Pinin/DRS/memA Interacts with SRp75, SRm300 and SRrp130 in Corneal Epithelial Cells

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PURPOSE. Pinin (Pnn/DRS/memA) is a cell-adhesion-related and nuclear protein that has been identified as central in the establishment and maintenance of corneal epithelial cell–cell adhesion. To begin the elucidation of the role of Pnn within the nucleus of corneal epithelial cells, this study was undertaken to identify the proteins that bind to Pnn.

METHODS. Yeast two-hybrid analyses were performed. A human cDNA library in the pGAD-10 vector and C-terminal region of human Pnn (465-717) in a pAS2-1 vector were cotransformed into the PJ69-4A yeast strain, containing the lacZ, HIS3, and ADE2 reporter genes. To dissect domains of Pnn responsible for mediating the interaction with the identified proteins, PNN fragments were ligated with the DNA-binding domain of the pAS2-1 vector. Human corneal epithelial cells (HCE-T, RCB1384) and HEK-293 cells were cotransfected with mammalian expression vectors containing Pnn with identified interacting partners and subsequently immunostained and immunoblotted to determine expressed and endogenous proteins.

RESULTS. Pnn colocalized and copurified with serine-arginine (SR) proteins. Three SR-rich proteins were identified that interact with the C-terminus of Pnn: SRp75 and SRm300, known components of splicesome machinery, and a novel 130-kDa nuclear protein, SRrp130. All of these proteins colocalized and coimmunoprecipitated with one another and exhibited speckled nuclear distribution that aligned with components of the pre-mRNA splicing machinery. The cDNA for SRrp130 encoded a protein of 805 amino acid residues and contained multiple arginine-serine (RS) repeats but had no RNA recognition motif. Analysis of the Pnn motifs using two hybrid system assays demonstrated that the polyserine/RS motif within Pnn plays a central but not exclusive role in mediating molecular interactions with identified SR-rich proteins.

CONCLUSIONS. The results suggest that Pnn and SR-rich proteins may be part of a multiprotein complex within the nucleus and may be involved in pre-mRNA processing. (Invest Ophthalmol Vis Sci. 2003;44:4715–4723) DOI:10.1167/iovs.03-0240

The cells of the corneal epithelium possess dynamic means by which to adhere to the underlying matrix and their neighboring cells to allow for alterations in the attachments, affording the cells the ability to undergo transitions from quiescence to active migration subsequent to wounding.1 We have studied a molecule, pinin (Pnn/DRS/memA) a 140-kDa phosphoprotein associated with the desmosome and localized in the nucleus of various tissues and cultured cell lines,2,3 which seems to play a key role in the establishment and maintenance of corneal epithelial adhesion.4 We have shown that Pnn alters its intracellular distribution after wounding and that overexpression of Pnn interferes with successful epithelial migration and wound closure.1 We have also shown that expression of Pnn is linked to the expression of genes such as E-cadherin, p21cip/waf, MIG-1, and Rho-A, which impact epithelial adhesion, proliferation, and cell motility.5 These observations suggest that Pnn may play a central role in epithelial cell gene expression. As part of determining the mechanism by which Pnn exerts its influence on corneal epithelial cell gene expression, we embarked on a study to determine the Pnn-binding partners.

Whereas the N-terminal portion of Pnn harbors some motifs characteristic of intermediate filament-associated proteins, the C-terminal portion contains two distinctive domains including the glutamine-proline glutamine-leucine (QPQL) repeat domain, the polyserine domain, and several SR-repeat motifs,6 characteristic of proteins that are involved in splicing. The original characterization of Pnn demonstrated its association with intermediate filaments at their convergence onto desmosomal plaques,6 and subsequent two-hybrid analyses determined that the N terminus of Pnn binds directly to keratins.7 The expression of exogenous Pnn in transformed cells dramatically alters the recipient cells’ morphology, driving them to a more epithelial phenotype. However, nearly all of the expressed Pnn is found within the nucleus, exhibiting a diffuse nucleoplasmic and speckle distribution. Little if any of the exogenous Pnn is localized to cell–cell adhesion sites, raising the possibility that Pnn may exert its effect through interaction with nuclear components, perhaps involving mRNA transcription and processing. Pnn’s localization at nuclear speckles has been described previously.2 Furthermore, Wang et al.8 have suggested that Pnn may function directly in alternative splicing, specifically the regulation of 5′ splice site choice. Dellaire et al.9 have reported that PRP4 kinase, which binds to Pnn, is part of an N-CoR–containing deacetylase complex, potentially linking Pnn to transcriptional regulation. We suggest that Pnn may belong to the growing family of cell-adhesion–related proteins also involved in nuclear functions such as transcription and mRNA processing.10

In the nucleus, Pnn displays both a diffuse nucleoplasmic and a speckled distribution.2,3,7 Speckles are discrete foci within the mammalian nucleus, numbering 20 to 50. It has been proposed that the function of speckles is storage of splicing and transcription-related factors, which are recruited from the speckles to active sites of gene transcription.11 At the ultrastructural level, they correspond to interchromatin granular...
ular clusters (IGCs) and perichromatin fibrils (PFs). Biochemical isolation and analysis of IGC components have revealed approximately 75 proteins. Many of these proteins have known functions in splicing and contain a distinctive region of arginine-serine (RS) repeats. In addition to splicing, some of the speckle-associated proteins are involved in other steps of pre-mRNA biogenesis, whereas others seem to play structural roles, such as protein 4.1, which may provide a scaffold for the assembly of large multiprotein complexes.

To begin to address the potential role of Pnn in nuclear processes, we performed yeast two-hybrid assays. These studies revealed that the C-terminal portion of this protein interacts with the SR-rich nuclear proteins SRp75, SRm300, and the novel SRp130, reaffirming Pnn’s possible involvement in splicing. Dissection of the domain of Pnn that mediates binding with SR-rich proteins suggests specific, yet overlapping regions within Pnn’s C-terminus. We confirmed the interaction of Pnn with these proteins by coimmunoprecipitation and immunoblot assay and by localizing Pnn and Pnn-binding proteins in corneal epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-Hybrid Screening and Generation of Pnn Truncation and Deletion Constructs**

A yeast two-hybrid system was used, including the *Saccharomyces cerevisiae* strainPJ69-4A, which contains three reporter genes (lacZ, HIS3, and ADE2), the yeast plasmids pAS2-1 and pGAD-10 (Matchmaker II system; BD Biosciences-Clontech, Palo Alto, CA), and a human fetal kidney cDNA library (Matchmaker; BD Biosciences-Clontech) in the pGAD-10 vector, as described previously. Briefly, to construct the bait plasmid, the PCR-generated C-terminal region of human Pnn (465-717) was cloned into the yeast expression vector pAS2-1 as a fusion to the DNA-binding domain of the yeast transcription factor GAL4. Yeast was cotransformed with the bait plasmid, and a human fetal kidney cDNA library (BD Biosciences-Clontech) fused to the activation domain of GAL4 in the pGAD10 yeast expression vector. Of the approximately 10^6 transformants, 22 subsequently passed -His and -Ade selection and were positive in a β-galactosidase liquid culture assay performed according to the manufacturer’s procedure (ortho-nitrophenyl-β-D-galactopyranoside reagent [ONPG]; BD Biosciences-Clontech). After isolation of the plasmids and sequencing, four distinct clones that coded for SR-related proteins were selected for further analysis. The pGAD-10 plasmids from these positive clones were reintroduced into the yeast strain PJ69-4A either with GAL4-DNA-BD-PNN(465-717), or controls consisting of GAL4-DNA-BD-PPN(1-480), GAL4-DBP55, or GAL4-DNA-BD alone. All transformants were sequentially reselected with -His and -Ade, and all the controls failed to grow on the second selection medium (-Ade). ONPG β-galactosidase assays were performed on the four positive clones: C-158, C-54-1, C-34-5 and C-25-10, which revealed values in β-galactosidase units of 47.4, 31.5, 59.3, and 32.2, respectively. These values represent averages of 3 separate measurements from three independent colonies and are significantly higher than the baseline level of β-galactosidase activity, which is obtained from control yeast cotransformed with a Pnn bait construct and an “empty” prey (pGAD-10) plasmid.

To dissect domains of Pnn responsible for mediating the interaction with the identified SR proteins, truncation constructs were PCR generated with primers introducing restriction sites Ndel at the 5’ terminus and Sall at the 3’ terminus. These were used to clone PNN fragments in frame with the DNA-binding domain of the pAS2-1 vector. The deletion Pnn mutants, missing RS repeats from the polyserine/RS domain (construct 465-717Δ2256Δ411), or missing the entire polyserine/RS domain (construct 465-717Δ2559Δ411), were also generated by PCR amplification. The primers introduced internal *Bam*HI sites, thus replacing deleted domains and fusing together the remaining 5’ and 3’ Pnn domains with the *Bam*HI site. The detailed truncations of the polyserine/RS domain were obtained by introducing stop codons at residues 579, 606, 619, or 650 with a site-directed mutagenesis kit (Quick Change; Stratagene, La Jolla, CA), therefore terminating translation of the Pnn construct fused with GAL4-DNA-BD in the pAS2-1 vector.

**cDNA Isolation, Sequencing, and Analysis**

The full-length PCR cDNA clone of SRrp130 was generated with a primer pair located outside the predicted open reading frame (ORF), SRmus-1F (5’-CGA AAG CGT TGG AGA GGT-3’) and Scrb-1R (5’-CTT GCC ACA TCA TTC CTC AGC-3’). Template cDNA was obtained by reverse transcription of total RNA from the HEK-293 cell line using a kit (Superscript II; BD Biosciences-Clontech). Amplified cDNA consisting of 2865 bp was subsequently subcloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and vector sequenced. Sequence analysis of the PCR cDNA clone, overlapping IMAGE clone (IDG11987; http://www.infoimage.nlm.nih.gov/ the Integrated Molecular Analysis of Genomes and Their Expression Consortium; hosted by The Lawrence Livermore National Laboratory, Livermore, CA) and the clone isolated from two-hybrid screening, allowed the assembly of a 3173 bp contig containing the SRrp130 ORF. Oligonucleotide primers were purchased from GenoMechanix (Alachua, FL). PCR-amplified cDNA fragments were generated with a commercial PCR system (Expand Long Template; PCR System; Roche Molecular Biochemicals, Mannheim, Germany).

Sequencing was then performed (Big Dye Terminator and Prism 310 Genes Analyzer; Applied Biosystems, Foster City, CA). For assembly of contigs, predictions of the SRrp130 protein’s secondary structures and sequence motifs, two commercial software packages were used (Lasergene; DNastar, Inc., Madison, WI, and MacDNAsis; Hitachi, Tokyo, Japan).

A human multiple-tissue Northern blot filter (BD Biosciences-Clontech) was prehybridized in 50% formamide, 5× SSC, 5× Denhardt’s solution, 0.2% SDS, and 200 μg/ml salmon sperm DNA overnight at 42°C. Next, the filter was hybridized in the same solution with a 32P-labeled PCR-generated C-terminal probe (196-586 bp) at 42°C for 16 hours. After a double wash in 0.1% SDS at 60°C for 2 hours, the filter was exposed to x-ray film at −80°C with an intensifying screen. Subsequently, the same filter was stripped and hybridized to the N-terminal probe (1832–2408 bp).

**Pnn Complex Isolation**

To begin biochemical characterization of how Pnn is associated with nuclear components, we generated stable HeLa cells expressing human Pnn tagged with both Flag and hemagglutinin (HA) epitopes at its amino terminus. The expression levels of the tagged Pnn were comparable to that of endogenous Pnn. The detailed procedure has been described previously. In brief, recombinant retrovirus expressing a bicistronic messenger RNA containing ORFs of Flag-HA-tagged human Pnn and interleukin-2 receptor (IL-2R)α was constructed and transduced into HeLa cells. The infected HeLa cells were sorted by anti-Flag M2 mAb-conjugated agarose beads (Sigma-Aldrich, St. Louis, MO).

**Epitope-Tagged Expression Plasmids**

The GFP and myc-epitope–tagged human PNN cDNA has been described. The HA epitope–tagged, full length SRrp130 was constructed by amplifying an SRrp130 cDNA with specific primers, introducing the HA tag with an EcoRI site at the 5’ end and a NotI site at the 3’ end. Reconstruction of the full-length clone Srm300 was accomplished in the vector pGAD10, using a BamHI site present in the overlap of C-25-10 and C-34-5 clones and an EcoRI cloning site. After this, the

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SRm300 (1-714) PCR product was generated with a forward primer introducing an EcolR site and an HA tag, SRm-Eco-HA-F, and a reverse primer, introducing a NolI site and stop codon, SRm-Not-R. The HA-tagged, full-length SRP75 was constructed by amplifying an SRP75 cDNA with specific primers introducing the SpeI site at the 5′ end and an HA tag with a stop codon and SpeI site at the 3′ end. Subsequently, introduced restriction sites were used for cloning the PCR product into the pc3-neo mammalian expression vector (Promega, Madison, WI).

**Cell Lines, Cell Culture, and Transfections**

Human corneal epithelial cells (HCE-T, RCB1384) were kindly provided by Kaoru Araki-Sasaki (Osaka University, School of Medicine, Osaka, Japan). HCE-Ts were cultured in DMEM/F12 (BioWhittaker; Walkersville, MD) containing 5% FBS (Cellgro; Mediatech, Herndon, VA), 5 μg/mL insulin (Sigma-Aldrich), 0.1 μg/mL cholera toxin (Sigma-Aldrich), 10 ng/mL human epidermal growth factor (hEGF; Invitrogen) and 0.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich). The HEK-293 cells were cultured in DMEM (BioWhittaker) containing 10% FBS (Cellgro; Mediatech), 2 mM glutamine, and 200 U/mL each of streptomycin and penicillin-G. Cells were transfected at 60% to 70% confluence (Lipofectamine TM2000; Invitrogen-Gibco, Gaithersburg, MD) according to the manufacturer’s recommendations.

**Antibodies**

The Pnn 215 pAb was generated by injecting rabbits with the GST-Pnn fusion protein, as described previously. This Pnn antibody was used at a 1:200 dilution factor. Mouse anti-HA epitope 16B12 antibody (used at 1:1000) and anti-myc epitope polyclonal (used at 1:200) were purchased from Babco/Covance (Richmond, CA). SR proteins were identified using a monoclonal antibody that recognizes a subset of non-snRNP splicing factors (SR proteins) such as SRP75, SRP95, SRP40, and SRP20 but not SRP30a or -b (SC-55), SRm300, or Pnn (Zymed, South San Francisco, CA). The previously described, polyclonal anti-SRm300 antibody was used at a 1:200 dilution factor, was a generous gift from Benjamin J. Blencowe (University of Toronto, Ontario). The secondary antibodies included Alexa Fluor 488 IgG H+L (1:2000) goat anti-mouse (Molecular Probes, Eugene, OR), Alexa Fluor 488 IgG H+L (1:2000) goat anti-rabbit (Molecular Probes) and rhodamine IgG/IgM (1:200) goat anti-mouse (Chemicon International, Temecula, CA).

**Immunoprecipitation and Immunoblot Analysis**

HEK-293 cells in 100-mm dishes were transfected with 6 μg Pnn-myc and 6 μg SRm300-HA, SRp130-HA, or SRp75-HA. After 24 hours, cells were extracted in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholine, 0.1% SDS, and 50 mM Tris [pH 8.0]), and the resultant extracts were centrifuged at 14,000 g for 30 minutes. Supernatants were incubated for 4 hours in 1.5 μg polyclonal anti-myc antibody (rabbit), and 15 μL protein-A–conjugated Sepharose was added for an additional 2 hours. Beads were centrifuged at 700 rpm for 2 minutes and washed in 10 mL of RIPA buffer. Subsequent to washing, the Sepharose pellets were boiled in Laemmli’s sample buffer. Protein samples were run on 4% to 15% gradient polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes for immunoblot analysis. The membranes were blocked with 5% nonfat dry milk and incubated in monoclonal β1 anti-HA or 9B11 anti-myc antibodies (Cell Signaling Technology, Beverly, MA). Peroxidase-conjugated goat anti-mouse IgG (Amersham Biosciences, Arlington Heights, IL) was used at 1:5000 dilution, and chemiluminescence detection (ECL; Amersham) was performed according to the manufacturer’s instructions.

**Immunostaining**

Cells cultured on coverslips were fixed with acetone (-20°C), methanol (-20°C), or 4% paraformaldehyde (followed by 0.1% Triton). Primary and secondary antibodies were incubated on coverslips for 1 hour and washes were performed with PBS. For deconvolution and volume-rendering, the SR/215 slide was optically sectioned under a microscope (DM IRBE; Leica, Deerfield, IL) with software support (Openlab ver. 5.1; ImproVision, Lexington, MA). The imaging process was performed under three channels with a 0.2 μm step-width at a 65% magnification. A total of 111 slices were retrieved under each channel. Each channel was subjected to a background-reduction step, pseudocolored, and then subjected to deconvolution (Volocity 1.4.1; ImproVision). With the deconvolution software (Volocity; ImproVision) a point-spread function was acquired for each of the channels at different wavelengths. A three-dimensional (3D) restoration of the three channels was performed at a single time point and for 12 iterations. Images were then rotated about the y-axis, and snapshots of the computer-generated 3D model were acquired.

**RESULTS**

**Distribution of Pnn within Corneal Epithelial Cells**

Examination of cultured human corneal epithelial cells (HCE-T) with routine immunofluorescence, using a polyclonal antibody (UF215) against Pnn, revealed both cell adhesion-associated and intranuclear distribution (Fig. 1A). The cell–cell associated staining decorated keratin filaments near their points of convergence at the lateral cell surfaces, consistent with numerous previous studies of corneal epithelia from the chicken23 and rabbit.24 The intranuclear Pnn was localized to speckles and distributed diffusely throughout the nucleoplasm. Double immunostaining with UF215 and a monoclonal antibody against a shared epitope on SR proteins was used for better determination of Pnn’s association to constitutive components of the nuclear speckle. Deconvolution microscopic imaging indicated a partial colocalization of Pnn and SR proteins (Figs. 1B–E). Pnn immunostaining was also obvious at sites of cell adhesion. Little diffuse cytoplasmic or nucleoplasmic Pnn was shown because of the nature of deconvolution microscopy. Digital rotation of the image stacks revealed that Pnn did not align to all SR-containing speckles, although a number of these speckles exhibited Pnn immunoreactivity (Figs. 1B, 1E, white arrows). Furthermore, there were large nuclear deposits of Pnn that exhibited no corresponding SR immunostaining (Figs. 1B, 1E, outlined arrows), perhaps suggestive of a non-SR-associated intranuclear compartment for Pnn.

**Pnn Coimmunopurified with SR Proteins**

Creation of stable cell lines (HeLa) expressing epitope-tagged Pnn enabled the isolation and identification of Pnn-associated proteins. Nuclear extracts from these cells were subjected to purification with anti-Flag antibody columns. Western blot analyses of the eluted material were performed with a monoclonal antibody directed against a shared epitope found on a subset of approximately 20 non-snRNP splicing factors termed SR proteins, such as SRP55, SRp40, and SRP75.21 The Western blot revealed that the affinity-purified Pnn complex contained an array of endogenous SR proteins (Fig. 2). Prominent SR-immunoreactive bands, with Mr(K) of approximately 55, 75, 100, and 200, were copurified with Pnn. These results are consistent with Pnn’s localization at the nuclear speckle.

**Identification of the Pnn Binding Partners SRP75, SRm300, and SRp130**

In an effort to identify specific nuclear proteins interacting with pinin, we performed a yeast two-hybrid screening.7 Sequence analyses of four of the positive clones revealed that...
they encoded proteins belonging to the family of SR-rich proteins. Clone C-54-1 encoded an RS-repeat domain of SRp75 (residues 117-494). Clones C-34-3 (residues 1-278), and C-25-10 (residues 129-714) overlapped, and both showed identity to the N-terminal portion of the sequence coding for an RNA-binding protein (GenBank Accession Number AB016092; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), defined later as the SR-related nuclear matrix protein of Pnn (green), which aligned to convergence of keratin filaments (red) with diffuse and speckled intranuclear staining. In (B) a monoclonal antibody was used to visualize SR proteins within the nuclear speckles. Immunostaining for Pnn (green) aligned to many (yellow), but not all, of the SR rich speckles (red). Pnn was also observed in intranuclear aggregations, which appeared to be devoid of SR proteins. (C–E) A three-dimensional rendering of a nucleus immunostained for SR-proteins and Pnn rotated 180°. Again, Pnn can be seen aligned to a number of the SR-containing speckles (white arrows). In addition Pnn can be seen alone within intranuclear aggregations (outlined arrows).

The clones encoded overlapping portions of SRm300 N-terminus (residues 1-278 and 129-714), of which only residues 129-714 contained an extensive RS-repeat domain, which suggests that interactions between Pnn and SR-rich proteins may involve distinct motifs, in addition to the SR dipeptides. An intriguing finding is that the overlapping stretch coded by these clones was the same portion of SRm300 that was shown to be necessary for binding to the 5′ end of AT-rich element binding factor 1 (ATBF1) mRNA.27

Dissection of Protein Interactions between the C-terminal Portion of Pnn and SRm300, SRp75, and SRrp130

To determine specific regions of Pnn that mediate protein-protein interactions with SRm300, SRp75, and SRrp130, we performed additional two-hybrid assays using truncations and deletions of Pnn (Fig. 3A). SRm300 (1-278) exhibited interaction with the polyserine/RS domain of Pnn, whereas SRm300 (129-714) required both the PQPL and the polyserine/RS domains. SRm300 (1-714) exhibited interactions only with the full-length Pnn C-terminus. SRp75 interacted with the polyserine/RS-DRK domains (Figs. 3A–C). SRrp130 interacted with the polyserine/RS domain by itself or in conjunction with either flanking PQPL or DRK domains (Figs. 3A–C). These results emphasized the importance of the polyserine/RS domain in mediating interactions between Pnn and SRm300, SRp75, or SRrp130, yet suggested a complementary role for the flanking sequences. Two-hybrid experiments testing further deletions and truncations of the C-terminal domain of Pnn suggested that the polyserine/RS portion of Pnn is integral to binding for the SRm300, SRp75, and SRrp130 proteins, and the residues flanking the SS/RS domain may also participate in binding to SR proteins (Fig. 3D).

FIGURE 1. Pnn is localized to desmosomes and nuclear speckles. HCE-T cells were immunostained for Pnn and keratin (A) and Pnn and SR proteins (B). The routine double immunofluorescence counterstained with 4′,6′-diamino-2-phenylindole (DAPI; blue) showed the typical desmosome-associated staining of Pnn (green), which aligned to convergence of keratin filaments (red) with diffuse and speckled intranuclear staining. In (B) a monoclonal antibody was used to visualize SR proteins within the nuclear speckles. Immunostaining for Pnn (green) aligned to many (yellow), but not all, of the SR rich speckles (red). Pnn was also observed in intranuclear aggregations, which appeared to be devoid of SR proteins. (C–E) A three-dimensional rendering of a nucleus immunostained for SR-proteins and Pnn rotated 180°. Again, Pnn can be seen aligned to a number of the SR-containing speckles (white arrows). In addition Pnn can be seen alone within intranuclear aggregations (outlined arrows).

FIGURE 2. Pnn copurified with SR proteins. Nuclear extracts from HeLa cells expressing human Pnn tagged with both Flag and HA epitopes were immunoprecipitated with Flag antibody resin. The original nuclear extract and eluted Pnn-associated polypeptides were resolved on SDS-PAGE and immunoblotted (IB) with either anti-HA antibody (HA) for Pnn or a monoclonal antibody against a shared epitope on SR proteins (SR). Prominent SR-immunoreactive bands with Mr (K) approximately 55, 75, 100, and 200 were resolved in the immunoblots of Pnn immunoprecipitates.

Cloning, Sequence Analysis, and Expression of the SRrp130 Gene

We cloned the cDNA encoding full-length of SRrp130 ORF, using the partial clone C-15-8 identified in the two-hybrid screening. The contig assembled from human expressed se-
sequence tag (EST) overlapping clones present a continuous ORF encoding 805 amino acid residues beginning from the first putative start codon (Fig. 4A). Confirmation of the database-derived cDNA sequence was achieved by sequencing of two IMAGE Consortium (LLNL) cDNA clones: ID611987 (1.7 kb GenBank accession number AF314186) and ID209774 (0.7 kb). The presence of 5' sequence coding for CpG-island (GenBank accession number Z64565) and multiple stop codons in all three frames preceding the continuous ORF (GenBank accession number Z64565) raises the possibility that the N-terminal domain of Pnn binds to Pnn near the polyserine stretch but each requires a different extent of flanking sequences. Epitope-tagged Pnn (Pnn-myc) was cotransfected with epitope-tagged SRp130-HA, SRm300-HA, or SRp75-HA into HEK-293 cells. After 24 hours, the cells were extracted and the extracts immunoprecipitated with polyclonal anti-myc. Immunoprecipitates were immunoblotted (IB) with monoclonal anti-HA. Whereas the expression levels of each SR protein varied, with SRp75 being the most highly expressed, SRp130, SRm300, and SRp75 all coimmunoprecipitated with Pnn. The immunoreactive band migrating around Mr(K) 55 is the heavy chain of the immunoprecipitating antibody.

Northern blot analysis with 390 bp N-terminal and/or 576 bp C-terminal probes from the SRp130 cDNA showed up to four mRNA species, ranging from approximately 3.5 to 7.5 kb, detected in most of the human tissues (Fig 4C). Comparison of the cDNA sequence with the genomic DNA sequence revealed the presence of 11 introns of various lengths, from 720 to 8786 bp. The SRp130 cDNA sequence reported herein corresponds to approximately 3.2-kb transcript, with the C-terminal part of which is encoded by two-hybrid positive clone C-15-8, demonstrating the usage of the second polyadenylation signal (see Fig. 4D). In addition, the SRp130 C-terminus shows similarity to splicing factors belonging to the SR family proteins, such as ASF/SF2, SC35, SRp75, and SRp20 (Lipman-Pearson similarity indexes 35, 31, 29, and 27, respectively). The N-terminal region of SRp130 protein harbors well conserved motifs present in the putative Mus musculus protein (GenBank accession number BAB30779), Caenorhabditis elegans protein (GenBank accession number Y53H1A.1), and the Drosophila melanogaster CG31211 gene product (GenBank accession number AA54719). A motif discovery tool (http://meme.sdsc.edu/meme/website/intro.html; developed by Timothy Bailey, Charles Elkan, and Bill Grundy, UCSD Computer Science and Engineering Department, San Diego, CA), we identified the three motifs in SRp130 conserved in the above four species: motif 1 (residues 11-25) repeated at residues 33-47; motif 2 (residues 224-264) repeated at residues 416-455, and motif 3 (residues 324-362). The presence of these conserved motifs raises the possibility that the N-terminal domain of Pnn has been evolutionarily conserved.

Results of a BLAST search of the human genome database using assembled SRp130 cDNA as a query indicated the presence of a single locus encoding an approximately 25-kb SRp130 gene located on chromosome 6 between 6q22.3-q23.1. The SR-rich C-terminal portion of SRp130 contains two well-conserved, and seven degenerated RRSRSXXS repeats that are characteristic of proteins in the SR family (Fig. 4A). There is no canonical RNA recognition motif within SRp130; therefore, it is classified as an SR-related protein (SRrp). SRp130 exhibits an intriguing homology to another recently identified rat novel SR-rich 86-kDa protein (Fig. 4B). In addition, the SRp130 C-terminus shows similarity to splicing factors belonging to the SR family proteins, such as ASF/SF2, SC35, SRp75, and SRp20 (Lipman-Pearson similarity indexes 35, 31, 29, and 27, respectively). The N-terminal region of SRp130 protein harbors well conserved motifs present in the putative Mus musculus protein (GenBank accession number BAB30779), Caenorhabditis elegans protein (GenBank accession number Y53H1A.1), and the Drosophila melanogaster CG31211 gene product (GenBank accession number AA54719). Using the MEME-motif discovery tool (http://meme.sdsc.edu/meme/website/intro.html; developed by Timothy Bailey, Charles Elkan, and Bill Grundy, UCSD Computer Science and Engineering Department, San Diego, CA), we identified the three motifs in SRp130 conserved in the above four species: motif 1 (residues 11-25) repeated at residues 33-47; motif 2 (residues 224-264) repeated at residues 416-455, and motif 3 (residues 324-362). The presence of these conserved motifs raises the possibility that the N-terminal domain of Pnn has been evolutionarily conserved.

Image 1: Interaction of Pnn with SRp75, SRm300, and SRrp130. The C-terminal portion of Pnn (residues 657-677) and derivatice truncation and deletion Pnn constructs were fused to GAL4-DNA-BD and cotransformed individually with the GAL4-AD-SRm300 (residues 1-278, 129-714, or 1-714), GAL4-AD-SRp75 (residues 117-494), or GAL4-AD-SRrp130 (residues 536-805) fusions into the yeast strain PJ69-4A. Transformants were subjected subsequently to two selection assays, His and Ade. Plates show growth on second selection medium (Ade) after 3 days. Pnn constructs (A) include truncations (1-6) and two deletion mutants: 1A, deletion of the RS repeat from the polyserine/RS domain (construct 465-717Δ625-641); 1B, deletion of the polyserine domain (construct 465-717Δ559-641); and 2A, SR-DRK truncation (Δ66-630). Constructs 2B-2D (not shown on the schematic) are further SR-DRK truncations. (B) Typical growths on selection plates. (C) Tabular presentation of data: +, growth on the plates for each Pnn construct; −, lack of growth; *not shown on plates depicted in (B); and ND, experiments not performed. (D) Schematic representation of the Pnn and SRp75 all coimmunoprecipitated with Pnn. The immunoreactive band migrating around Mr(K) 55 is the heavy chain of the immunoprecipitating antibody.
AL080186) from a database entry contains 1.3-kb additional 3'-untranslated sequence, suggesting the usage of a sixth polyadenylation signal.

SRrp130 protein has a calculated molecular mass of 92.6 kDa. After the transient transfections of the HEK-293 cell line with the HA epitope-tagged SRrp130 construct, Western blot analysis revealed higher molecular weight resolved on SDS-PAGE at approximately 130 kDa, which most likely is due to protein conformation and/or phosphorylation. This shift has consistently been observed in a number of SR-rich proteins including Pnn.26 Based on a PROSITE analysis (http://www.ncbi.nlm.nih.gov/PROSITE/ provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland), the SRrp130 protein bears 11 predicted cAMP- and cGMP-dependent PK phosphorylation sites, 42 PKC phosphorylation sites, and 23 CKII phosphorylation sites.

SRp75, SRm300, and SRrp130 Colocalize with Pnn in the Nuclear Speckles in Corneal Epithelial Cells

Immunostaining of endogenous and expressed proteins (Pnn, SRm300, SRp75, and SRrp130) in the human corneal epithelial cell line HCE-T revealed strong codistribution (Fig. 5). As shown previously,5 most of the expressed Pnn was within nucleus in a diffuse nucleoplasmic and speckled pattern (Figs. 5A–D). Immunostaining with the polyclonal-215 antibody against Pnn coaligned with the nuclear Pnn-GFP, but also revealed Pnn along cell–cell adhesion sites (Figs. 5A, 5C, 5E, 5I, and 5M). The question of why expressed Pnn does not efficiently accumulate at the sites of cell–cell adhesion is currently under investigation.

The expression of exogenous SRm300, SRp75, and SRrp130 altered the distribution of endogenous Pnn, indicative of possible in vivo protein–protein interactions. Transfections of HA epitope-tagged SRm300 (1-700) into HCE-T corneal epithelial cells revealed multiple prominent rounded nuclear speckles (Figs. 5E–H). Endogenous Pnn coaligned with the HA-tagged SRm300 at the rounded nuclear speckles (Figs. 5E, 5F), whereas it remained in a speckled and diffuse nucleoplasmic pattern in the nontransfected cells. Coexpressing both Pnn and SRm300 revealed similar patterns (Figs. 5G, 5H). Transfections with HA-tagged SRp75 resulted in an abundant accumulation of protein throughout the interchromatin space. Endogenous Pnn (Fig. 5I), expressed Pnn (Fig. 5K), and endogenous SRm300 (Fig. 5Q) with expressed SRp75 (Figs. 5J, 5L, 5R) demonstrated a more diffuse nucleoplasmic staining. Transfections with HA-tagged SRrp130 revealed prominent codistribution with Pnn (Figs. 5M–P) and endogenous SRm300 (Figs. 5S, 5T) at the speckles. Taken together, these data suggest that indeed Pnn, SRp75, SRm300, and SRrp130 may interact within HCE-T cells and the overexpression of one component may affect the nuclear distribution of the other moieties.

**DISCUSSION**

In this study, we showed that Pnn interacts with two known SR-rich proteins involved in splicing, SRp75 and SRm300, and also with a new SR-related protein, SRrp130. Pnn was found to...
colocalize with these proteins within the interchromatin space and at nuclear speckles. Detailed Pnn truncation and deletion analyses revealed that the polyserine/SR motif played a central role in maintaining molecular interactions between Pnn and SR-rich proteins. These results show that Pnn C-terminus interactions with three identified SR-rich proteins are complex and unique for each Pnn-binding partner. Although the polyserine/SR domain seems to play a central role, multiple motifs distributed along the C-terminus of Pnn may also be involved. It remains to be established whether Pnn binds to multiple SR-partners at the same time, or whether there is competition for a binding site(s) among these proteins. However, based on the results reported herein and the data implicating Pnn’s function in splicing complexes from other laboratories,8,9,34,35 it is tempting to speculate that Pnn may play a structural and/or functional role in mRNA processing.

Pnn and its binding partners were localized to the nucleus where they exhibited a prominent characteristic speckled and diffuse nucleoplasmic distribution. Speckles are nuclear domains that are highly enriched by splicing factors. It is suggested that speckles are the sites of mRNA processing component storage and/or assembly that supply splicing factors to the actively transcribed genes, as needed.11,34,36,37 Despite intensive studies of the speckled domains, the basis for compartmentalization of splicing factors into such nuclear domains has not been determined. Because of Pnn's ability to bind intermediate filaments through its amino terminus, it is tempting to speculate that Pnn may provide a molecular scaffold or linkage to the putative nuclear matrix. Indeed, many investigators have speculated that protein-based framework is responsible for organization of splicing factors.38,39 It has been demonstrated that some splicing factors associate with the proteinaceous structure of the nuclear speckles.40 Pnn, SRp75, SRm300, and SRrp130 colocalize to, and are partially retained in, nuclear speckles after extraction with detergent, high salt, and DNAase digestion (data not shown) suggesting an association with the nuclear matrix. However, Sacco-Bubulya and Spector35 have recently shown that speckles in living cells are quite dynamic structures. Overexpression of Clk/STY (cdc2-like kinase) induces the disassembly of the speckle and the dispersal of speckle components, including Pnn. Therefore, it is unlikely that Pnn provides a stable structural scaffold for speckle assembly.

**Figure 5.** Immunocolocalization study of Pnn and its SR-rich binding partners. SRm300(1-700), SRp75, and SRrp130 were shown to colocalize with Pnn and SRm300 antigens in the nucleus of HCE-T cells. After the transient transfections with Pnn-GFP, SRm300(1-700)-HA, SRp75-HA, or SRrp130-HA into HCE-T cells, the distributions of specific SR proteins were examined in double-labeling experiments. The localization of SR proteins was determined with the monoclonal anti-HA antibody 16β12, and endogenous Pnn and SRm300 antigens were detected with the polyclonal antibody 215 and anti-SRm300-sera70, respectively. Distribution of Pnn-GFP antigen was detected directly (B, D, G, K, O). SRm300(1-700)-HA (F, H), SRp75-HA (J, L, R), and SRrp130-HA (N, P, T) colocalized with endogenous Pnn (A, C, E, I, M), as well as with the endogenous SRm300 antigen (Q, S). Pnn-GFP coaligns with nuclear endogenous Pnn (A–D). The expression of exogenous SRm300 (E, F) and SRrp130 (M, N) induced a greater accumulation of Pnn at nuclear speckles, whereas SRp75 caused Pnn (I, J) and SRm300 (Q, R) to change to a more diffuse nucleoplasmic pattern. Final magnification, ×750.
Although Pnn does not contain a canonical RNA binding motif, it binds to the splicing factor SRp75, which is a member of the SR family proteins that has well conserved modular RNA-binding motifs and RS domains. SRp75 can complement HeLa cell S100 extract and enhance both constitutive splicing and regulated alternative splicing. 22 In ongoing studies, expression of Pnn in the HEK-293 cell line did not alter the rate of exclusion of the exon 4 of CD45 in the cells transfected with pSV-mini-LCA18. This result, however, does not eliminate the possibility of Pnn’s involvement in constitutive and/or alternative splicing of other substrates. In fact, Wang and colleagues have provided data in support of a role for Pnn in alternative splicing. Another member of the Pnn/SR protein putative complex is SRm300, a nuclear matrix antigen component of the SRm160/300-splicing coactivator that functions in splicing by promoting critical interactions between splicing factors such as snRNP and SR family proteins. 22,41 Although the precise role of SRm300 in splicing is SRm300, a nuclear matrix antigen component of the SRm160/300-splicing coactivator that functions in splicing by promoting critical interactions between splicing factors such as snRNP and SR family proteins, 22 although the precise role of SRm300 in splicing is not yet fully understood, it may influence the nuclear distribution of SRm160 and/or other associated SR proteins and thereby participate in the general functional organization of splicing factors. 22

The amino acid sequence of SRp130 exhibited an RS-rich C-terminus, suggesting that this protein may also be classified as SR-rich protein. The presence of conserved RS motifs tempts speculation that SRp130 interacts with SR-rich proteins and is involved in pre-mRNA biogenesis. In particular, the SRp130 C-terminus shows significant homology to SRp86. This splicing regulatory factor SRp86 is involved in both inhibition and activation of splicing, perhaps by the pair-wise combination of SRp86 with other SR proteins, resulting in altered splicing efficiency and differential splice site selection. 23

These data are now added to the growing body of evidence that indicate the coordination of pre-mRNA processing and transcriptional regulation, including chromatin-remodeling events. It is therefore conceivable that Pnn is associated with central regulatory protein complexes that function in transcriptional regulation and pre-mRNA maturation, or spatial organization of these processes in the nucleus of epithelial cells.

Acknowledgments

The authors thank Lei Zhou of the University of Florida College of Medicine for providing help in using the MEME-motif discovery tool, Marguerite Hunt for technical assistance.

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