Characterization of an ankyrin repeat-containing Shank2 isoform (Shank2E) in liver epithelial cells

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Shank proteins are a family of multidomain scaffolding proteins best known for their role in organizing the postsynaptic density region in neurons. Unlike Shank1 and Shank3, Shank2 [also known as Pro-SAP1 (proline-rich synapse-associated protein 1), CortBP1 (cortactin binding protein 1) or Spank-3] has been described as a truncated family member without an N-terminal ankyrin repeat domain. The present study utilized bioinformatics to demonstrate the presence of exons encoding ankyrin repeats in the region preceding the previously described *Shank2* gene. cDNA sequencing of mRNA from epithelial cells revealed a novel spliceoform of Shank2, termed Shank2E, that encodes a predicted 200 kDa protein with six N-terminal ankyrin repeats. Shank2 mRNA from epithelial tissues was larger than transcripts in brain. Likewise, the apparent mass of Shank2 protein was larger in epithelial tissues (230 kDa) when compared with brain (165/180 kDa). Immunofluorescence and membrane fractionation found Shank2E concentrated at the apical membrane of liver epithelial cells. In cultured cholangiocytes, co-immunoprecipitation and detergent solubility studies revealed Shank2E complexed with actin and co-distributed with actin in detergent-insoluble lipid rafts. These findings indicate epithelial cells express an ankyrin repeat-containing Shank2 isoform, termed Shank2E, that is poised to co-ordinate actin-dependent events at the apical membrane.

Key words: actin cytoskeleton, apical domain, cortactin binding protein 1 (CortBP1), lipid raft, proline-rich synapse-associated protein 1 (ProSAP1A).

INTRODUCTION

Specialized membrane regions in polarized cell types utilize scaffolding proteins to co-ordinate the organization, movement and activities of receptor, regulatory and effector proteins. Scaffolding proteins often contain multiple domains that mediate a variety of distinct protein–protein interactions. Proteins containing PDZ (an acronym formed from the names of three proteins: PSD-95, Dlg and ZO-1) domains have emerged as central figures in organizing events in submembranous regions within cells. The PSD (post-synaptic density) region in neurons has a well-characterized complex of interacting proteins that includes several PDZ-domain-containing scaffolding proteins. PSD95 protein binds the cytoplasmic tail of the NMDA (*N*-methyl-D-aspartate) receptor via a PDZ domain interaction, while the protein Homer binds the cytoplasmic tail of the mGluR (metabotropic glutamate receptor) [1,2]. Both of these complexes reside within 12 nm of the PSD membrane, and serve to tether these integral membrane proteins on to a protein scaffold. The PSD95-NMDA receptor and Homer–mGluR proteins are clustered further into an interactive macromolecular complex by another PDZ domain protein, Shank1 [3,4]. In addition to their membrane protein interactions, Shank proteins interact with a variety of cytoplasmic proteins, including cortactin [5,6], dynamin2 [7], insulin-receptor tyrosine kinase substrate p53 [8] and Sharpin [9]. Importantly, Shank-mediated macromolecular complexes are pivotal to the formation, structure and co-ordinated activities of the PSD. Distributed between the membrane protein complexes and the cytoplasm, Shank is positioned to serve as a 'central co-ordinator' and integrate membrane and cytoplasmic protein interactions [10].

There are three distinct mammalian *Shank* genes, each gene sharing a similar domain organization (see Figure 1) [3,4,6,11,12]. The prototypical Shank protein has six ankyrin repeat domains, an SH3 (Src homology 3) domain, a single PDZ domain, a proline-rich domain and a SAM (sterile α-motif) domain. The complexity of the Shank family of proteins is diversified further through mRNA splicing. While Shank1 expression appears to be brain-specific, Shank3 is expressed at various levels in all tissues examined [8]. In addition to the brain, Shank2 protein is expressed in glial and endocrine cells [13], and Shank2 mRNA is present in the kidney, lung and liver [8,14]. Two Shank2 spliceoforms have been described. Both spliceoforms, ProSAP1A (proline-rich synapse-associated protein 1) [12] and CortBP1 (cortactin binding protein 1) [6], lack the N-terminal ankyrin repeat domains seen in Shank1 and Shank3. ProSAP1A, but not CortBP1, contains an SH3 domain. The lack of ankyrin repeats in Shank2 mRNA and protein may be due either to truncation of the duplicated *Shank2* gene or to alternative splicing. In the present study, bioinformatic analysis identified the presence of an ankyrin repeat domain upstream of the previously described *Shank2* gene, and found an ankyrin repeat-containing Shank2 spliceoform in epithelial cells.

Abbreviations used: Arp2/3, actin related protein 2/3 complex; CFTR, cystic fibrosis transmembrane conductance regulator; CortBP1, cortactin binding protein 1; EBP50, ezrin/radixin/moesin-binding phosphoprotein 50; ENaC, epithelial sodium channel; IB, isolation buffer; MBS, Mes-buffered saline; mGluR, metabolic glutamate receptor; Mrp2, multidrug resistance protein 2; NMDA, *N*-methyl-D-aspartate; NRC, normal rat cholangiocyte; PpII, polyproline type II; ProSAP1A, proline-rich synapse-associated protein 1; PSD, post-synaptic density; RT-PCR, reverse transcriptase-PCR; SAM, sterile α-motif; SH3, Src homology 3; UCHSC, University of Colorado Health Sciences Center.

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Similar to neurons, epithelial cell function requires polarized membrane domains. Junctional complexes demarcate the apical and basolateral domains, and are essential for establishing and maintaining the barrier function of an epithelium. Within the basolateral and apical membranes, specific receptor, transporter and channel proteins are discretely distributed to permit efficient, specific vectorial movement of a multitude of substances across the epithelium. Epithelial transport capacity is dynamic and responsive to changes in need. Regulation of these capacities includes moderating both the density and activity of the specific integral membrane proteins. In junctional, basolateral and apical regions of epithelial cells, PDZ domain proteins have emerged as key contributors to the development, maintenance and regulation of these signalling and transport events. These distinct transport events are often co-ordinately regulated. For example, in pulmonary epithelial cells, increased intracellular cAMP increases CFTR (cystic fibrosis transmembrane conductance regulator)-dependent Cl^- secretion and inhibits ENaC (epithelial sodium channel)-dependent Na^+ reabsorption across the apical membrane. Although the molecular mechanism is undetermined, CFTR is required for cAMP-dependent inhibition of ENaC Na^+ reabsorption [15]. Although the potential need for 'central co-ordinator' proteins within epithelial cells is clear, their contribution to epithelial cell function has not been elucidated. Indeed, the expression of Shank proteins in epithelial cells has been only modestly described. The present study identifies and characterizes a novel epithelial Shank2 spliceoform that is concentrated at the apical membrane of hepatocytes and cholangiocytes, interacts with the actin cytoskeleton, and resides predominantly within an actin-containing population of detergent-insoluble lipid rafts. Shank2E expression provides epithelial cells with a PDZ domain protein known to cluster and co-ordinate the activities of functionally related protein complexes within microdomains of cells.

EXPERIMENTAL

Bioinformatics, genome screening and cDNA sequencing

Reference sequences (RefSeq) derived from predicted mRNA sequences using GenomeScan and ESTs (expressed sequence tags) were utilized to determine putative exons in and around the *Shank2* gene. Analysis was focused primarily to RefSeq on the positive strand, upstream of the known CortBP1 sequence [6,12]. Examinations included the genome contig sequences, NW_043404, NW_043312 and NW_043405 (GenBank®) and sequenced cDNA products. Exon-specific primers were designed using LocusLink in conjunction with MapViewer (National Center for Bioinformatics) to determine the full-length sequence for *Shank2E*. Putative exons were derived using the AT/GT rule of exon splicing [16,17]. cDNAs were sequenced in the UCHSC (University of Colorado Health Sciences Center) Cancer Center DNA Sequencing and Analysis core (using an ABI-Prism 377 sequencer).

Cell culture and animal handling

Normal rat cholangiocytes (NRCs) were originally isolated by Vroman and LaRusso [18], and were maintained in culture under modified conditions [19]. Cultures used for RNA isolation, protein isolation and immunoprecipitation studies were grown on collagen-treated flasks (Biocoat; Becton Dickinson). Cholangiocyte cultures were used out to 25 passages. Sprague–Dawley rats (male; 250 g) were used in immunofluorescence studies, harvesting RNA and protein from whole organs, isolation of hepatocytes and enrichment of sinusoidal and canalicular membranes. All animals were handled humanely, as described in an Institutional Animal Care and Use Committee-approved protocol.

RNA isolation, RT-PCR (reverse transcriptase-PCR) analysis and Northern blotting

Total RNA was isolated from rat tissues or NRC monolayers using Trizol (Life Technologies) or RNeasy (Qiagen), as described by the manufacturers. For RT-PCR reactions, total RNA was reverse-transcribed (Superscript II; Invitrogen) with oligo(dT) with standard reaction mixtures and under standard conditions (Platinum *Taq* Polymerase; Invitrogen). For Northern blot analysis, 10–30 μg of total RNA was separated on a 1 % (w/v) agarose denaturing gel, transferred on to nylon membranes (MagnaGraph; Micron Separations) in 10 × SSC (where 1 × SSC is 0.15 M NaCl/0.03 M sodium citrate) and cross-linked with UV light. Using NRC cDNAs, RT-PCR products from probe-1 and probe-2 primers (see Table 1) were radiolabelled with [^{32}P]dCTP by random priming (DecaPrime II; Ambion), as described by the manufacturer. The probes were diluted in hybridization buffer [50 % (v/v) formamide/5 × SSC/5 × Denhardt's solution (where 1 × Denhardt's solution is 0.1 % Ficoll 400/0.1 % polyvinylpyrrolidone/0.1 % BSA)/5 % (w/v) SDS/250 mM Tris/25 mM EDTA/18.8 mM sodium pyrophosphate/100 μg/ml denatured herring-sperm DNA/50 μg/ml yeast tRNA] and incubated with the blot overnight at 42 °C. After washing, ^{32}P labelling was detected by autoradiography.

Western blot analysis

Proteins from rat tissues and cultured cholangiocytes were assayed by Western blotting [20]. Briefly, tissues or cells were solubilized in 5× PAGE solution [5 % (w/v) SDS/25 % (w/v) sucrose/5 mM EDTA/5 % 2-mercaptoethanol/50 mM Tris/HCl (pH 8.0)], separated on a 4–14 % gradient gel and transferred on to nitrocellulose membranes (Osmonics, Westborough, MA, U.S.A.). Primary antibodies for Western blot analysis included CE9 (kindly provided by Professor Anne Hubbard at Johns Hopkins University, Baltimore, MD, U.S.A.), Mrp2 (multidrug resistance protein 2; supplied by Dr Franz Simon at UCHSC, Denver, CO, U.S.A.), EBP50 (ezrin/radixin/moesin-binding phosphoprotein 50; supplied by Professor Chris Yun at Emory University School of Medicine, Atlanta, GA, U.S.A.), cortactin [5], CortBP1 (*Shank2*; provided by Dr Tom Parsons at the University of Virginia, Charlottesville, VA, U.S.A.), actin (Oncogene), and dynamin-2 (Affinity Bioreagents). Bound primary antibody was labelled using horseradish-peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch; 1:60 000 dilution) and detected by chemiluminescence (Pierce Biochemicals).

Immunofluorescence studies

The subcellular localization of Shank2 in native hepatocytes and cholangiocytes was determined by immunofluorescence labelling of whole-liver sections [20]. Briefly, livers were fixed by retrograde arterial perfusion with 3 % (w/v) paraformaldehyde in PBS. Blocks of fixed liver (10 mm × 5 mm × 3 mm) were infused with 5 %, 10 % and 25 % sucrose, frozen in liquid nitrogen and cryosectioned (6 μm). Sections were rehydrated, blocked (10 % serum and 1 % albumin in PBS), incubated with Shank2 antibody (1:200 dilution), washed, incubated with Alexa 488-tagged anti-rabbit IgG secondary anitbody (1:400 dilution; Molecular Probes), washed and mounted. Immunofluorescence localization was observed and refined by digital deconvolution

microscopy (Delta Vision; UCHSC Light Microscopy Core Facility).

Hepatocyte preparation

Hepatocytes were prepared by a recirculating collagenase digestion technique [21]. Briefly, Sprague–Dawley rats were anaesthetized (50 mg/kg pentobarbital), and collagenase was recirculated through the liver via portal vein perfusion and inferior vena cava recovery. Hepatocytes were combed from the liver, filtered through a gauze and subjected to repeated pelleting and resuspension (50 ***g***, 1 min, three times). Hepatocytes were washed and resuspended in Krebs–Ringers–Hepes buffer [115 mM NaCl/5 mM KCl/1 mM KH_2PO_4 (pH 7.4)/1.2 mM $MgSO_4$/25 mM sodium Hepes/0.2 % albumin]. Trypan Blue exclusion found cell viability to be > 90 % in all utilized preparations.

Sinusoidal and canalicular membrane isolation

Sinusoidal and canalicular membrane fractions were isolated from rat livers [22]. Briefly, liver (2 g) was excised, homogenized in isolation buffer [IB; 15 mM Tris/HCl/300 mM mannitol/5 mM EGTA/1 mM PMSF (pH 7.4) containing protease-inhibitor cocktail tablets (Roche Diagnostics)] and pelleted (48 000 ***g*** for 30 min). The pellet was resuspended in IB, $MgCl_2$ was added to a final concentration of 15 mM and then centrifuged (2500 ***g*** for 15 min). The resultant pellet containing crude sinusoidal membrane was subjected to a second $MgCl_2$ precipitation, resuspended in water, homogenized with a loose fitting Dounce homogenizer and centrifuged (700 ***g***) to remove the nuclei. The supernatant was centrifuged at 48 000 ***g*** for 30 min. The remaining pellet was resuspended in a 50 % sucrose cushion, and the sinusoidal membrane was floated on a 41–37.5 % sucrose step gradient ($\omega^2 t = 7.2 \times 10^{10}$). The recovered sinusoidal membrane was then washed in IB and centrifuged (48 000 ***g*** for 30 min). The supernatants from the first and second Mg^{2+} precipitations were pelleted (48 000 ***g*** for 30 min) and subjected to an additional Mg^{2+} precipitation and centrifuged (2500 ***g*** for 20 min). Re-centrifugation (48 000 ***g*** for 30 min) of the supernatant resulted in the canalicular membrane fraction.

Immunoprecipitation and identification of co-precipitated proteins

Shank2 was immunoprecipitated from solubilized cultured cholangiocytes [23]. Briefly, cholangiocyte monolayers were lysed [immunoprecipitation buffer, consisting of 50 mM Tris, pH 7.4, 75 mM NaCl, 0.5 % (v/v) Triton X-100, 0.5 % (w/v) deoxycholate and protease-inhibitor cocktail tablets at 4 °C], solubilized at 25 °C for 10 min, and the insoluble material was then pelleted (10 000 ***g*** for 10 min). The resultant supernatant was precleared (2 h for 4 °C) with washed Protein A/G–Sepharose beads (Pierce Biochemicals). The resultant supernatant was immunoprecipitated (overnight at 4 °C) with washed Protein A/G–Sepharose beads (Pierce Biochemicals) and 0.34 mg/ml Shank2 rabbit polyclonal antibody or 0.96 mg/ml non-immune rabbit serum (Pierce Biochemicals). Pre-cleared and immunoprecipitation beads were washed with PBS and eluted with 5× PAGE solution. Proteins in the immunoprecipitate were identified by Western blot analysis.

Triton solubilization and isolation of detergent-resistant membranes

Extraction of Triton-soluble proteins was performed as described previously [24]. Briefly, confluent NRC monolayers on semipermeable supports (Corning, New York, NY, U.S.A.) were washed with ice-cold PBS and incubated with 400 μl of Triton extraction buffer [0.1 % Triton X-100/300 mM sucrose/2 mM EGTA/5 mM Tris/HCl (pH 7.4)] for 12 min at 4 °C with gentle rocking. The supernatant (soluble fraction) was collected. The remaining material (insoluble fraction) was solubilized in 200 μl of solubilization solution [10 % (w/v) SDS/50 % (w/v) sucrose]. Volumes were normalized in the soluble and insoluble fractions with the addition of 200 μl of solubilization solution and 400 μl of Triton extraction buffer respectively. Equivalent volumes were assayed by Western blotting, and densitometry was used to quantify the percentage solubility for Shank2E, actin and caveolin-1. The actin cytoskeleton was disrupted by pre-treatment with latrunculin A (1 μM; treatment for 60 min at 37 °C; Calbiochem).

Triton-insoluble membrane microdomains were isolated on sucrose gradients [25]. Confluent NRC monolayers on collagen-treated flasks (BioCoat; Becton Dickinson) were solubilized with 0.5 % Triton X-100 in MBS [Mes-buffered saline; composition: 150 mM NaCl/2 mM EDTA/25 mM Mes (pH 6.5)], scraped from the flask and loose-Dounce-homogenized. This material was diluted with 3 ml of 53.3 % sucrose in MBS, and overlaid with 5 ml of 30 % sucrose in MBS and 3.5 ml of 5 % sucrose. The gradient was subjected to ultracentrifugation (200 000 ***g*** for 20 h at 4 °C), fractionated, and individual protein contents were assayed by Western blotting. The actin cytoskeleton was disrupted by pre-treatment with 1 μM latrunculin A (for 60 min at 37 °C; Calbiochem). Cholesterol was depleted by pre-treatment in serum-free media with methyl-β-cyclodextrin (10 mM; treatment for 120 min at 37 °C; Sigma).

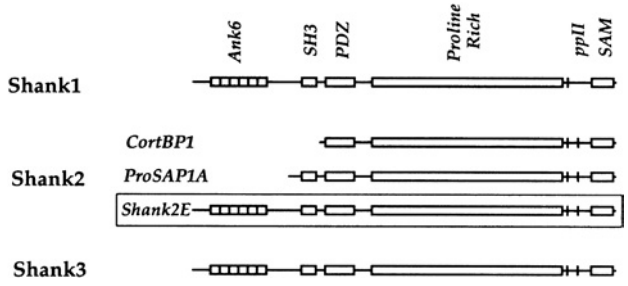

Figure 1 Domain structures of Shank family members

The three Shank protein family members share common domain structures. From the C-terminal end, these domains include a SAM domain, a proline-rich domain, a PDZ domain and an SH3 domain. Shank2 and Shank3 have a PpII domain that enables these two isoforms to bind cortactin. Shank1 and Shank3 proteins also express ankyrin repeats, but reported Shank2 isoforms, ProSAP1A and CortBP1, do not express an ankyrin repeat domain. The present study demonstrates that, similar to *Shank1* and *Shank3*, the *Shank2* gene codes for ankyrin repeats, and an elongated isoform of *Shank2* that includes ankyrin repeats is expressed in epithelial tissues (shown by the boxed domain map). This isoform was termed *Shank2E*.

RESULTS

Shank2 protein is expressed in the liver

The three families of Shank proteins share a common structural organization (Figure 1), and are broadly expressed within neuronal cell types. Shank2 and Shank3 expression has been observed in non-neuronal cell types [8,13]. Given their pivotal role in the function of the PSD, we sought to assess Shank expression in liver epithelia. Using gene-specific primers (Table 1), the presence of Shank1, Shank2 and Shank3 mRNA in whole liver and intrahepatic bile duct epithelial cells (i.e. cholangiocytes) was evaluated (Figure 2). Brain mRNA served as a positive control for all three Shank family members. mRNA from whole

Table 1 Nucleotide sequences of gene-specific primers

The expression of Shank family members (**a**) and the tissue-specific expression of *Shank2* isoforms (**b**) are shown. Those probes marked with an asterisk were subsequently used for Northern blot analysis. GADPH, glyceraldehyde-3-phosphate dehydrogenase.

(**a**)

Protein	Primer	Sequence
Shank1	Sense	GCAGCACAGACAGCCACCACGGAGGT
	Antisense	GTTTAGGCCAGTTCTGAAAGAATGCT
Shank2*	Sense	CTTTGTGGTTGACAAGCCCCCAGTAC
	Antisense	CCTGAGGTCCTGCTTTCATAGTC
Shank3	Sense	GGAGGTCAGTGGCGTTGTCCAA
	Antisense	GGGTCTTGATTACCTCGGCAAG
GAPDH	Sense	CCCCTTCATTGACCTCAAGTAC
	Antisense	CCAAAGTTGTCATGGATGAC

(**b**)

Protein	Primer	Sequence
ProSAP1A	Sense	GTCTTTGTTAAATGCCTTC
	Antisense	TGCTTGGCTGTCCCGGGG
CortBP1	Sense	ATGAGCGTCCCCGGCC
	Antisense	CGGAGTTCACATCAGAGGC
Shank2E*	Sense	CCAGCTTGATGATTCCATGGAG
	Antisense	CACAGCTGTCCTGATTGTAGAGG

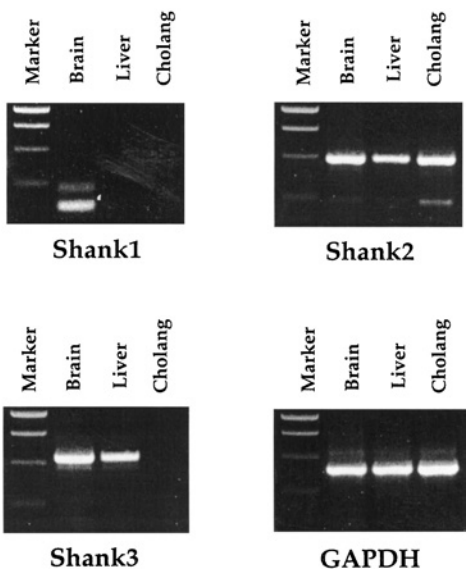

Figure 2 Differential expression of Shank mRNAs

The presence of Shank1, Shank2 and Shank3 mRNAs was evaluated in rat brain, liver and cholangiocyte (Cholang) tissues by RT-PCR. Brain mRNA contained all three Shank isoforms, and the liver had Shank2 and Shank3 mRNAs, but no mRNA for Shank1. In cultured rat cholangiocytes, Shank2 was the only Shank message detected. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase served as a positive control for mRNA integrity.

liver contained both Shank2 and Shank3 transcripts. Likewise, hepatocytes, which comprise ≈80 % of the liver cell mass, also had Shank2 and Shank3 mRNA (results not shown). In contrast, cholangiocytes, which comprise 2–4 % of the liver, only expressed Shank2 mRNA (Figure 2).

To evaluate the relative abundance, size and translation of the Shank2 mRNA, epithelial tissues were subjected to Northern and Western blot analyses (Figure 3). Initial Northern blot analyses of

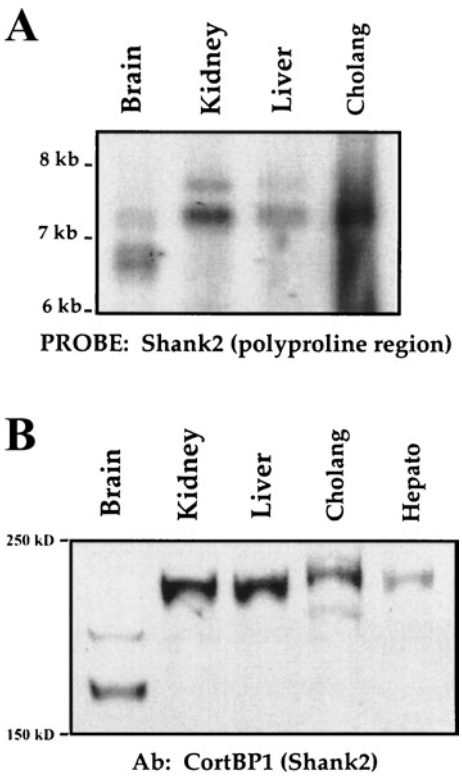

Figure 3 Characterization of Shank2 transcript and protein in epithelia

(**A**) Total RNA from brain (10 μg), kidney (20 μg), liver (20 μg) and cholangiocytes (20 μg; Cholang) was probed for Shank2 mRNAs. Epithelial tissues (kidney, liver and cholangiocytes) had a larger transcript (7.2 kb) when compared with Shank2 mRNA in the brain (6.6 kb). (**B**) Western blotting of brain (5 μg), kidney (30 μg), liver (30 μg), cholangiocyte (30 μg; Cholang) and hepatocyte (30 μg; Hepato) protein detected Shank protein in each tissue. The apparent Shank2 protein mass in brain tissue (the 180/165 kDa doublet) was significantly smaller than Shank2 in epithelial tissues (230 kDa).

equivalent mRNA amounts from brain compared with epithelial tissues showed that *Shank2* mRNA was expressed in epithelial tissues, but the levels were significantly greater in brain tissue (results not shown). Subsequent analyses included 10 μg of brain mRNA and 30 μg of mRNA from epithelial tissues (Figure 3A). In comparison with brain mRNA, Shank2 transcripts from kidney, liver and cholangiocytes were markedly larger (6.6 kb compared with 7.2 kb). Results from Western blot analysis of Shank2 protein expression in brain compared with epithelial tissues paralleled these observations (Figure 3B). As previously shown [6], Shank2 in brain migrated as a 165/180 kDa doublet protein. In contrast, Shank2 in epithelial tissues migrated as a 230 kDa protein.

Shank2 gene encodes ankyrin repeats

Since the diversity of Shank proteins is enhanced through alternative splicing, genomic analysis of the regions around the known *Shank2* locus was conducted to determine the sites and sequences that account for the increased size of the Shank2 transcript in epithelial tissues. The rat *Shank2* gene resides approx. 163 cM (centimorgans) from the centromere on the long arm of chromosome 1 (1q41). Bioinformatic searches of this region found three non-overlapping genomic contig sequences (Figure 4A) that encode and surround the previously described *Shank2* coding sequence. Putative exons derived from reference sequences within these contigs were mapped (Figure 4A). The

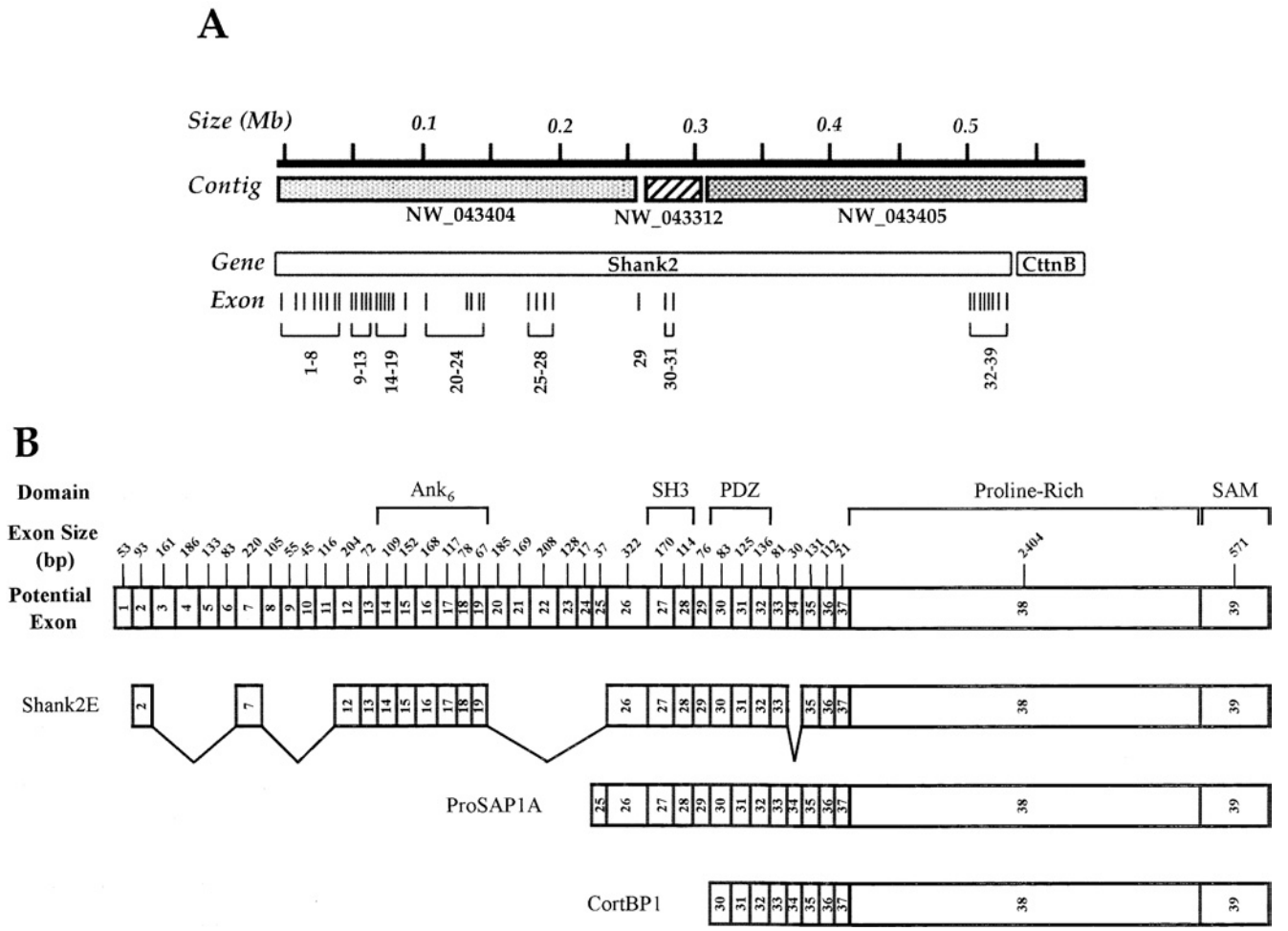

Figure 4 Exon mapping of the rat *Shank2* gene

The rat *Shank2* gene is located on the long arm of chromosome 1 at 1q41. (**A**) Dispersed over a 0.5 Mb span of genomic DNA, the *Shank2* gene is contained in three non-overlapping genomic contigs (NW_043404; NW_043312 and NW_043405). Using the AG/GT rule of exon splicing, 39 potential exons were identified throughout the three contigs. One exon (exon 29) is predicted to reside within the intervening sequence between the NW_043404 and NW_043312 contigs. (**B**) Derived from the cDNA sequence, an exon map of *Shank2E* was developed. The position of the distinct domains within the exons is listed. This includes exons 14–19 that code for ankyrin repeats that are expressed in *Shank2E*. For comparison, exon maps of ProSAP1A and CortBP1 are provided. Exon 34 may represent more than one exon.

known sequence for CortBP1 (accession number NM_133440) was compared with the sequences in the predicted exons in the contig sequences. CortBP1 is encoded by ten of the predicted exons within the NW_043312 and NW_043405 contigs (Figure 4). Applying a similar approach for the *Shank2* isoform ProSAP1A (accession number: AJ249562), an additional five exons were spliced into the initial CortBP1 exon. This splicing accounts for the distinct start sites in ProSAP1A and CortBP1. The first four exons for ProSAP1A are encoded in the NW_043404 contig. The fifth exon is not found in either the NW_043312 or NW_043404 contigs, and is probably encoded in the interim sequences between these two non-overlapping contigs. It should be noted that there could be additional exons that reside within the interim regions between contig sequences, as well as small potential exons within the contig sequences. There were, however, no putative exons present in the gene coding for ProSAP1A that could account for the increased mass of Shank2 observed in the epithelial tissues.

Upstream of these exons, and still within the NW_043404 contig, were an additional 24 predicted exons. Sequence analysis of these 24 upstream exons found that ankyrin repeats with 74 % sequence identity to Shank3 were encoded in exons 14–19 (Figure 4B). Given that the Shank1 and Shank3 proteins both have ankyrin repeat domains, it was postulated that these putative exons were indeed part of the *Shank2* gene, and accounted for the increase in Shank2 mass observed in epithelial tissues. An exon map of 39 predicted exons in the three contigs and one intervening region was generated to show the breadth of the *Shank2* gene, and display the distribution and clustering of exons (Figure 4).

The epithelial Shank2 isoform includes ankyrin repeat domains

To confirm that these upstream exons were part of the *Shank2* gene and accounted for the increased Shank2 mass in epithelial tissues, the epithelial isoform of Shank2 was fully sequenced from RT-PCR products (accession number AY298755). Using overlapping primers designed from rat ProSAP1A, the *Shank2* sequence from cholangiocytes was found to be essentially identical in exons 26–39. The lone exception was the absence of exon 34. Exon 34, which may include more than a single exon, is variably spliced in

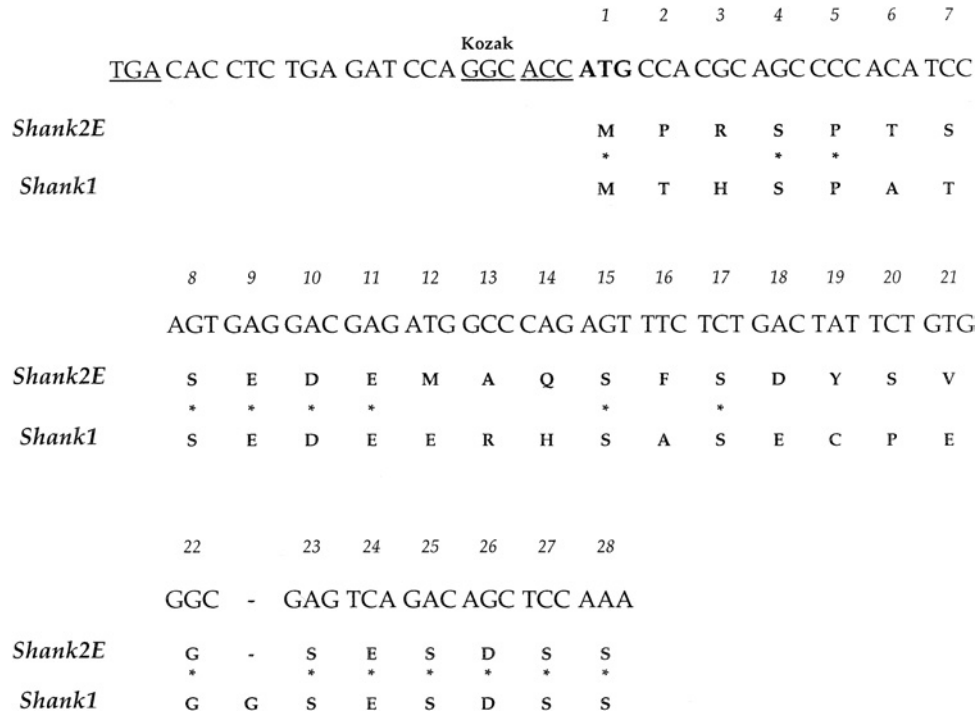

Figure 5 Sequence analysis of the putative start site for Shank2E

An in-frame stop codon (TGA) resides within exon 7. A Kozak sequence (GGCACC) followed by an in-frame ATG (shown in bold) resides immediately downstream of this stop codon. Furthermore, the predicted amino acid sequence of the initial 28 amino acids from this start site has 54 % identity (denoted by *) with the first 29 amino acids of the long form of *Shank1*. Downstream methionine residues do not have discernible Kozak sequences. These observations support the role of this ATG as the start site for *Shank2E*. The Shank2E amino acids are numbered from the initiating methionine residue.

the Shank2 isoforms, and can code for 10 amino acids [8]. An in-frame TAA stop codon is located in exon 39, and is identical in CortBP1, ProSAP1A and the epithelial Shank2 isoforms.

To determine sequences in cholangiocyte mRNA that might reside upstream of the known Shank2 isoforms, overlapping primer sets were designed within the 24 predicted upstream exons. Sequencing of the RT-PCR products from these exons demonstrated that mRNA sequences in exons 2, 7 and 12–19 were expressed in the epithelial Shank2 isoform. This sequenced isoform was denoted 'Shank2E'. The putative start site is in exon 7 (Figure 5). An in-frame stop codon resides within exon 7, defining the sequence above this codon as part of the 5′-untranslated region. Located 16 bases downstream of this in-frame stop codon is a Kozak sequence (GGCACC), followed directly by an ATG codon. The sequence of the first 30 amino acids downstream from this ATG codon has 54 % identity with the first 28 amino acids of full-length *Shank1* (Figure 5). Thus, like the *Shank1* and *Shank3* genes, the *Shank2* gene codes for ankyrin repeats. These ankyrin repeats are probably omitted from ProSAP1A and CortBP1 transcripts by alternative splicing, whereas the entire ankyrin repeat sequence coded for in exons 14–19 is expressed in the epithelial Shank2E spliceoform.

The total *Shank2* gene encompasses approx. 0.5 Mb of genomic DNA. The Shank2E isoform has a calculated molecular mass of 200 kDa, and a pI value of 6.7. Comparison of the predicted amino acid sequence from the Shank2E isoform with full-length Shank1 and Shank3 shows 44 % and 49 % identity respectively. Sequence identity is markedly higher within the specific domains. From the N-terminus, the expressed domains within Shank2E include six ankyrin repeats, an SH3 domain, a PDZ domain, a proline-rich domain that includes a PpII (polyproline type II) motif and the terminal SAM domain. The PpII motif is important, since this motif is responsible for Shank2 binding to cortactin [6,26] (incidentally, the gene for cortactin resides only 15 kb downstream of the *Shank2* gene).

Shank2 isoforms have distinct tissue expression patterns

To directly evaluate the expression of the three Shank2 isoforms in the brain compared with cholangiocytes, mRNAs from brain and cholangiocytes were analysed by RT-PCR and Northern blotting. The distinct start sites for Shank2E, ProSAP1A and CortBP1 permitted the design of Shank2-spliceoform-specific primers (Table 1). Primers in the proline-rich domain, which is present in all three isoforms, served as a common control. RT-PCR products for the proline-rich domain were readily detected in both brain and cholangiocyte samples (Figure 6A). Both ProSAP1A- and CortBP1-specific primers readily amplified mRNA from the brain, but little or no signal was amplified from cholangiocyte mRNA. Conversely, Shank2E-specific primers readily detected mRNA in cholangiocytes. In brain mRNA, faint bands were detected in some, but not all, samples. These observations were supported further by Northern blot analysis. Probes against the common proline-rich domain detected transcripts in brain (6.6 kb) and cholangiocytes (7.2 kb; Figure 6B). The larger cholangiocyte transcript reflects the presence of the ankyrin repeat sequence in Shank2E. A probe designed against the ankyrin repeat domain probe readily detected a 7.2 kb transcript in cholangiocytes, but failed to detect an mRNA transcript in brain (Figure 6B). These data confirm that the Shank2E isoform contains an ankyrin repeat domain, and is not expressed in the brain, but is expressed in cholangiocytes.

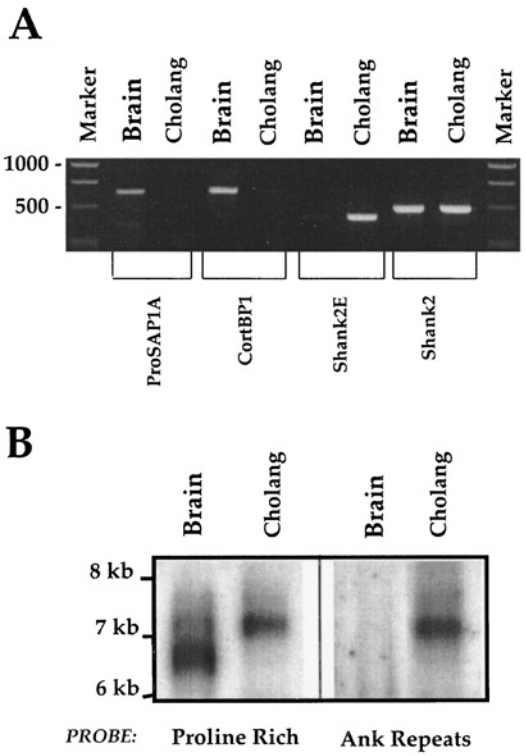

Figure 6 Neuronal compared with epithelial distribution of Shank2 isoforms

Primers directed against the unique start sites of ProSAP1A, CortBP1 and Shank2E isoforms of *Shank2* were used to detect the distribution of the three Shank2 isoforms in brain compared with cholangiocyte (Cholang) tissues. (**A**) RT-PCR products of ProSAP1A and CortBP1 were readily detected in brain mRNA samples, but were at very low or undetectable levels in cholangiocyte mRNA. Conversely, little or no Shank2E transcript was observed in brain RNA, but was readily amplified from cholangiocyte RNA. Primers against the common C-terminus readily detected Shank2 mRNA in both brain and cholangiocytes. (**B**) Northern blotting with a probe against the common C-terminus of Shank2 detected Shank2 mRNA in both the brain and cholangiocyte samples. The signal in brain was markedly smaller (6.6 kb compared with 7.2 kb). A probe against the ankyrin repeat domain, found only in the Shank2E isoform, failed to detect a transcript in the brain, but did detect a 7.2 kb transcript in cholangiocyte mRNA.

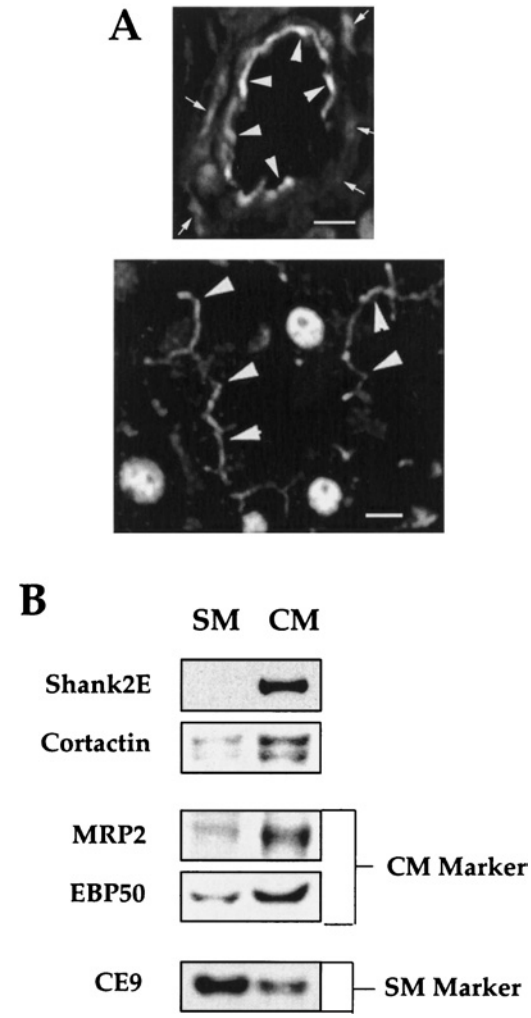

Figure 7 Shank2E is enriched in the apical region of liver epithelial cells

(**A**) Immunofluorescence analysis showed that Shank2 is concentrated at the apical region of cholangiocytes and hepatocytes. Arrowheads within the lumen of the intrahepatic bile duct (upper panel) point to the apical aspect of cholangiocytes that line these ducts. Shank2E was concentrated in the apical domain, but faint staining was also observed along the basal aspect of these cells (shown by the arrows). In hepatocytes (lower panel), Shank2E was distributed in a 'chicken-wire' pattern, demonstrating that Shank2E was concentrated at the canalicular (i.e. apical) membrane (shown by the arrowheads). The bars in both panels represent 10 μm. (**B**) Sinusoidal (i.e. basolateral) and canalicular (i.e. apical) membranes (SM and CM respectively) were enriched from hepatocytes. Western blotting for basolateral (CE9) and apical (EBP50 and Mrp2) markers confirmed the enrichment of the two fractions. Shank2E was highly enriched in the CM fraction. Cortactin, a known Shank2 binding partner, was similarly enriched in the CM fraction.

Shank2E is apically polarized in rat epithelial cells

The subcellular localization of Shank2E in liver epithelial cells was evaluated by immunofluorescence and membrane fractionation. Negative control rat liver sections, incubated in 10% non-immune serum, showed no specific staining, and were used to set the background level of detection. Sections incubated with *Shank2* antibody showed *Shank2E* concentrated at the apical membrane of both cholangiocytes and hepatocytes (Figure 7A). Cholangiocytes are the epithelial cells that line the intrahepatic bile ducts. Whereas faint staining was observed in the basolateral domain, immunofluorescence staining was concentrated at the luminal (i.e. apical) aspect of cholangiocytes. Arrows positioned within the lumen of the bile duct in Figure 7(A) point to Shank2E that is concentrated along the apical domain. In hepatocytes, Shank2E staining displayed the distinctive 'chicken wire' pattern of the canalicular (i.e. apical) membrane, with little or no staining detected along the sinusoidal (i.e. basolateral) membrane. Although nuclear staining was observed, this was probably due to non-specific interactions. Previously, nuclear staining with this antibody was not inhibited by pre-incubation with the original antigen [6]. Furthermore, Western blotting of isolated nuclei from hepatocytes failed to detect Shank2E protein in isolated nuclei preparations (results not shown). The concentration of Shank2E at the canalicular membrane was confirmed by Western blot analysis of enriched canalicular and sinusoidal membrane fractions from rat hepatocytes (Figure 7B). Relative enrichment of these fractions was confirmed by Western blotting for Mrp2 and EBP50, two proteins concentrated at the canalicular membrane [27,28], and CE9, which is concentrated in the sinusoidal membrane [29]. Shank2E was highly enriched in the canalicular membrane fraction with little or no immunoreactivity present in the sinusoidal membrane fraction. The retention of Shank2 with the canalicular membrane fraction suggests it is complexed with one or more integral membrane proteins. Cortactin, a cortical actin binding protein that directly binds Shank2 [6], was

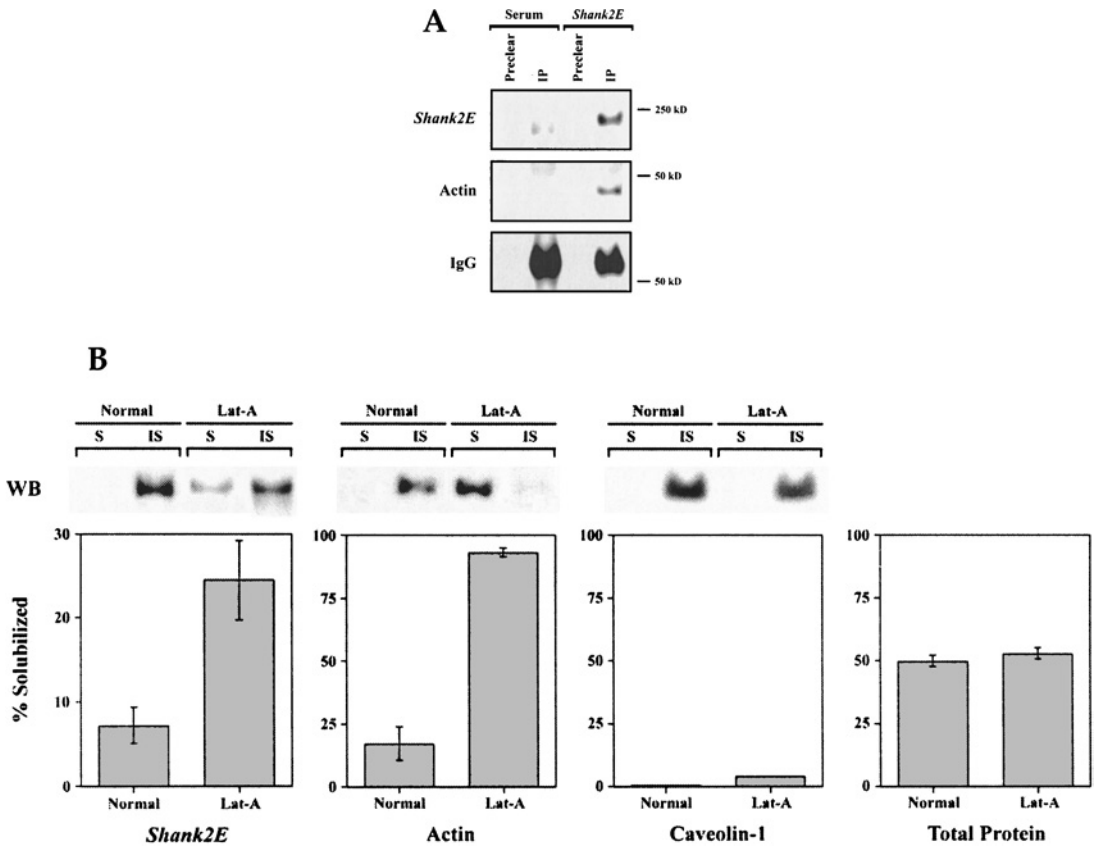

Figure 8 Shank2E complexes with actin

(**A**) Shank2E was specifically immunoprecipitated from cultured rat cholangiocytes and assayed for co-precipitation of actin. Shank2E was specifically immunoprecipitated (lanes labelled 'IP') from solubilized NRC cells (top panel). Actin was not observed in non-immune serum controls or in pre-clear samples, but did co-precipitate with Shank2E (middle panel). Relative amounts of IgG in the four samples were shown using horseradish-peroxidase-labelled anti-rabbit IgG (lower panel). (**B**) The association of Shank2E with the actin cytoskeleton was further demonstrated by Western blotting (WB) for the distribution of Shank2E in Triton X-100-soluble (S) and insoluble (IS) fractions following treatment of NRC cells with latrunculin A, an actin depolymerizing agent. Latrunculin A treatment resulted in almost-complete Triton solubility of actin. Similarly, latrunculin A treatment resulted in a significant increase in the Triton solubility of Shank2E (7 ± 2 % to 25 ± 5 %; $P < 0.05$). The Triton X-100 solubility of caveolin-1, a Triton-insoluble lipid-raft protein, and the distribution of total proteins were largely unaffected by latrunculin A treatment.

also enriched with the canalicular membrane fraction (Figure 7B). Collectively, these data parallel previous observations in tanycytes, choroid plexus and pancreatic duct epithelial cells [13], and show that Shank2E is closely associated with the apical plasma membrane of liver epithelia.

Shank2E complexes with the actin cytoskeleton

Similar to the PSD of neurons, the apical aspect of epithelial cells is a complex intracellular region in which a number of signalling and transport events occur simultaneously in a co-ordinated fashion. In the PSD, Shank proteins are complexed with the actin cytoskeleton [6]. In the apical domain of epithelial cells, the actin cytoskeleton forms the structural backbone of microvilli and the terminal web, anchors various transporter and channel proteins, contributes to regulating the activity of membrane proteins and has an integral role in the molecular machinery for exocytosis and endocytosis. To determine whether Shank2E formed a complex with the actin cytoskeleton, co-precipitation of actin with Shank2E was evaluated in NRC cells. In six out of eighteen Shank2E immunoprecipitates, actin was co-immunoprecipitated with Shank2E (Figure 8). Paired precipitations with non-immune serum did not show specific precipitation of either Shank2E or actin. To investigate further the interaction of Shank2E with the actin cytoskeleton, the solubility of Shank2E in Triton X-100 was evaluated in control and latrunculin A-treated cells (Figure 8B). Triton X-100 insolubility is a measure of a protein's association with the actin cytoskeleton and/or with lipid-raft domains. As anticipated, latrunculin A treatment resulted in a marked increase (17 ± 7 to 93 ± 2%; $n = 9$, $P < 0.05$) in the solubility of actin. Conversely, caveolin-1 was not dramatically shifted to the soluble fraction by latrunculin A treatment (control: 1 ± 1 %; latrunculin A: 4 ± 1 %; $n = 13$). For Shank2E, latrunculin A treatment resulted in a significant increase (7 ± 2 % compared with 25 ± 5 %; $n = 15$, $P < 0.05$) in Triton X-100 solubility, consistent with an association with the actin cytoskeleton. Because the increase in solubility was not of the same magnitude as actin, this suggested that Shank2E may sequester within a membrane-raft population.

To test the distribution of Shank2E in lipid rafts directly, lipid rafts were floated from Triton X-100-solubilized NRC monolayers under control and cholesterol-depleted conditions (Figure 9). Under both conditions, > 94 % of the total cellular protein was not raft-associated. The predominant distribution of caveolin-1, a known raft-associated protein, in fractions 4 and 5 validated the established gradient. Immunoblotting revealed that a significant portion of Shank2E was also distributed in low-density raft fractions. Interestingly, actin and Shank2E were similarly distributed within fractions 4, 5 and 6. Disruption of lipid rafts by cyclodextrin treatment resulted in a marked failure of Shank2E,

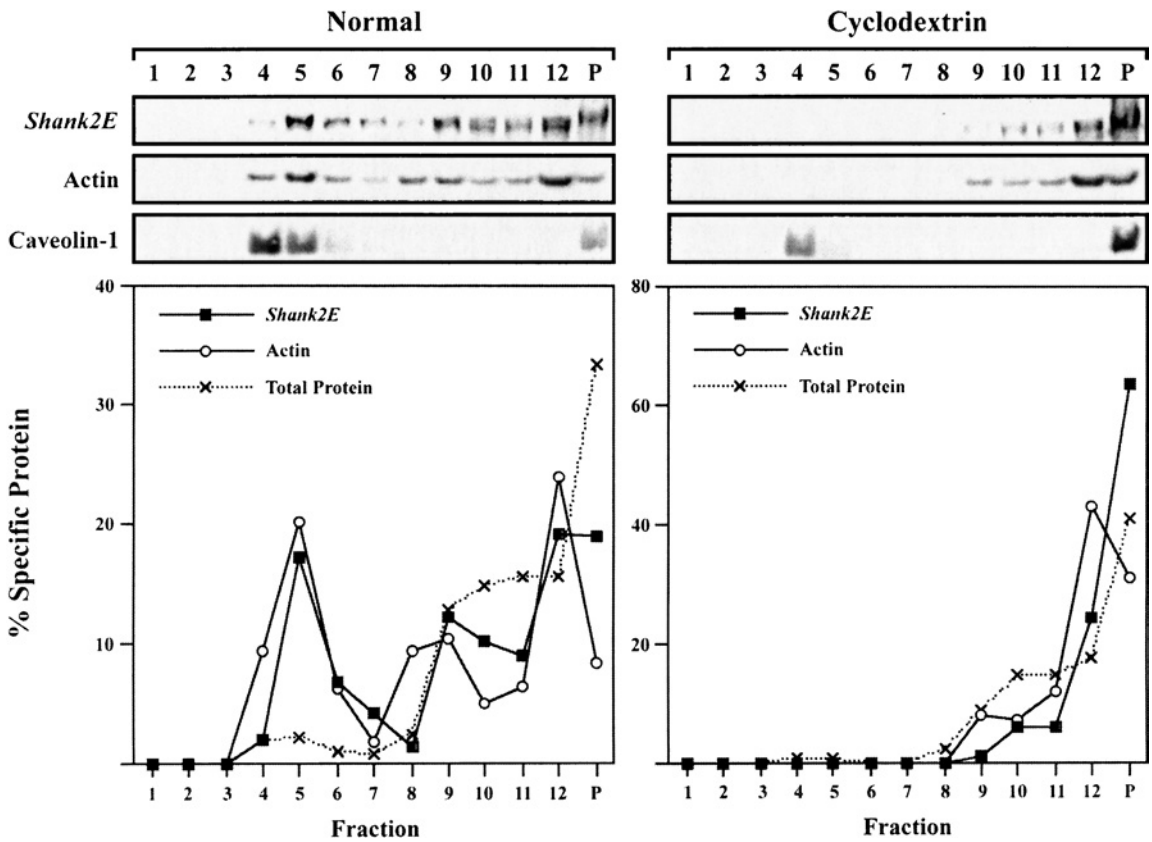

Figure 9 Shank2E and actin co-distribution in lipid rafts

The distribution of Shank2E and actin in lipid-raft populations was evaluated by sucrose-density separations of NRC cell suspensions. Caveolin-1, a known lipid-raft protein, floated to fractions 4 and 5 under normal conditions (left panel) and shifted into the pellet fraction (P) when rafts were disrupted with cyclodextrin treatment (right panel). A significant portion of Shank2E (20 % of the total in the example shown) also showed a clear distribution within raft fractions under normal conditions. Interestingly, actin was also partially distributed in raft fractions (30 % of the total in the example shown). The distribution of both Shank2E and actin was found in fraction 5, whereas caveolin-1 was concentrated in fraction 4, with some distribution in fraction 5. Cyclodextrin disruption of lipid rafts resulted in complete shifts of Shank2E and actin from the lipid raft fractions.

actin and caveolin-1 to float in the sucrose gradient and detection of the vast majority of these proteins in the 40 % sucrose (fraction 12) and pellet fractions. These studies demonstrate that Shank2E is associated with the actin cytoskeleton and is distributed within a population of lipid rafts. It suggests further that the Shank2E–actin association may occur within this raft population.

DISCUSSION

First described in 1999 [3], 'SH3 domain and ankyrin repeat-containing proteins' were termed 'Shank' proteins. Ironically, members of the Shank2 family had not previously been found to contain ankyrin repeats. The highly homologous Shank1, Shank2 and Shank3 family members probably arose from gene duplication with a prototypical, full-length Shank protein having ankyrin repeats, an SH3 domain, a PDZ domain, a proline-rich domain and a SAM domain (Figure 1). The complexity of the expressed Shank isoforms is greatly amplified by alternative splicing of Shank transcripts [8]. Although the ankyrin repeat domain could have been omitted during gene duplication, it remained an attractive hypothesis that the *Shank2* gene retained the ankyrin repeats, and that the repeats were spliced from the two known *Shank2* isoforms, CortBP1 and ProSAP1A [6,12]. Exploring the contig gene sequences surrounding the ProSAP1A gene, the present study located a region 420 kb upstream from the known gene sequence that codes for six ankyrin repeats. *Shank1* and *Shank3* are located on chromosomes 1q21 and 7q34 respectively, indicating that these ankyrin repeats are not part of the *Shank1* or *Shank3* genes. Unlike CortBP1 and ProSAP1A, this novel, ankyrin repeat-containing Shank2 isoform was not detected at any significant level in brain (see Figures 3 and 6), but was expressed in epithelial tissues. The discovery of this novel epithelial isoform underscores the importance of alternative splicing in creating cell- and tissue-type-specific Shank proteins.

Functional role of Shank proteins in neurons

Within the PSD, Shanks are key elements in the scaffolding and regulation of large receptor and effector complexes. Structurally, Shank proteins are associated with the development of dendritic spines and enlargement of spine heads [30]. Shank is interposed between the synaptic membrane and the underlying actin cytoskeleton [10]. This anatomic positioning enables Shank to sequester the PSD95–NMDAr and Homer–mGluR complexes into a co-ordinated complex and anchor the grouping on to the underlying actin cytoskeleton. Shank linkage to the actin cytoskeleton occurs through association with actin-binding proteins, including α-fodrin [31], cortactin [6] and dynamin-2 [7]. Rather than static scaffolding complexes, proteins within the Shank2 macromolecular complex suggest that Shank2 contributes to dynamic responses within cells. For example, cortactin enhances Arp2/3

(actin-related protein 2/3) complex activity. Arp2/3 complex initiates new actin filament formation and drives the motility of membranes and intracellular vesicles [32,33]. Dynamin-2, a pivotal protein in early endocytosis and vesicular trafficking, binds with cortactin and with Shank2 and Shank3 [7]. Insulin-receptor tyrosine kinase substrate p53 and β-PIX, a guanine nucleotide exchange factor, are also Shank2 binding partners and moderate the activation of Rac1 and Cdc42 [34–36]. Rac1 and Cdc42 are small GTPases that serve as molecular switches in regulating actin dynamics. In neurons, Shank proteins serve as a 'central co-ordinator' of the PSD by bringing smaller membrane–PDZ protein complexes into a co-ordinately regulated macro-complex, tethering the macro-complex on to the cytoskeleton and associating the macro-complexes with the dynamic aspects of the actin cytoskeleton and endocytic machinery. NRC cells express both cortactin and dynamin-2. In preliminary studies, both cortactin and dynamin-2 were co-precipitated with Shank2E immunoprecipitates in two of eighteen studies. Although these studies were not isolated with non-immune serum controls, these co-precipitates suggest that Shank2E in NRC cells may also associate with protein complexes involved in vesicle and protein dynamics.

Functional role of Shank2E in epithelial cells

Epithelial cells have subcellular regions of the cell that require sophisticated organization to co-ordinate their specialized molecular events. PDZ-domain-containing scaffolding proteins have come to the forefront as key organizers of these specialized epithelial domains [37]. From its known functions in neurons, its apical distribution in a variety of epithelial cell types [13] (see Figure 7) and observations regarding association with the actin cytoskeleton and lipid-raft domains made in the present study (Figure 8 and 9), Shank2 could impact three distinct pathways at the apical membrane of epithelial cells. First, PDZ proteins directly regulate the activities of specific apical membrane proteins, and Shank2E may interact with and regulate such proteins. Secondly, the activities of functionally related transporters are often co-ordinately regulated in epithelial cells. For example, CFTR not only has its own Cl^- channel activity, but also inhibits ENaC activity [15]. CFTR is sequestered into an actin-linked protein complex through EBP50, a PDZ domain-containing protein, and ezrin, which binds EBP50, actin and protein kinase A into a signalling-effector cluster. How this complex is able to interact with and regulate other ion-conductance pathways, such as that involving ENaC, is currently unknown. It has been postulated that 'central co-ordinator' proteins, such as Shank2, are expressed in these domains and co-ordinate the activities of the functionally related proteins. Finally, epithelial cells utilize regulated exocytosis and endocytosis to regulate activities in their membrane domains. Cultured cholangiocytes exchange an equivalent of 1.5% of their plasma membrane per minute, recycle 40% of the endocytosed membrane back to the plasma membrane and regulate these rates via distinct signalling pathways [38,39]. In polarized epithelial cells, raft-associated proteins are preferentially targeted to the apical membrane. Recently, direct comparison of raft-associated and raft-independent protein trafficking in polarized MDCK cells found that the targeted apical membrane delivery required an intact actin cytoskeleton [40]. It will be of interest to determine, in polarized cholangiocytes, whether Shank2E, which complexes with actin and is co-distributed with actin in a lipid raft population (Figures 8 and 9), is required for co-ordinating the targeted delivery of proteins to the apical membrane. Actin polymerization and dynamin-2 are central players in the vesicle-trafficking activities at the apical membrane of epithelial cells [41]. Given the known association of dynamin-2 and cortactin with Shank2E, and the expression of both cortactin and dynamin-2 in cultured cholangiocytes (R. R. McWilliams and R. B. Doctor, unpublished work), it will be important to determine whether Shank2E plays a pivotal role in co-ordinating the regulated retrieval and internalization of membrane and membrane proteins at the apical region of epithelial cells.

Ankyrin repeat domains in epithelial function

The principal difference between the Shank2 isoforms in neurons and epithelial cells is the expression of the ankyrin repeats in the epithelial cells. Ankyrin repeats are a protein–protein binding motif best known for their ability to bind integral membrane proteins [42]. Thus the ankyrin repeats expressed in Shank2E may provide another means to tether specific integral membrane proteins to the Shank2 complex. However, the ankyrin repeat domain of Shank in the PSD has not been shown to bind directly to integral membrane proteins. Shank ankyrin repeats have been shown to bind both α-fodrin [31] and Sharpin [9]. α-Fodrin binds and cross-links actin filaments, and serves as a molecular scaffold for site-specific protein anchorage [42]. In the apical domain of epithelial cells, α-fodrin is localized to the sub-apical terminal web. Thus the ankyrin repeats expressed in Shank2E may provide a cytoskeletal linkage for the Shank2E complex when residing within or traversing through the terminal web. Broadly distributed, the protein Sharpin is expressed in epithelial tissues and has the capacity to bind the ankyrin repeats of Shank and homodimerize [9]. Functionally, Sharpin is postulated to promote the clustering of Shank proteins that express the ankyrin repeats domain.

In summary, the present study has demonstrated the presence of ankyrin repeat domains within the *Shank2* gene, described a novel *Shank2* isoform that expressed these ankyrin repeats, demonstrated that this isoform, termed *Shank2E*, was expressed preferentially in epithelial tissues, and has shown that Shank2E associates with an actin cytoskeletal complex and lipid-raft population at the apical domain of epithelial cells. Given the role of Shank proteins in providing higher-order protein complexes in the PSD of neurons, the present description of Shank2E provides great incentive to investigate the role of Shank2E in co-ordinating physiological events at the apical domain of epithelial cells.

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