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PACSIN 2, a novel member of the PACSIN family of cytoplasmic adapter proteins[1]

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Abstract The PACSIN-related proteins are cytoplasmic adapter proteins with a common arrangement of domains and conserved regions. Here we report the cloning, sequencing, and expression of PACSIN 2, a novel member of the PACSIN protein family and accordingly rename the original PACSIN to PACSIN 1. The sequences of the murine and human cDNAs reveal an open reading frame encoding a putative protein of 486 residues. Despite its high sequence similarity to PACSIN 1, PACSIN 2 is encoded by distinct transcripts in human and mouse, in particular displaying a ubiquitous expression pattern. Immunofluorescence microscopy of PACSIN 2-transfected NIH3T3 fibroblasts reveal a broad, vesicle-like cytoplasmic staining. In contrast to FAP52, another PACSIN-related protein derived from chicken brain, PACSIN 2 could not be detected at focal contacts. Taken together, these findings suggest that PACSIN 2 is a novel PACSIN isoform with similar domain and motif arrangement, but an unrestricted expression pattern, which may participate in the organization of the actin cytoskeleton and the regulation of vesicular traffic.

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Key words: PACSIN; Syndapin; SH3 domain; NPF motif; Adapter protein

1. Introduction

We previously identified PACSIN 1 as a member of a novel class of cytoplasmic adapter proteins [1], which share characteristic structural features such as a well conserved C-terminal protein binding SH3 domain and a CDC15-NT domain which includes an N-terminal RAEYL motif and a central coiled-coil region. The latter domain represents an uncharacterized profile (PS50133) in the PROSITE database with the RAEYL motif being the highly conserved region at its N-terminus. PACSIN 1 exhibits a highly restricted expression pattern and is detected predominantly in terminally differentiated neural tissues, with maximal expression in the adult. During mouse brain regeneration the expression of PACSIN 1 drops dramatically. Recently Qualmann et al. [2] reported the identification of the rat homolog of PACSIN 1, syndapin 1, based on its ability to bind to the proline-rich domain (PRD) of dynamin. Syndapin 1 was found to be enriched in synapses and to bind to three major nerve terminal proteins implicated in the trafficking of synaptic vesicles, synaptojanin 1, dynamin 1 and synapsin 1, via its C-terminal SH3 domain. The same protein binding domain of syndapin 1 interacts with N-WASP, a multidomain homolog of the Wiskott-Aldrich syndrome protein (WASP) also found in nerve terminals. This was shown to associate with actin filaments and thereby regulates the dynamics of the actin cytoskeleton. Several other PACSIN 1-related sequences have been described. One, FAP52, recovered from chicken brain, is a phosphoprotein with 70% identity to PACSIN 1, which is localized in focal adhesion contacts [3]. A second encodes an as yet incompletely characterized *Echinococcus* antigen, EM13, with 34% sequence identity to PACSIN 1 [4]. The third predicts the less homologous PSTPIP which is thought to be involved in the control of cleavage furrow formation during cytokinesis and appears to be the mammalian homolog of the *Schizosaccharomyces pombe* phosphoprotein CDC15p [5,6]. PSTPIP has recently been shown to bind to WASP in an SH3-mediated and tyrosine phosphorylation-dependent manner [7]. All family members appear to be involved in signaling pathways associated with the organization of cytoskeletal structures.

Assuming the existence of more members of this novel family of cytoplasmic phosphoproteins, we attempted to identify further gene products with a similar modular structure by sequence comparison and search of EST databases. Here we report the deduced primary structure of murine and human PACSIN 2, which are highly homologous to PACSIN 1 and FAP52, differing mainly by the inclusion of a 41 amino acids long PACSIN 2-specific region.

2. Materials and methods

2.1. Clones and libraries

A murine fetal EST clone, IMAGE Consortium clone ID no. 373221, representing a fragment of PACSIN 2, was obtained from the UK HGMP Resource Center. Two human EST clones derived from adult retina, clone ID no. 220502 (IMAGp998I15437), and infant brain, clone ID no. 50687 (IMAGp998H17283), were obtained from the Resource Center of the German Human Genome Project (RZPD). Four filters with dotted cDNA derived from a 9 days post-coitum murine embryo cDNA library (RZPD no. 559) and corresponding positive clones were obtained from the RZPD.

2.2. Isolation of clones and DNA analysis

Filter hybridization of a murine cDNA library (see Section 2.1) was performed in 50% formamide, 5×Denhardt's solution (50×Denhardt's solution is 1% BSA, 1% Ficoll 400, and 1% polyvinylpyrrolidone), 5×SSPE (1×SSPE is 0.15 M NaCl, 10 mM sodium phosphate (pH 7.6), and 1 mM EDTA), 1.5% SDS (sodium dodecyl sulfate), and 300 μg/ml salmon sperm DNA with a 520 bp probe specific for mouse PACSIN 2. The cDNA fragment was derived from the EST clone

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[1] The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers AF128535 and AF128536, respectively.

Abbreviations: EST, expressed sequence tag; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EH, eps15 homology; SH3, src homology 3; PRD, proline-rich domain; CDC15NT, CDC15 N-terminal; UTR, untranslated region

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373221 by *Eco*RI/*Xmn*I digestion and radiolabeled by random priming (TaKaRa). The filter were finally washed with 0.1×SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.5) and 0.1% SDS at 65°C for 20 min, and subjected to autoradiography. Positive clones from the library as well as EST clones were sequenced in both directions with universal and internal primers using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, and products resolved on an ABI Prism 377 Automated Sequencer (Perkin Elmer/Applied Biosystems). DNA and protein sequence analysis were performed using the GCG software package (University of Wisconsin, Madison, WI, USA) and multiple gene databases were searched using the BLAST programs [8].

2.3. Northern blot analysis

Poly(A)$^+$ RNA was isolated from freshly prepared murine tissues by guanidinium thiocyanate lysis using the Oligotex mRNA Kit (Qiagen). 5 μg poly(A)$^+$ RNA per tissue was electrophoresed on a 1% agarose gel containing formaldehyde and transferred onto Hybond XL membrane (Amersham) by capillary transfer. Hybridization was performed in the same formamide mix as used for cDNA library filter hybridization using the radiolabeled (TaKaRa) 520 bp probe specific for mouse PACSIN 2 (see Section 2.2). The filter was washed stringently with 0.1×SSC and 0.1% SDS at 65°C for 10 min, and subjected to autoradiography. Prior ethidium bromide staining of the gel and hybridization with GAPDH cDNA were used to control equal loading and checking RNA integrity.

2.4. Immunofluorescence

The complete open reading frame of murine PACSIN 2 was cloned into the eukaryotic expression vector pMyc-CMV (Clontech) in order to express myc-tagged PACSIN 2. NIH3T3 fibroblasts were grown to 70% confluence and transfected with 2 μg DNA and 6 μl FuGENE 6 (Roche) per well of a 6-well dish. After 24 h, cells were plated on circular 12 mm glass coverslips in 24 well dishes. 48 h after transfection cells were fixed in 2% paraformaldehyde in PBS for 10 min and permeabilized by incubation in 0.2% Triton X-100 in PBS for 1 min. All antibody incubations and washing steps were performed in TBS containing 0.1% Tween 20. Myc-tagged mouse PACSIN 2 was detected using a polyclonal rabbit serum against c-myc (A-14, Santa Cruz, dilution 1:1000) in combination with a Cy2-conjugated anti-rabbit immunoglobulin serum (Jackson ImmunoResearch Laboratories, dilution 1:400). F-actin was stained using TRITC-conjugated phalloidin (Sigma, dilution 1:80). Microtubules were detected with a mouse anti-α-tubulin monoclonal antibody (N356, Amersham, dilution 1:50), the focal adhesion proteins with a monoclonal anti-paxillin antibody (clone 349, Transduction Laboratories, dilution 1:100), and the monoclonal anti-vinculin antibody (clone VIIFS, gift from Dr. M. Glukhova, Institute Curie, Paris, France, dilution 1:2). For visualization of microtubules, paxillin, and vinculin, a Cy3-conjugated goat anti-mouse immunoglobulin serum (Jackson ImmunoResearch Laboratories, dilution 1:200) was used. Confocal laser scanning microscopy (Leica) was used to scan sections 16–32 times. Processing of the resulting pictures was performed using Adobe Photoshop 3.0.

3. Results

3.1. Isolation of PACSIN 2 cDNA

A search of EST databases yielded several murine and human EST clones with sequence identity to PACSIN 1 and FAP52 of about 73% and 78%, respectively. Using a fragment of EST clone 373221 representing the 5′-region of murine PACSIN 2 as a probe, we obtained two clones on screening a mouse embryo cDNA library. Each contained the entire coding region (position 251–1711) and comprised the complete cDNA of mPACSIN 2 with a length of 3217 bp (Fig. 1A). The first in-frame ATG is located 251 nt from the 5′-end and fulfills Kozak's criteria for a translation initiation site showing conservation of six of 10 bases in the consensus [9]. This putative initiator was preceded by two in-frame stop codons located 173 and 42 nucleotides upstream (Fig. 1A). For the complete human PACSIN 2 transcript of 3255 bp the sequences of the EST clones 220502 and 50687 were combined. The putative translation initiation site is located at nucleotide positions 209–211 and also preceded by two in-frame stop codons (nucleotide positions 59 and 173, respectively).

The predicted protein products encoded by the open reading frames are both 486 residues long (Fig. 1) with a calculated molecular weight of 55 833 (mouse) and 5 905 Da (human), respectively. The human and murine PACSIN 2 sequences show significant homology at both the cDNA and protein levels with 79.8% and 93.6% identity, respectively (Fig. 2A). Furthermore, the murine PACSIN 2 protein is 89% identical to chicken FAP52 and 70% to murine PACSIN 1, but compared to these PACSIN 2 contains a unique 41 amino acid insertion (Fig. 2B). This insertion is part of a region which individually characterizes each PACSIN-related protein and contains an additional third NPF motif besides the two NPF motifs also found in PACSIN 1 and FAP52. The general domain organization of PACSIN 2 is consistent with the arrangement found in other related proteins (Fig. 2B). A CDC15 N-terminal domain is localized between residues 47 and 245, and the C-terminus is characterized by a well conserved SH3 domain.

3.2. Expression of PACSIN 2

In contrast to the restricted neural expression of PACSIN 1 [1], Northern blot analysis revealed a more ubiquitous distribution of PACSIN 2 (Fig. 3). The specific transcript of 3.5 kb was detected in all tissues tested with highest levels in brain, heart, skeletal muscle, and ovaries. A search of the EST database yielded a high number of human and murine clones that could be identified as fragments of PACSIN 2 cDNA. These EST clones originate from a large number of different tissues, a finding which further supports the ubiquitous expression. Additionally PACSIN 2 was found in several tumors and pathologically altered tissues (data not shown). Furthermore, PACSIN 2 is expressed in adult tissues as well as in tissues and cells derived from very early stages of development (e.g. EST clone J0701E04, accession number AU014698, derived from a murine two-cell embryo).

3.3. Intracellular localization of PACSIN 2

The intracellular localization was studied employing NIH3T3 fibroblasts transfected with myc-tagged PACSIN 2 (Fig. 4). In contrast to FAP52, PACSIN 2 shows a vesicle-like distribution throughout the cell. The costaining of cytoskeletal structures with phalloidin (Fig. 4A–C) and anti-α-tubulin (Fig. 4D–F), showing the actin filament and microtubule networks, respectively, revealed that PACSIN 2 distribution seems to overlap at least partially with both cytoskeletal networks. As a negative control NIH3T3 cells transfected with a myc-tagged vector without insert were processed for indirect immunofluorescence, but no staining was detected using an anti-myc antibody (data not shown).

Since PACSIN 2 is highly similar to the focal adhesion protein FAP52, which localizes together with paxillin and vinculin in focal contacts of chicken embryo fibroblasts [3], we used antibodies against both proteins, but failed to localize PACSIN 2 in focal contact sites of NIH3T3 cells (Fig. 4G–L).

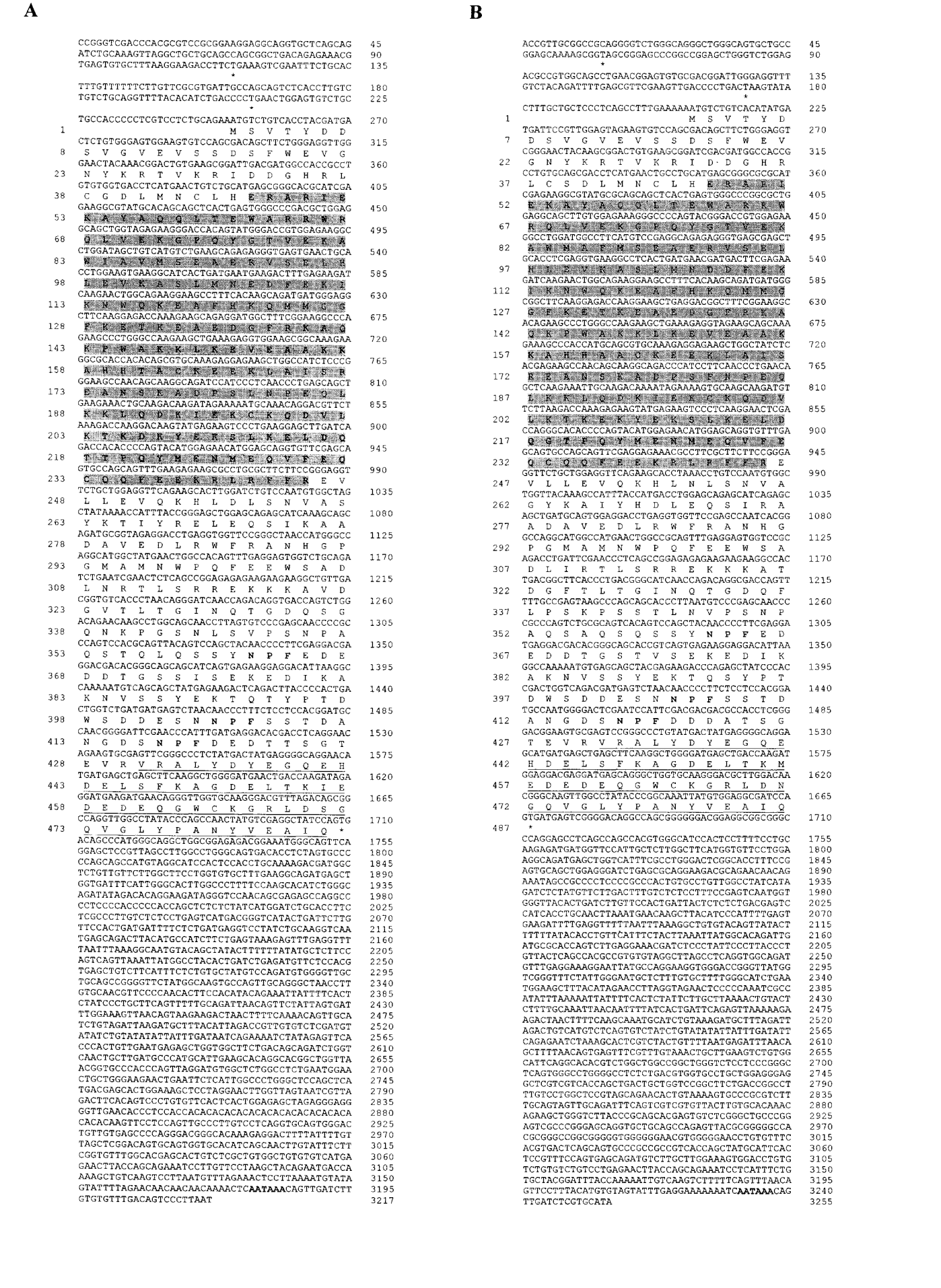
A
B

Fig. 1. Annotated nucleotide sequences of full-length mouse (A) and human (B) cDNAs encoding PACSIN 2. The numbers on the left and right sides of the sequences indicate amino acid residues and nucleotide positions, respectively. Within the nucleotide sequence the in-frame stop codons are marked by asterisks, and the putative poly(A) signals (AAUAAA) are indicated by bold letters at the end of the 3′-UTR (untranslated regions). Within the protein sequence the CDC15 NT domains are highlighted by gray shading, the EH domain binding NPF motifs by bold letters, and C-terminal SH3 domains are underlined.
←

4. Discussion

It was recently demonstrated that syndapin 1, the rat homolog of murine PACSIN 1, binds to dynamin 1, synaptojanin 1, synapsin 1 and N-WASP via its C-terminal SH3 domain [2]. We now report the identification of a novel isoform, PACSIN 2, which in contrast to PACSIN 1 appears to be ubiquitously expressed. Despite its high similarity to PACSIN 1 (70% identity), and therefore comparable arrangement of motifs and domains, PACSIN 2 contains an additional peptide sequence including a third NPF motif. The presence of an SH3 domain, three NPF motifs, and a central coiled-coil region located within the CDC15-NT domain suggests a function as an adapter protein. This interpretation is supported by the fact that syndapin 1 binds to four different proteins via its SH3 domain [2]. Some of these proteins have been shown to participate in endocytic processes [10–12]. Additionally, several proteins that are involved in vesicle formation during endocytosis contain either EH domains or their corresponding NPF binding motifs (reviewed in [13]). Within PACSIN 2 three of these motifs are present between the CDC15-NT domain and the C-terminal SH3 domain, all of which are

A

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mPACSIN2    1 MSVTYDDSVGVEVSSDSFWEVGNYKRTVKRIDDGHRLCGDLMNCLHERARIEKAYAQQLT
hPACSIN2    1 MSVTYDDSVGVEVSSDSFWEVGNYKRTVKRIDDGHRLCSDLMNCLHERARIEKAYAQQLT
gFAP52      1 MSGSYDDSVGVEVSSDSFWEVGNYKRTVKRIDDGHRLCNDLMNCTHERARIEKVYAQQLT
mPACSIN1    1 MSGSYDEA..SEEITDSFWEVGNYKRTVKRIDDGHRLCNDLMSCVQERAKIEKAYAQQLT

mPACSIN2   61 EWARRWRQLVEKGPQYGTVEKAWIAVMSEAERVSELHLEVKASLMNEDFEKIKNWQKEAF
hPACSIN2   61 EWARRWRQLVEKGPQYGTVEKAWIAFMSEAERVSELHLEVKASLMNEDFEKIKNWQKEAF
gFAP52     61 EWAKRWKQLVEKGPQYGTVERAWCAFMSEAERVSELHLEVKGSLMNEDFEKIKNWQKEAF
mPACSIN    59 DWAKRWRQLTEKGPQYGSLERAWGAMMTEADKVSELHQEVKNSLTNEDLEKVKNWQKDAY

mPACSIN2  121 HKQMMGGFKETKEAEDGFRKAQKPWAKKLKEVEAAKKAAHTACKEEKLAISREANSKADP
hPACSIN2  121 HKQMMGGFKETKEAEDGFRKAQKPWAKKLKEVEAAKKAAHAACKEEKLAISREANSKADP
gFAP52    121 HKQMMGGFKETKEAEDGFRKAQKPWAKKLKEVEAAKKAYHAACKEEKLAISRETNSKADP
mPACSIN1  119 HKQTMGGFKETKEAEDGFRKAQKPWAKKMKELEAAKKAYHLACKEERLAMTREMNSKTEQ

mPACSIN2  181 SLNPEQLKKLQDKIEKCKQDVLKTKGKYEKSLKELDQTTPQYMENMEQVFEQCQQFEEKR
hPACSIN2  181 SFNPEQLKKLQDKIEKCKQDVLKTKEKYEKSLKELDQGTPQYMENMEQVFEQCQQFEEKR
gFAP52    181 ALNPEQLKKLQDKVERSKQDVLKTKAKYEKSLKELDNATPQYMENMEQVFEQCQQFEEKR
mPACSIN1  179 SVTPEQQKKLVDKVDKCRQDVQKTQEKYEKVLEDVGKTTPQYMEGMEQVFEQCQQFEEKR

mPACSIN2  241 LRFFREVLLEVQKHLDLSNVASYKTIYRELEQSIKAADAVEDLRWFRANHGPGMAMNWPQ
hPACSIN2  241 LRFFREVLLEVQKHLNLSNVAGYKAIYHDLEQSIRAADAVEDLRWFRANHGPGMAMNWPQ
gFAP52    241 LRFFREVLLEVQKHLDLSNVASYKNIYRELEQNLKTADAVEDLRWFRANQGPGMSMNWPQ
mPACSIN1  239 LVFLKEVLLDIKEHLNLAENSSYMHVYRELEQAIRGADAQEDLRWFRSTSGPGMPMNWPQ

mPACSIN2  301 FE..EWSADLNRTLSRREKK.KAVDGVTLTGINQTGDQSG...QNKEGSNLSVPSNPAQS
hPACSIN2  301 FE..EWSADLIRTLSRREKK.KATDGETLTGINQTGDQFL...PSKESSTLNVPSNPAQS
gFAP52    301 FEDDEWSADLNRTLSRREKK.KASDGVTLTGINQTGDQVS..QPNKHSS..........
mPACSIN1  299 FE..EWNPDLPHTTAKKEKQPKKAEGATLS..NATGAVESTSQAGDRGS..........

mPACSIN2  355 TQLQSSYNPFEDEDDTGSSISEKEDIKAKNVSSYEKTQTYPTDWSDDESNNPFSSTDANG
hPACSIN2  355 AQSQSSYNPFEDEDDTGSTVSEKEDIKAKNVSSYEKTQSYPTDWSDDESNNPFSSTDANG
gFAP52    347 ..............................VSSYEKNQSYPTDWSDEESNNPFSSTDAKG
mPACSIN1  344 ..............................VSSYDRGQTYATEWSDDESGNPFGGNEANG

mPACSIN2  415 DSNPFDEDTTSGTEVRVRALYDYEGQEHDELSFKAGDELTKIEDEDEQGWCKGRLLSGQV
hPACSIN2  415 DSNPFDEDATSGTEVRVRALYDYEGQEHDELSFKAGDELTKMEDEDEQGWCKGRLLNGQV
gFAP52    377 DTNPFDEDTSPVMEVRVRALYDYEGQEQDELSFKAGDELTKMENEDEQGWCKGRLLNGQV
mPACSIN1  374 GANPFEDDAKG...VRVRALYDYDGQEQDELSFKAGDELTKLGEEDEQGWCRGRLLSGQL

mPACSIN2  475 GLYPANYVEAIQ*
hPACSIN2  475 GLYPANYVEAIQ*
gFAP52    437 GLYPANYVEPIQ*
mPACSIN1  431 GLYPANYVEAI*
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Fig. 2. Comparison of the murine and human PACSIN 2 deduced amino acid sequences with those of related proteins. A: Sequence alignments of both PACSIN 2 proteins (mPACSIN2 and hPACSIN2) with murine PACSIN 1 (mPACSIN1) and chicken FAP52 (gFAP52). Identical amino acids are shown in white against black, and conservatively substituted ones are shaded. Gaps in the sequences, needed to optimize the alignment, are represented by dots. B: Comparison of the modular structure of PACSIN-related proteins. All proteins contain a CDC15 NT domain, including a coiled-coil region and a C-terminal SH3 domain, indicated as black modules. Additionally members of the PACSIN protein family contain up to three conserved regions, specific for the PACSIN family, which are shaded gray. The more distantly related PSTPIP instead contains regions specific for the PSTPIP family at these positions. Individually distinct modules are indicated by different stripe patterns. The overall amino acid identity and similarity (including conservatively substituted residues) of the individual proteins to murine PACSIN 2 is given at the right.

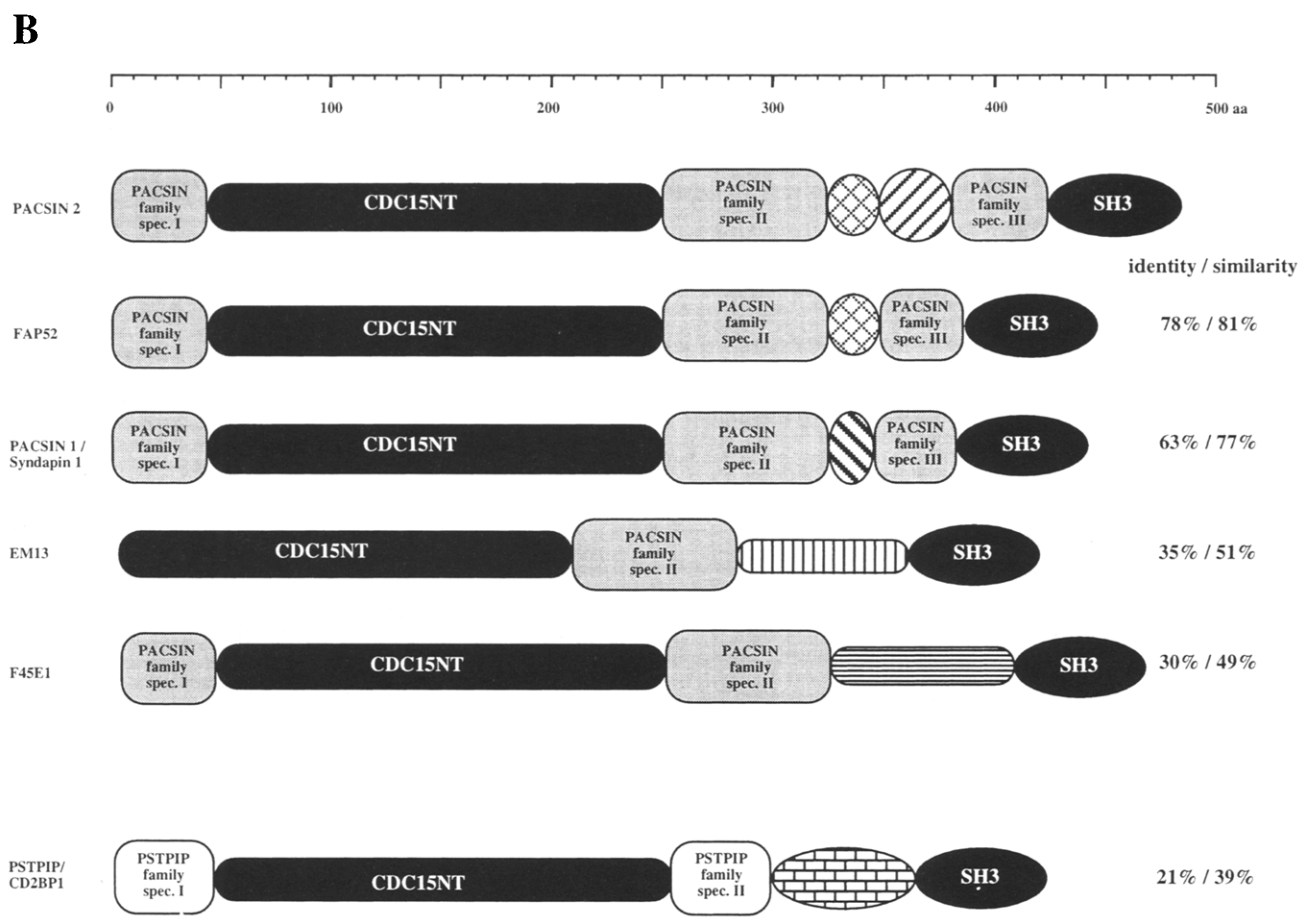

Fig. 2 (*continued*).

potentially able to bind to proteins containing EH domains [14]. Also the aspartic acid residue adjacent to the first NPF motif at residue 366 exactly matches the consensus sequence NPFxD characterizing a new class of endocytosis signals in *Saccharomyces cerevisiae* [15]. While expression of synapsin 1 and synaptojanin 1 is restricted to the nervous system [16,17], other interaction partners of syndapin 1, e.g. N-WASP, or isoforms of interaction partners, e.g. dynamin 2, are also expressed in non-neural tissues [18,19]. These may interact with the PACSIN 2 SH3 domain in other tissues.

PACSIN 2 shows a high similarity to FAP52, a PACSIN family member with a broad tissue distribution and an intracellular localization to focal contacts [3]. Although PACSIN 2 appears to be the mammalian homolog of FAP52, PACSIN 2 shows no colocalization with paxillin and vinculin in NIH3T3 fibroblasts. Also when compared to FAP52, the human and murine PACSIN 2 proteins contain an insertion of 41 amino acids, which leads to a theoretical molecular weight of 56 kDa. Such an insertion could explain the discrepancy between the FAP52 63 kDa signal seen in Western blots compared to the calculated molecular weight of 52 kDa [3]. Although we have found no evidence for alternative splicing of PACSIN 2 transcripts in Northern blots and the EST database, we can-

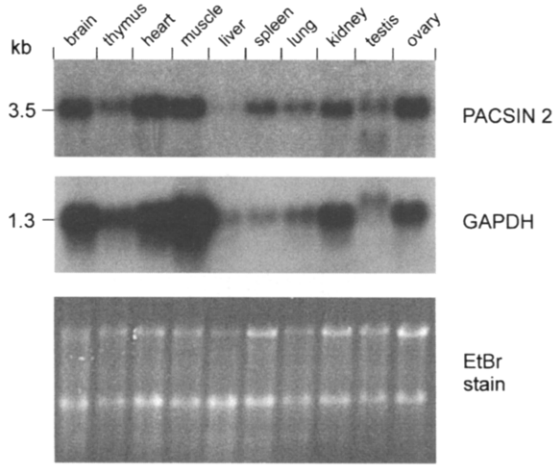

Fig. 3. Expression of mouse PACSIN 2 in adult murine tissues. Northern blot was performed with approximately 5 μg of poly(A)$^+$ RNA per lane. The filter was hybridized with a fragment of clone 373221 containing the 5′ untranslated region and parts of the open reading frame. Integrity of RNAs and standardization of loaded amounts were checked by ethidium bromide staining of the gel and reprobing with a GAPDH control probe.

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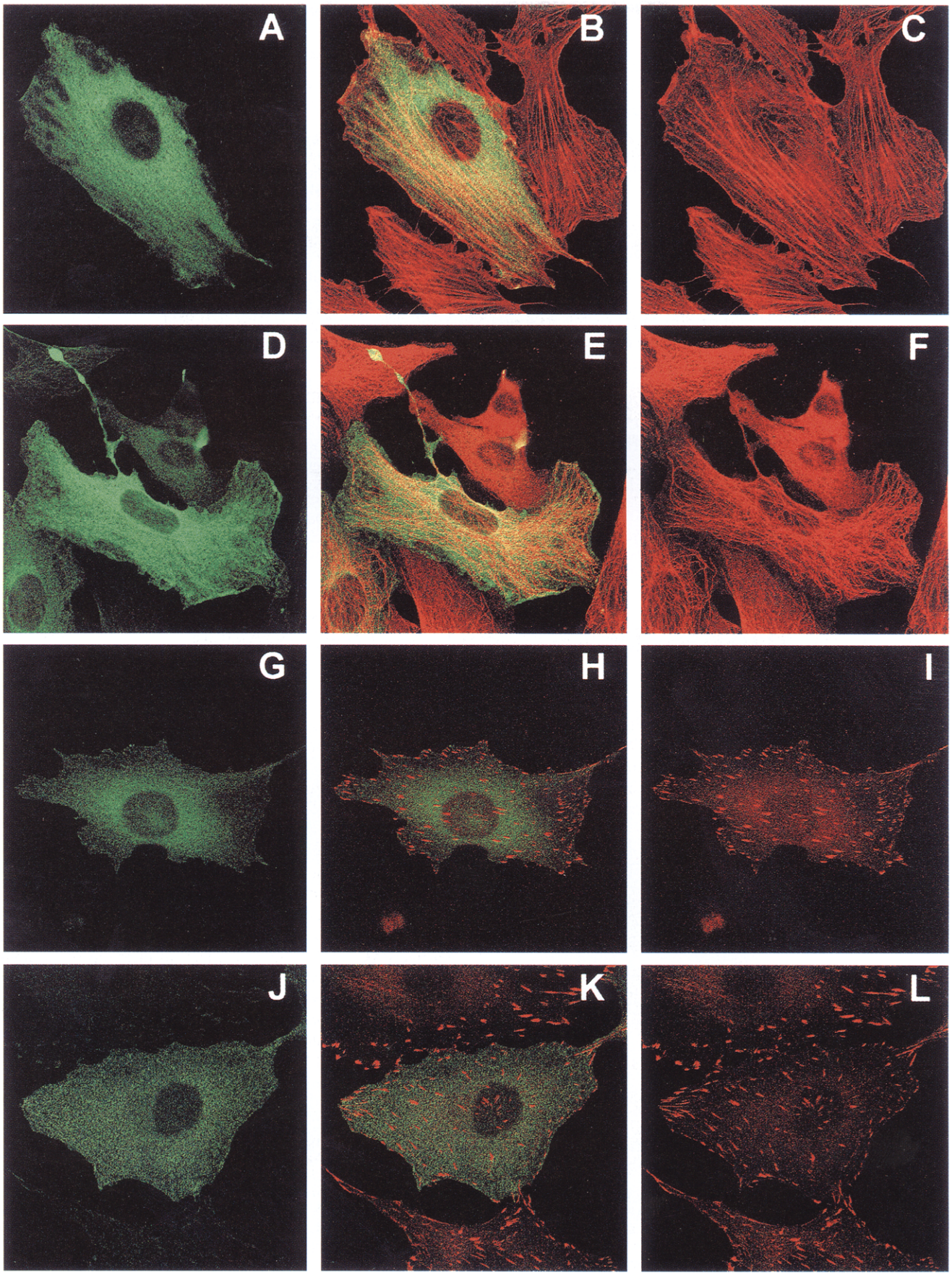
Fig. 4. Immunofluorescence analysis of the localization of PACSIN 2 in fibroblasts. Transiently transfected NIH3T3 cells expressing myc-tagged PACSIN 2 were fixed with 2% PFA and processed for indirect immunofluorescence as described in Section 2. After permeabilization, PACSIN 2 distribution was determined using an anti-myc antibody (A, B, D, E, G, H, J, and K). Cytoskeletal networks were visualized using TRITC-labelled phalloidin for actin (B and C) and an anti-α-tubulin monoclonal antibody for microtubules (E and F). Focal adhesions were stained by using antibodies against paxillin (H and I) and vinculin (K and L).


A
B
C
D
E
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K
L

not rule out that FAP52 might represent another splice variant occurring in chicken.

Immunofluorescence microscopy for PACSIN 2 in NIH3T3 fibroblasts revealed a vesicle-like cytoplasmic distribution, that seems to partially overlap with that of microtubules and the actin network. Both participate on vesicular transport.

In conclusion, we have identified PACSIN 2 as a novel member of the PACSIN protein family that in contrast to the closely related PACSIN 1, which only occurs in neural tissues, appears to be ubiquitously expressed. The similar arrangement of domains and motifs, together with features of the intracellular localization suggest a participation in endocytic processes. Although most proteins binding to syndapin 1, the rat homolog of PACSIN 1, are localized in nerve terminals, many are also members of protein families with some members having a more widespread expression pattern. These other members may show interactions with PACSIN 2.

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