  G  G  R  G  W  F  P  K  S  Y  V  K  I  I  P  G  S  E  V  K  R  960

GAAGAACCAGAAGCTTTGTATGCAGCTGTAAATAAGAAACCTACCTCGGCAGCCTATTCAGTTGGAGAAGAATATATTGCACTTTATCCATATTCAAGTGTGGAACCTGGAGATTTGACT  3240
 E  E  P  E  A  L  Y  A  A  V  N  K  K  P  T  S  A  A  Y  S  V  G  E  E  Y  I  A  L  Y  P  Y  S  S  V  E  P  G  D  L  T  1000
   IT2-16f                  IT2-4r
TTCACAGAAGGTGAAGAAATATTGGTGACCCAGAAAGATGGAGAGTGGTGGACAGGAAGTATTGGAGATAGAAGTGGAATTTTTCCATCAAACTATGTCAAACCAAAGGATCAAGAGAGT  3360
 F  T  E  G  E  E  I  L  V  T  Q  K  D  G  E  W  W  T  G  S  I  G  D  R  S  G  I  F  P  S  N  Y  V  K  P  K  D  Q  E  S  1040

TTTGGGAGTGCTAGCAAGTCTGGAGCATCAAATAAAAAACCTGAGATTGCTCAGGTAACTTCAGCATATGTTGCTTCTGGTTCTGAACAACTTAGCCTTGCACCAGGACAGTTAATATTA  3480
 F  G  S  A  S  K  S  G  A  S  N  K  K  P  E  I  A  Q  V  T  S  A  Y  V  A  S  G  S  E  Q  L  S  L  A  P  G  Q  L  I  L  1080

ATTCTAAAGAAAAATACAAGTGGGTGGTGGCAAGGAGAGTTACAGGCCAGAGGAAAAAAGCGACAGAAAGGATGGTTTCCTGCCAGTCATGTTAAACTTTTGGGTCCAAGTAGTGAAAGA  3600
 I  L  K  K  N  T  S  G  W  W  Q  G  E  L  Q  A  R  G  K  K  R  Q  K  G  W  F  P  A  S  H  V  K  L  L  G  P  S  S  E  R  1120

GCCACACCTGCCTTTCATCCTGTATGTCAGGTGATTGCTATGTATGACTATGCAGCAAATAATGAAGATGAGCTCAGTTTCTCCAAGGGACAACTCATTAATGTTATGAACAAAGATGAT  3720
 A  T  P  A  F  H  P  V  C  Q  V  I  A  M  Y  D  Y  A  A  N  N  E  D  E  L  S  F  S  K  G  Q  L  I  N  V  M  N  K  D  D  1160
   IT2-18f
CCTGATTGGTGGCAAGGAGAGATCAACGGGGTGACTGGTCTCTTTCCTTCAAACTACGTTAAGATGACGACAGACTCAGATCCAAGTCAACAGTGGTGTGCTGATCTGCAAACCCTGGAC  3840
 P  D  W  W  Q  G  E  I  N  G  V  T  G  L  F  P  S  N  Y  V  K  M  T  T  D  S  D  P  S  Q  Q  W  C  A  D  L  Q  T  L  D  1200
```

Fig. 1.

ACAATGCAGCCAATTGAGAGGAAAAGACAGGGCTATATTCATGAGCTGATTCAGACCGAAGAGCGGTACATGGCTGACCTTCAGCTCGTCGTCGAGGTTTTTCAGAAACGCATGGCAGAG 3960
T M Q P I E R K R Q G Y I H E L I Q T E E R Y M A D L Q L V V E V F Q K R M A E 1240

TCAGGCTTTCTCACTGAAGGGGAGATGGCCCTGATTTTTGTTAACTGGAAGGAGCTCATCATGTCCAACACAAAGCTGCTGAAGGCTTTGCGGGTGCGGAAGAAGACCGGGGGCGAGAAG 4080
S G F L T E G E M A L I F V N W K E L I M S N T K L L K A L R V R K K T G G E K 1280

ATGCCGGTGCAGATGATTGGGGACATCCTGGCCGCTGAGCTGTCCCACATGCAGGCTTACATCAGGTTCTGCAGCTGCCAGCTTAATGGAGCAGCTCTGTTACAGCAGAAGACAGATGAA 4200
M P V Q M I G D I L A A E L S H M Q A Y I R F C S C Q L N G A A L L Q Q K T D E 1320

GACACAGATTTCAAAGAATTTTTAAAGAAGCTGGCATCTGACCCGCGGTGTAAAGGAATGCCCCTCTCCAGCTTCCTGCTGAAACCCATGCAGAGGATCACCCGCTACCCACTGCTCATC 4320
D T D F K E F L K K L A S D P R C K G M P L S S F L L K P M Q R I T R Y P L L I 1360

AGAAGTATTCTGGAGAACACCCCGGAGAGCCATGCAGACCATTCCTCCCTAAAGCTGGCCCTCGAGCGGGCAGAGGAGCTGTGCTCTCAAGTGAATGAGGGAGTTCGGGAGAAGGAAAAC 4440
R S I L E N T P E S H A D H S S L K L A L E R A E E L C S Q V N E G V R E K E N 1400

TCGGACCGACTGGAGTGGATCCAGGCGCACGTGCAGTGTGAAGGCCTCGCGGAGCAACTTATTTTCAACTCTCTCACCAACTGCCTGGGGCCCCGGAAGCTCTTACACAGTGGGAAATTA 4560
S D R L E W I Q A H V Q C E G L A E Q L I F N S L T N C L G P R K L L H S G K L 1440

TACAAGACCAAGAGCAMCAAGGAACTGCACGGATTCCTCTTCAATGACTTCCTGCTTCTTACCTACATGGTCAAGCAGTTTGCTGTTTCCTCTGGCTCTGAGAAACTTTTCAGCTCGAAG 4680
Y K T K S X K E L H G F L F N D F L L L T Y M V K Q F A V S S G S E K L F S S K 1480

TCCAATGCTCAATTCAAAATGTATAAAACGCCCATTTTCCTGAATGAAGTCTTGGTGAAACTGCCCACAGACCCTTCCAGCGATGAGCCTGTCTTCCACATTTCCCACATTGATCGGGTC 4800
S N A Q F K M Y K T P I F L N E V L V K L P T D P S S D E P V F H I S H I D R V 1520

IT2-20f IT2-17r
TACACCCTCCGAACAGACAACATTAATGAGAGGACCGCCTGGGTGCAGAAGATCAAGGCGGCGTCTGAGCAGTACATCGACACCGAGAAGAAGAAGCGTGAGAAAGCTTACCAAGCCCGC 4920
Y T L R T D N I N E R T A W V Q K I K A A S E Q Y I D T E K K K R E K A Y Q A R 1560

TCCCAAAAGACTTCAGGCATTGGGCGCCTGATGGTGCATGTCATTGAAGCTACAGAATTAAAAGCCTGCAAACCAAATGGAAAGAGCAACCCATACTGTGAAATCAGCATGGGCTCCCAG 5040
S Q K T S G I G R L M V H V I E A T E L K A C K P N G K S N P Y C E I S M G S Q 1600

AGCTACACCACCAGGACCATCCAGGACACACTCAATCCCAAGTGGAATTTTAACTGCCAGTTCTTTATTAAGGATCTCTACCAAGACGTGCTGTGTCTCACCCTGTTTGACAGAGACCAG 5160
S Y T T R T I Q D T L N P K W N F N C Q F F I K D L Y Q D V L C L T L F D R D Q 1640

IT2-22f
TTTTCACCAGATGATTTCCTGGGTCGTACTGAAATTCCAGTGGCAAAAATTCGAACAGAACAGGAAAGCAAAGGCCCTATGACCCGCCGACTGCTGCTGCATGAGGTCCCCACCGGGGAG 5280
F S P D D F L G R T E I P V A K I R T E Q E S K G P M T R R L L L H E V P T G E 1680

GTCTGGGTCCGTTTTGACCTGCAGCTTTTTGAGCAAAAAACTCTCCTG**TAG**GGGTTCTAAAGGACAGCACCAGCGGGACAGCCCACAAGGCTGGGGCTGGAGAATGAGAGACTGCGCTCT 5400
V W V R F D L Q L F E Q K T L L * 1696

CTTGGGGCTGAGGGAGCACCATGCAGCTTCACCCCTCACAAAGCCATGCACGCTGGGGGCTCTGTTTTCCTGCACACTAAATAGCTAGCAATCTATGCAAACACCTTTCCCATAAAGAAA 5520
CCAAACCCCATAGTACAGTGCCTTGTCCTAGTGTTCACATGTTCAGCTCTGTTTGTTTAGATGCCAAGGTTTCCATTTTCAGGGCTATAAAAAGTATTACTTGGAAATGAGGCATCAGAC 5640

IT2-23r
CACCAGATGTTACCGCTCGGTTGAATGTGTCCACCGTGGAGTGGTTTGGTGACGCTGTAACCATTCCACGCCAGTGACCTCTGCTGGGTCACAGCCACTCAGGAGGGGAAGGGTCAGGAT 5760
GAGAGGCTGCAGCCTCGACACTTGGCGCGGCCTGATACTGAAATAGCGTCTACTCGTGCACTGAATAAAAACAGAAACTTGATCATTTTATTCCTGATTAGATTTTATCACTCTCTGCTA 5880
AGACAATATAGTCTGGAGTATAAGTGGGAAAGCTTGATTTAAATACTGTGAACTCTAATAATGTGGAAAATATTTTTCAACTTTAATTTTCTGAAGTATAAATTATTTATGTAAATTCAT 6000
TGTTTTTGCATATTTCTTAGGACATGCATCTTTAAGCTTTATCATTGCCCATATGTACAGAAAGAGAATAAAGACATATGTTTATGGATGG 6091

B

...TATGACTATGCAGCAAATAATGAAGATGAGCTCAGTTTCTCCAAGGGACAACTCATTAATGTTATGAACAAAGATGAT 3720
...Y D Y A A N N E D E L S F S K G Q L I N V M N K D D 1160

IT2-18f
CCTGATTGGTGGCAAGGAGAGATCAACGGGGTGACTGGTCTCTTTCCTTCAAACTACGTTAAGATGACGACAGACTCAGATCCAAGTCAACAGTGGTGTGCTGATCTGCAAACCCTGGAC 3840
P D W W Q G E I N G V T G L F P S N Y V K M T T D S D P S Q Q W C A D L Q T L D 1200

ACAATGCAGCCAATTGAGAGGAAAAGACAGGGCTATATTCATGAGCTGATTCAGACCGAAGAGCGGTACATGGCTGACCTTCAGCTCGTCGTCGAGGTGTGGAGGCTGCTGCTGGCTAGC 3960
T M Q P I E R K R Q G Y I H E L I Q T E E R Y M A D L Q L V V E V W R L L L A S 1240

TCTCGGGGTATCTGCTGTCTCTCA**TGA**GAGATGGTGGGCATCAGACTCAGGGCTGCCTCCACGCAGAGTCAAAGCAAGGCATCACTTTTGATGTGTGAATTCACAAATAGTGACGGAAGC 4080
S R G I C C L S * 1248

TCTCACATCCTCCAAATGCTGTCTCTGCCTGCCGGATAATGCTTGAGATTGAAAGTCTCTAATGAGCTCTTTCCCCCAGATGAGGTCACTCAGAGTGAAGCGGGAGAACAAGAGGGCATT 4200
CGCATGGCTTCGTTGGATGTGGCAGGAGCCCCATCAAGGAAGGACGGGGATAGAGTGGATGGGAAGGGCCTATGGCAGACCGTAGCTTCCTTGGATATTTGCCTAATATCTGTTTTAACA 4320
TCTGACATTTTCATAAACTGGTATCTCTGGAGGAACTGTGAAACAGTGAAAGTGTTCACCTCATGGTTGTATCAGTTTGGAAAACCCAATGGGAGCATACTGTAAAATAGTTCCCAAATA 4440
TCATATAGCTACTGTTTGTTTATACCAGTGACCTCTACTCTGATGACAGCTATCCTTATTCGAGTAGCACTTTAAAATGATTTGTGCTTGAGTGAACAAAAGAAGACTTTCCATTTC 4557

C

...TATGACTATGCAGCAAATAATGAAGATGAGCTCAGTTTCTCCAAGGGACAACTCATTAATGTTATGAACAAAGATGAT 3720
...Y D Y A A N N E D E L S F S K G Q L I N V M N K D D 1160

CCTGATTGGTGGCAAGGAGAGATCAACGGGGTGACTGGTCTCTTTCCTTCAAACTACGTTAAGATGACGACAGACTCAGATCCAAGTCAACAG**TGA**CCCAATGTTGTCTTCCAGTTGTGA 3840
P D W W Q G E I N G V T G L F P S N Y V K M T T D S D P S Q Q * 1190

AAGCACCCCAGAGACCCACTATCCAAGTTTCACTCTAGCGTGGAGGCAGGGCAGGCAGCCCTGATCAAATATCTCCTACACAATTCGTTTACTTCGTTTGAATGTTAGAGCCACTTGTGA 3960
TTATTTTTTTGTGTTTCTAACTTACAGTTTAAATTTATTTGTAAAAAGTTAAAGGATAGTGGGTCTTTGTGTGGCTTTCCCTGCTGTTCACTCTGGCATCTTTAGCATTTTTCTTCTTTT 4080
TTAATTTGATAATTGTAGGTCATTAGCATGCATATTGAGTTTGCCCTTATGTGGTGGGAGTTCAAACACACAAAGACCCACTATTTGCACAAACTATTCT...

Fig. 1. Nucleotide and amino acid sequence of human *ITSN2* isoforms. A: Nucleotide and deduced amino acid sequence of *ITSN2-L*. Nucleotides and amino acid residues are numbered in the right margin. Potential initiation and stop codon and putative first methionine are in bold. The stop codon preceding the initiating ATG is indicated with a star. Putative polyadenylation signals are double underlined. Lines above the nucleotide sequence indicate primers used for the cloning strategy. B: Nucleotide and amino acid sequence of the 3′-end of *ITSN2-S1* showing the alternative 3′-UTR. The short fragment of 15 amino acids specific of intersectin 2-S1 is waved underlined. C: Partial nucleotide and amino acid sequence of the 3′-end of *ITSN2-S2* showing the alternative 3′-UTR. The 3′-UTR full sequence has 3268 nucleotides and corresponds to the SH3P18 cDNA sequence (GenBank accession number U61167).

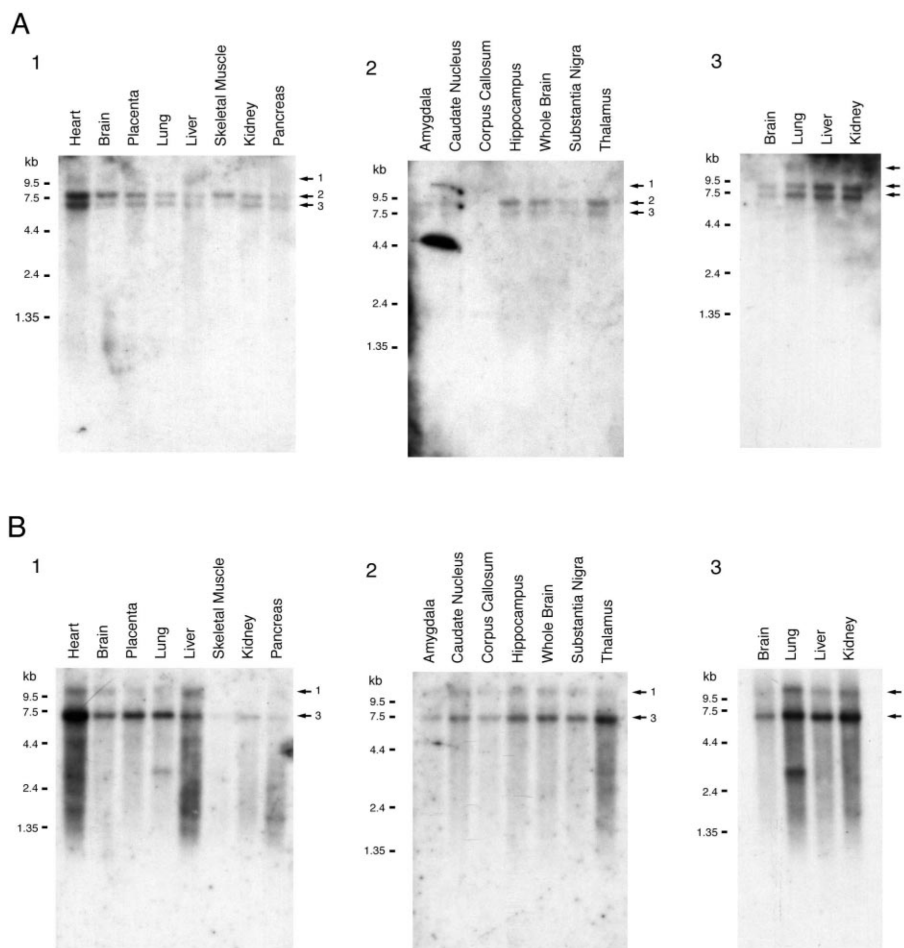


Fig. 2. Expression analysis of *ITSN2*. A: Northern blots containing 2 μg of poly(A)$^+$ RNA from the human adult tissues (panel 1), human brain sections (panel 2) and human fetal tissues (panel 3) were hybridized with probe S (nucleotides 304–1887). Arrows point to the three bands of approximately 6.5, 8 and 10 kb (arrows 1, 2 and 3, respectively) present in all the membranes. B: Membranes described above were hybridized with an *ITSN2-L*-specific probe spanning nucleotides 4906–5912 (probe L). Note the absence of the 8-kb transcript (arrow 2). A 3-kb mRNA is detected in adult and fetal lung.

tion of translation (CC[G/A]CCATGG [25]). Two consensus polyadenylation signals (AATAAA) are found at nucleotide positions 5824 and 6067, in the 763 bp long 3′-untranslated region (UTR) (Fig. 1A).

ITSN1 is expressed as two major alternative spliced transcripts that differ in their 3′-ends [16,17]. To look for the existence of a shorter isoform in *ITSN2*, we carried out a 3′-RACE reaction with primers IT2-16f and IT2-18f and isolated several clones with a different 3′-end. The new cDNA gave rise to an open reading frame of 1248 amino acids, with only 15 amino acids not present in the long open reading frame (Fig. 1B). In spite of all the isolated clones having poly(A) tails, no clear polyadenylation signals were found in their 3′-UTRs. A partial sequence of human *ITSN2* was represented in the cDNA clone SH3P18 ([26], GenBank accession number U61167), which would generate a different short open reading frame (Fig. 1C) lacking 57 amino acids and equivalent to the previously described short isoform of intersectin 1 (*ITSN1*). We will refer to the different protein isoforms as intersectin 2-L (*ITSN2-L*, GenBank accession number AF182198) and intersectin 2-S1 (*ITSN2-S*, GenBank accession number AF182199) for the long and short isoforms, respectively, and intersectin 2-S2 for the isoform represented by SH3P18.

3.2. *ITSN2 expression analysis*

To determine the expression pattern of *ITSN2*, we have hybridized Northern blots containing mRNA from different fetal and adult human tissues, using a probe spanning nucleotides 304–1887 (probe S) common to the short and long *ITSN2* isoforms. The assay revealed the presence of three major transcripts of approximately 6.5, 8 and 10 kb in all the human tissues tested, but with differences in the total amount and ratio among the three expressed bands (named 3, 2 and 1 in Fig. 2A, panels 1, 2 and 3). The 10-kb transcript, primarily expressed in adult heart and liver, is present in all the tissues, although it is only detectable with longer exposures (data not shown). To find out which of the transcripts corresponded to the long isoform, we hybridized the same set of blots with probe L (nucleotides 4906–5912 in Fig. 1A). In this case, only the 6.5- and 10-kb mRNA species were observed in all the tissues (bands 3 and 1 in Fig. 2B, panels 1, 2 and 3). Probe L also detected a minor band of approximately 3 kb in fetal and adult lung not present in probe S membranes (Fig. 2B, panels 1 and 3), suggesting that this transcript may contain only the C-terminal subset of the domains described below (Fig. 3A). Interestingly, despite both probes detecting the same band pattern in adult and fetal tissues, differences in the band ratio could be observed.

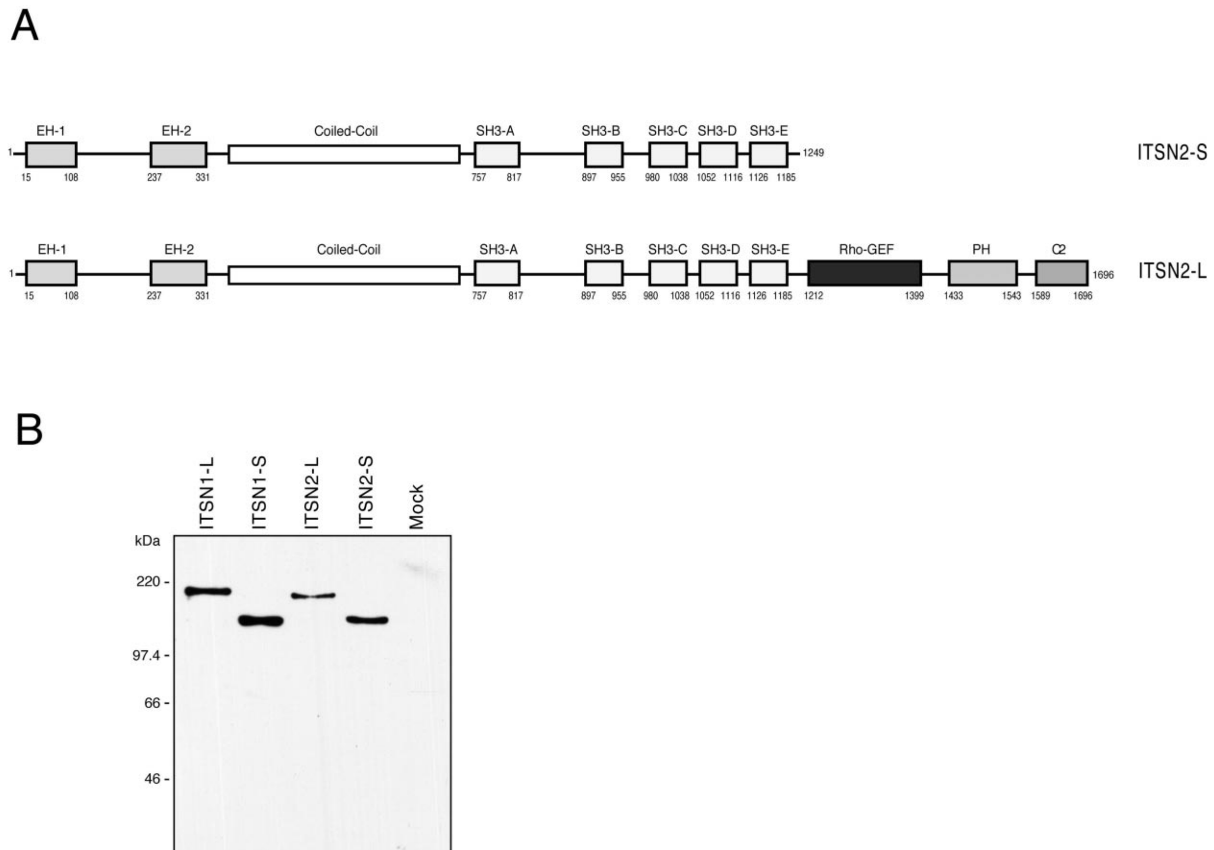


Fig. 3. Multimodular protein analysis and expression of intersectins. A: Schematic representation of intersectin 2-S and intersectin 2-L showing its multimodular structure. Domains are represented as boxes and numbers below them show the domain position in the amino acid sequence. B: Expression of human intersectins. Extracts of COS-7 cells transfected with pCDNA3-HA1/ITSN2-S, /ITSN2-L, /ITSN1-S and /ITSN1-L, or mock-transfected (−), were analyzed by Western blot. Tagged proteins were detected with an anti-HA antibody. Size markers in kDa are shown.

Thus, for instance, whereas in fetal brain bands 2 and 3 (short and long isoform, respectively) presented similar intensity, band 2 is clearly overexpressed with respect to band 3 in adult brain (Fig. 2A, panels 1 and 3). Altogether these results indicate that, although widely expressed, *ITSN2* expression is tissue-dependent and likely regulated during development.

Human *ITSN1* is expressed as three main transcripts of approximately 15, 6 and 4.5 kb [16]. In distinction to *ITSN2*, the longest one corresponds to the long isoform and it is expressed almost exclusively in brain, a fact that establishes a clear difference between both genes.

3.3. *ITSN2 protein primary sequence analysis*

The multidomain composition of intersectin 2 is schematically depicted in Fig. 3A. Intersectin 2-S possesses two EH domains at positions 15–108 and 237–331 which share 52.7% similarity between them, and 87.1% and 72.6% for EH1 and EH2, respectively, when compared with the equivalent EH domains in human intersectin 1. The EH domains are followed by a region almost entirely composed of glutamic acid (18.9%), lysine (15%), leucine (14.5%), glutamine (11.3%) and arginine (9.3%), which is predicted to be a coiled-coil region. Although this is one of the less conserved regions between intersectin 1 and 2, the amino acid composition is highly similar.

The central part of the protein has five consecutive SH3 domains named A–E, respectively (Fig. 3A). The open reading frame of intersectin 2-S finishes after the last SH3 domain (SH3-E), and may extend 57 amino acids when compared to intersectin 1-S, depending on which alternative splicing occurs (Fig. 1B,C). The level of conservation between each SH3 domain of intersectin 1 and 2 is high (more than 80% similarity),

→

Fig. 4. Subcellular localization of intersectins and transferrin uptake inhibition assay. A: COS-7 cells transiently transfected with pCDNA3-HA1/ITSN2-S, /ITSN2-L, /ITSN1-S and /ITNS1-L were analyzed by indirect immunofluorescence with an anti-HA antibody and a secondary antibody conjugated with FITC (left panels); TRICT transferrin uptake is detected by red staining (central panel). Overlapping images are shown (right panel). B: Co-localization of ectopically expressed intersectins with endogenous Eps15. COS-7 cells transiently transfected with pCDNA3-HA1/ITSN2-L and /ITNS1-L were analyzed by indirect immunofluorescence with anti-HA (green) and anti-Eps15 (red) antibodies as indicated. Overlapping images are shown (merge: yellow). Some of the co-localizing dots of intersectin 2-L are indicated with arrows. C: Quantitation of the effect of intersectin overexpression in the internalization of transferrin expressed as a percentage of transfected COS-7 cells showing normal transferrin uptake. More than 200 transfected cells were counted for each category. Error bars indicate standard deviation of three experiments.

A

Anti-HA

TRITC-Transferrin

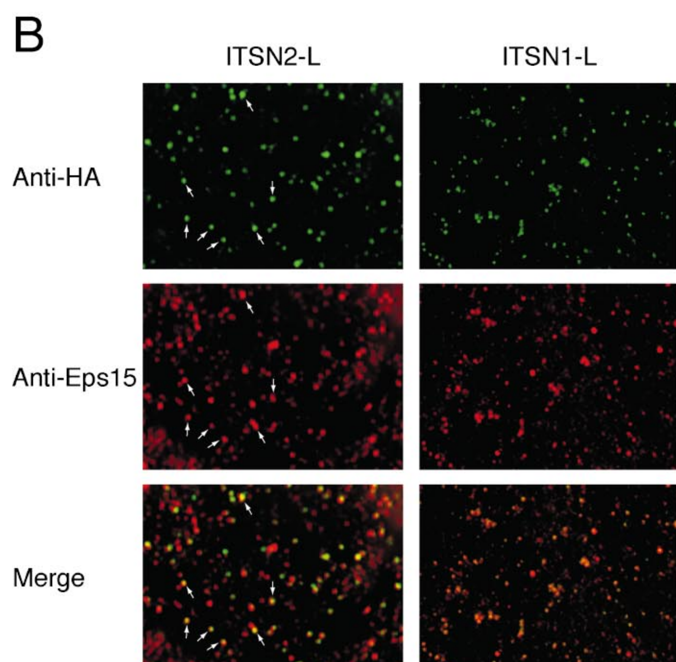
Merge

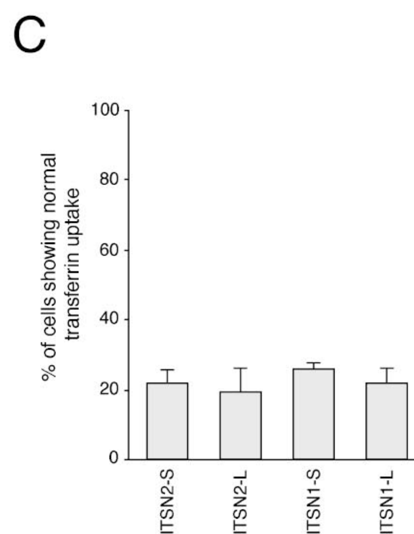
ITSN2-S

ITSN2-L

ITSN1-S

ITSN1-L





except in the case of the SH3A, which is noticeably less conserved (62% similarity).

The carboxy-terminus specific to the intersectin 2-L isoform contains three well-described domains. The first one, from residues 1212 to 1399, matches a DH domain which usually functions as the catalytic region of guanine nucleotide exchange factors (GEFs) for Rho-like GTPases [27]. Interestingly, all the residues which have been described as important for its enzymatic activity [28] are present in the intersectin 2 DH domain (L-1228, I-1253, F-1254, I-1287, L-1343, L-1347 and R-1353). As in all the other DH domain-containing proteins, the intersectin 2 DH domain is followed by a PH domain with 85.4% similarity between intersectin 1 and 2. Finally, the most carboxy region of the long isoform has sequence homology with C2 domains [29], a calcium binding motif widely distributed among eukaryotic proteins and thought to be involved in cellular membrane interaction.

We have generated four recombinant plasmids in which the coding regions of intersectin 2-L and -S, and the equivalent isoforms of intersectin 1 [17], were cloned as N-terminal HA-tagged proteins. To check intersectin expression, lysates of COS-7 cells transfected with the four plasmids were analyzed by Western blot with an anti-HA antibody. As is shown in Fig. 3B, expressed tagged proteins migrated in good agreement with their predicted molecular weights: intersectin 2-S, 141.7 kDa; intersectin 2-L, 193.3 kDa; intersectin 1-S, 137.6 kDa and intersectin 1-L, 195.4 kDa.

3.4. Intersectin 2 subcellular localization and endocytosis assay

The same constructs described above were used to transfect COS-7 cells in order to analyze the subcellular localization of the fusion proteins (Fig. 4A, left panels). Both intersectin 2 and 1 showed a fine punctate sparse throughout the cytoplasm that was very similar to the one reported for other components of CCVs such as AP-2, Eps15, clathrin or epsin [8,30]. In fact, all the intersectins tested (2-L, 2-S, 1-L and 1-S) co-localized with endogenous Eps15 (Fig. 4B and data not shown), comparable to what has been described for Ese1-S [19]. Despite both intersectins presenting a similar staining pattern, subtle differences could be appreciated, with a more marked perinuclear staining for intersectin 1 (two representative cells are shown in Fig. 4A, left panels), and concentrated in Golgi-like structures in agreement with what has been reported for the endogenous protein in rat hippocampal neurons [31]. Conversely, we observed no evident staining pattern differences between the short and long isoforms in either intersectin 1 or 2, as an indication that the protein domains present in the short isoforms are sufficient for mediating intersectin subcellular distribution. The EH domains have been pointed to as responsible for the subcellular localization of intersectin 1 [31]; however, the deletion of the SH3 domains in both intersectins promotes the nuclear accumulation of the resulting 60-kDa proteins (data not shown), suggesting that these domains may be involved in cytosolic retention and therefore contribute to the proper protein localization.

To test if intersectin 2 is involved in endocytosis, we have performed a transferrin endocytosis assay. Since endocytosis is dependent on a fine regulation of the interaction of the different proteins involved, altering the relative ratios of some of the components of the uptake mechanism can disrupt it. With this reasoning, clathrin-mediated endocytosis of transferrin has been shown to be inhibited by overexpression of amphiphysin I and II [10], epsins [32] and Ese1-S [19]. Therefore, we performed a transferrin inhibition uptake assay in COS-7 cells overexpressing the two isoforms of both intersectins. Untransfected cells gave a punctate, perinuclear red staining characteristic of the endosomal transferrin internalization pathway. As has been previously described for mouse Ese1 [19], intersectin 1-S behaved as an inhibitor of transferrin internalization in this assay. Similarly, overexpression of either intersectin 1-L, intersectin 2-S or intersectin 2-L caused a block of transferrin uptake, scored by the absence of perinuclear red staining (Fig. 4A). More than 200 cells were counted in the analysis, with reproducible results in different experiments as quantitated in the bar graph (Fig. 4C). The efficiency of the blockade was very similar for the distinct intersectins, with a slightly higher score for intersectin 2 isoforms. These data indicate that overexpression of any of the intersectin 2 isoforms can block clathrin-mediated endocytosis, probably by sequestering binding partners involved in the process.

In the case of intersectin 1 short isoform, this feature has been associated with its ability to bind CCV-related proteins such as dynamin [18–20] or synaptojanin [18,20]. In particular, the intersectin 1 SH3A domain has been described as the most potent inhibitor of CCV formation in permeabilized 3T3-L1 cells and as such participating in early events, while the SH3C and SH3E domains were involved in late events of CCV fission [33]. These differences probably reflect the binding abilities of each domain. We still do not know the binding partners of intersectin 2, although due to its high amino acid similarity, it is tempting to speculate that some will be the same as for intersectin 1. The co-localization of intersectin 2 with endogenous Eps15 indeed confirms that this is the case for this CCV-related molecule. At this point it is worthwhile to mention that the SH3A domain is the most divergent one when intersectin 1 and 2 are compared, and that this might impose differences in its ability of binding dynamin [18,20] or its recently described partner mSos1 [34]. Future efforts should be directed to finding out the partner specificity of each intersectin and the functional implication of the extra carboxy-end, a putative GEF for a Rho-like GTPase.

The number of proteins involved in clathrin-mediated endocytosis from the plasma membrane has grown dramatically in the past few years revealing a complex picture of protein–protein interactions. Intersectin 1 and its paralogous intersectin 2 described here belong to this group of proteins and likely act as scaffolding on providing a great variety of interacting domains.

Acknowledgements: We wish to thank Dr. M.L. Arbonés for critical reading of the manuscript. This study was supported by the European Union (BIOMED2 BMH4-CT98-3039), the Spanish CICYT (SAF99-0092-CO2-01), and the Fundació Catalana Síndrome de Down/Marató de TV3-1993. S.L. is supported by the Spanish Ministerio de Educación y Ciencia.

References

[1] Cremona, O. and De Camilli, P. (1997) Curr. Opin. Neurobiol. 7, 323–330.
[2] Schmid, S.L. (1997) Annu. Rev. Biochem. 66, 511–548.
[3] Marsh, M. and McMahon, H.T. (1999) Science 285, 215–220.
[4] Pishvaee, B. and Payne, G.S. (1998) Cell 95, 443–446.
[5] Kirchhausen, T., Bonifacino, J.S. and Riezman, H. (1997) Curr. Opin. Cell Biol. 9, 488–495.

[6] Fazioli, F., Minichiello, L., Matoskova, B., Wong, W.T. and Di Fiore, P.P. (1993) Mol. Cell. Biol. 13, 5814–5828.
[7] Benmerah, A., Begue, B., Dautry-Varsat, A. and Cerf-Bensussan, N. (1996) J. Biol. Chem. 271, 12111–12116.
[8] Chen, H., Fre, S., Slepnev, V.I., Capua, M.R., Takei, K., Butler, M.H., Di Fiore, P.P. and De Camilli, P. (1998) Nature 394, 793–797.
[9] David, C., McPherson, P.S., Mundigl, O. and De Camilli, P. (1996) Proc. Natl. Acad. Sci. USA 93, 331–335.
[10] Wigge, P., Kohler, K., Vallis, Y., Doyle, C.A., Owen, D., Hunt, S.P. and McMahon, H.T. (1997) Mol. Biol. Cell 8, 2003–2015.
[11] Owen, D.J., Vallis, Y., Noble, M.E., Hunter, J.B., Dafforn, T.R., Evans, P.R. and McMahon, H.T. (1999) Cell 97, 805–815.
[12] Wang, L.H., Sudhof, T.C. and Anderson, R.G. (1995) J. Biol. Chem. 270, 10079–10083.
[13] Hinshaw, J.E. and Schmid, S.L. (1995) Nature 374, 190–192.
[14] Stowell, M.H., Marks, B., Wigge, P. and McMahon, H.T. (1999) Nature Cell Biol. 1, 27–32.
[15] Schmid, S.L., McNiven, M.A. and De Camilli, P. (1998) Curr. Opin. Cell Biol. 10, 504–512.
[16] Guipponi, M., Scott, H.S., Chen, H., Schebesta, A., Rossier, C. and Antonarakis, S.E. (1998) Genomics 53, 369–376.
[17] Pucharcós, C., Fuentes, J.J., Casas, C., de la Luna, S., Alcántara, S., Arbonès, M.L., Soriano, E., Estivill, X. and Pritchard, M. (1999) Eur. J. Hum. Genet. 7, 704–712.
[18] Okamoto, M., Schoch, S. and Sudhof, T.C. (1999) J. Biol. Chem. 274, 18446–18454.
[19] Sengar, A.S., Wang, W., Bishay, J., Cohen, S. and Egan, S.E. (1999) EMBO J. 18, 1159–1171.
[20] Yamabhai, M., Hoffman, N.G., Hardison, N.L., McPherson, P.S., Castagnoli, L., Cesareni, G. and Kay, B.K. (1998) J. Biol. Chem. 273, 31401–31407.
[21] Roos, J. and Kelly, R.B. (1998) J. Biol. Chem. 273, 19108–19119.
[22] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[23] Schultz, J., Milpetz, F., Bork, P. and Ponting, C.P. (1998) Proc. Natl. Acad. Sci. USA 95, 5857–5864.
[24] Lee, C.W., Sorensen, T.S., Shikama, N. and La Thangue, N.B. (1998) Oncogene 16, 2695–2710.
[25] Kozak, M. (1991) J. Cell Biol. 115, 887–903.
[26] Sparks, A.B., Hoffman, N.G., McConnell, S.J., Fowlkes, D.M. and Kay, B.K. (1996) Nature Biotechnol. 14, 741–744.
[27] Cerione, R.A. and Zheng, Y. (1996) Curr. Opin. Cell Biol. 8, 216–222.
[28] Whitehead, I.P., Campbell, S., Rossman, K.L. and Der, C.J. (1997) Biochim. Biophys. Acta 1332, F1–F23.
[29] Nalefski, E.A. and Falke, J.J. (1996) Protein Sci. 5, 2375–2390.
[30] Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M. and Kirchhausen, T. (1996) J. Biol. Chem. 271, 28727–28730.
[31] Hussain, N.K., Yamabhai, M., Ramjaun, A.R., Guy, A.M., Baranes, D., O'Bryan, J.P., Der, C.J., Kay, B.K. and McPherson, P.S. (1999) J. Biol. Chem. 274, 15671–15677.
[32] Rosenthal, J.A., Chen, H., Slepnev, V.I., Pellegrini, L., Salcini, A.E., Di Fiore, P.P. and De Camilli, P. (1999) J. Biol. Chem. 274, 33959–33965.
[33] Simpson, F., Hussain, N.K., Qualmann, B., Kelly, R.B., Kay, B.K., McPherson, P.S. and Schmid, S.L. (1999) Nature Cell Biol. 1, 119–124.
[34] Tong, X.K., Hussain, N.K., de Heuvel, E., Kurakin, A., Abi-Jaoude, E., Quinn, C.C., Olson, M.F., Marais, R., Baranes, D., Kay, B.K. and McPherson, P.S. (2000) EMBO J. 19, 1263–1271.