Protein phosphatase 4 interacts with the Survival of Motor Neurons complex and enhances the temporal localisation of snRNPs

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Summary
Protein phosphatase 4 (PPP4) is a ubiquitous essential protein serine/threonine phosphatase found in higher eukaryotes. Coordinate variation of the levels of the catalytic subunit (PPP4c) and the regulatory subunit (R2) suggests that PPP4c and R2 form a heterodimeric core to which other regulatory subunits bind. Two proteins that specifically co-purify with Flag-epitope-tagged R2 expressed in HEK-293 cells were identified as Gemin3 and Gemin4. These two proteins have been identified previously as components of the Survival of Motor Neurons (SMN) protein complex, which is functionally defective in the hereditary disorder spinal muscular atrophy. Immunosedimentation of the epitope-tagged SMN protein complex from HeLa cells expressing CFP-SMN showed that the SMN protein interacts, as previously reported, with Gemin2 (SIP1), Gemin3 and Gemin4 and in addition associates with PPP4c. The SMN complex has been implicated in the assembly and maturation of small nuclear ribonucleoproteins (snRNPs). Expression of GFP-R2–PPP4c in HeLa cells enhances the temporal localisation of newly formed snRNPs, which is consistent with an association of R2-PPP4c with the SMN protein complex.

Key words: Gemin4, Gemin3, Spinal muscular atrophy, Spliceosome, Cajal bodies (coiled bodies), snRNP

Introduction
Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease characterised by progressive loss of motor neurons from the anterior horn of the spinal cord, resulting in paralysis and severe muscular atrophy (Melki, 1997). It is one of the leading hereditary causes of infant mortality (Pearn, 1978). Two Survival of Motor Neurons (SMN) genes are located in an inverted repeat on human chromosome 5 at q13, and SMA arises from deletions or mutations in the more highly expressed gene SMNI. The SMN protein (38 kDa), which can self oligomerize, is associated with other proteins. An SMN ‘core’ protein complex (~300 kDa) comprises several tightly associated proteins including the SMN protein Gemin2 (formerly called SIP1), Gemin3, Gemin4, Sm proteins and several proteins yet to be characterised (Charroux et al., 2000; Charroux et al., 1999; Liu et al., 1997; Meister et al., 2000). Additional proteins may associate with the SMN core complex, and it has been suggested that multiple SMN complexes may exist in vivo (Pellizzoni et al., 2001b).

SMN is expressed in all tissues and localises both in the nucleus and the cytoplasm. In the nucleus of most cultured cells and primary neurons, SMN and Gemin2 localise in the Cajal (also termed coiled) bodies (Carvalho et al., 1999; Matera, 1999; Sleeman and Lamond, 1999). These bodies are discrete nuclear structures that are known to contain components such as small nuclear ribonucleoproteins (snRNPs), small nucleolar ribonucleoproteins (snoRNPs) and transcription factors, which suggest that Cajal bodies have a role in snRNP and snoRNP biogenesis and metabolism. In a small proportion of rapidly proliferating cells in some cell lines in culture, SMN localises in discrete foci, often near the Cajal bodies called gems (gemini of coiled bodies) (Liu and Dreyfuss, 1996; Liu et al., 1997).

Several studies implicate SMN complexes in spliceosome assembly and regeneration of splicedosomal components. In the cytoplasm, the SMN complex appears to be involved in the binding of small nuclear uridine-rich (U) RNAs to Sm proteins for the assembly of spliceosomal snRNPs (Bühler et al., 1999; Fischer et al., 1997; Meister et al., 2000). The SMN complex is then believed to accompany the assembled snRNPs to the Cajal bodies in the nucleus, where it might be involved in the generation of active spliceosomes and the recycling of snRNPs after each round of pre-mRNA splicing (Matera, 1999; Pellizzoni et al., 1998). An SMN complex has also been implicated in the assembly and metabolism of snoRNPs required for pre-rRNA splicing (Pellizzoni et al., 2001a). A recent study indicates that an SMN complex may play a central role in the assembly of transcriptomes (Pellizzoni et al., 2001b), large complexes containing RNA polymerase I, II or III, transcription factors and spliceosomes for the coordinated synthesis and processing of mRNA and snRNA (Gall et al., 1999). The interaction between the SMN protein and RNA polymerase II was shown to be mediated by RNA helicase A,
which although not a ‘core’ component of the SMN complex, may be present in a subset of SMN complexes and is suggested to be a substrate of the SMN complex (Pelizzoni et al., 2001b). Interestingly, hnRNP-R, a protein that is involved in RNA processing, is predominantly expressed in the axons of motor neurons and interacts with SMN (Rossoll et al., 2002). To date there is no information on how the functions of the SMN complex are regulated or coordinated with other cellular functions.

Reversible protein phosphorylation is a key mechanism for the control of cellular processes in eukaryotes. PPP4 (originally termed PPX) is a ubiquitous protein phosphatase that dephosphorylates serine and threonine residues (Cohen, 1997). The catalytic subunit, PPP4c, is very highly conserved from mammals to Drosophila (91% amino acid identity) (Brewis and Cohen, 1992) and production of a D. melanogaster mutant deficient in PPP4c has demonstrated that this phosphatase is essential for the nucleation, growth and/or organisation of microtubules at centrosomes (Helps et al., 1998). Analysis of the homologue in Caenorhabditis elegans by RNA-mediated interference showed that PPP4c is also essential for formation of the mitotic spindle in mitosis and is required for sperm meiosis (Sumiyoshi et al., 2002). In accordance with these data, PPP4c exhibits a prominent localisation at centrosomes in cultured mammalian cells (Brewis et al., 1993). However, its high expression in the nucleus with weak expression in the cytoplasm suggests that PPP4c regulates additional cellular functions.

PPP4c is a member of the PPP family of protein phosphatases and is most closely related to PP2Ac (~65% amino acid identity) and PPP6c (~60% identity), with less similarity to PP1 (~45% identity). The PPP2A holoenzyme comprises a heterodimeric ‘core’ of PPP2Ac complexed to an A (PR65) regulatory subunit, and this dimer may then bind to one of a number of different B regulatory subunits (Janssens and Goris, 2001). However, PPP1c forms heterodimeric holoenzymes complexes in which the catalytic subunit is bound to one of more than 40 distinct regulatory subunits (Cohen, 2002). Interaction occurs via a short conserved motif that is present in many of the regulatory subunits, and the different regulatory subunits may target PPP1c to different subcellular locations. Sit4p, the S. cerevisiae homologue of mammalian PPP6c, also exists as heterodimeric complexes containing one variable subunit (Luke et al., 1996).

PPP4 exists as high molecular mass complexes of 450-600 kDa, and two putative regulatory subunits have been identified, R1 (Kloeker and Wadzinski, 1999) and R2 (Hastie et al., 2000). It is present in many of the regulatory subunits, and the different regulatory subunits may target PPP4c to different subcellular locations. Sit4p, the S. cerevisiae homologue of mammalian PPP6c, also exists as heterodimeric complexes containing one variable subunit (Luke et al., 1996).

PPP4 exists as high molecular mass complexes of 450-600 kDa, and two putative regulatory subunits have been identified, R1 (Kloeker and Wadzinski, 1999) and R2 (Hastie et al., 2000). However, the subunit composition of higher molecular mass complexes of PPP4 is unclear, and the structures of R1 and R2 regulatory subunits do not provide any additional information on the location or function of these PPP4 complexes. Here, we examine the PPP4 complexes that contain R2 and identify as novel ‘variable’ regulatory subunit(s) Gemin3 and/or Gemin4, which are components of the SMN complex. We also present data suggesting that R2-PPP4c enhances the maturation of snRNP, a function in which the SMN complex is implicated.

Materials and Methods
Preparation of expression constructs
Construction of the expression vector, Flag-R2 in pCMV5, was described previously (Hastie et al., 2000). To construct the pEGFP-R2 expression vector, DNA encoding R2 was amplified by PCR using R2 template DNA and oligonucleotides 5'-GCGGATCCGGCCACCA-TGGACTACAAGGACGACGA TGACAAGTGCCAGGCGCCA TGT-GG-3' and 5'-GCGTCGACTCAGAAGAACCACTTACTTACCAAGGCACCCAGGGCC-3' to incorporate a BamHI site and FLAG tag (DYKDDDK) at the 5' end and a SalI site at the 3' end. The BamHI/SalI fragment was ligated into pCR2.1 TOPO (Invitrogen) and then subcloned into the same sites in pEGFP-C1 (Clontech) to produce the plasmid pEGFP-R2. DNA encoding PPP4c was amplified by PCR using PPP4c as the template DNA and oligonucleotides 5'-GAATTCCGCCACCATGACCACTACGATGTCGACGTTGAGATCACGCCACCCAGGGCC-3' and 5'-GGATCTGACCTAGAAGAACCACTTACTTACCAAGGCACCCAGGGCC-3' to incorporate an EcoRI site and HA tag (YPYDVPDYA) at the 5' end and a BamHI site at the 3' end. The EcoRI/BamHI fragment was ligated into pCR2.1 TOPO and then subcloned into the same sites of pCMV5 to produce plasmid pCMV5-PPP4c. cDNA for the complete coding region of Gemin4 was obtained by PCR amplification using a human multi-tissue cDNA panel (Clontech, Palo Alto, CA) as template and primers corresponding to the known 5' initiating coding sequence (5'-ATGACCTAGAAGAACCACTTACTTACCAAGGCACCCAGGGCC-3') and 3' stop codon region (5'-TCAGAAGACTCAGTCTATCTGTGAACGGA-3'). The resulting 3472 bp cDNA fragment was ligated into pcDNA4/HisMax-TOPO to produce a construct of Gemin4 epitope tagged with Xpress (DLYDDDK) at the N-terminus (Invitrogen, Groningen, Netherlands). Constructs were verified by sequencing prior to transfection or injection in human cells. Sequence analysis was performed on an Applied Biosystems 373A automated DNA sequencer using Taq dye terminator cycle sequencing by the University of Dundee DNA sequencing service managed by Nick Helps (www.dnaseq.co.uk).

Fluorescence in situ hybridisation
The Vysis Nick Translation Kit was used to directly label purified R2 cDNA or BAC DNA with the fluorochrome Spectrum Red as described in the manufacturer’s protocol. The labelled probe was used for hybridisation of metaphase chromosome spreads.

Screening of LLNL human single chromosome libraries
Chromosome 3 and 5 libraries, constructed in the cosmid Lawrist 16 at Lawrence Livermore National Laboratory, USA, were kindly provided by the UK HGMP Resource Centre in the form of high-density gridded filters. The libraries had an average insert size of 30-50 kb and contained approximately 20,000 clones providing a three-to-four-fold coverage of each chromosome. The filters were screened by Southern blotting, using the R2 coding region cDNA (~0.8 kb) as a probe. Positive clones, kindly provided by the HGMP, were analysed using PCR with R2 specific primers or Topo shotgun cloning (Invitrogen, Groningen, Netherlands). Three chromosome 5 R2 positive clones were sequenced in both directions.

RNA analyses
Northern blots (Clontech, Palo Alto, CA) contained approximately 2 µg poly (A)+ RNA from different tissues. The blots were hybridised with R2 and PPP4c probes according to the manufacturer’s instructions, with the last wash in 15 mM NaCl/1.5 mM sodium citrate/0.1% SDS at 55°C. Following autoradiography, blots were stripped by washing the membrane in 0.5% SDS at 100°C for 5 minutes and subsequently reprobed with a control β-actin probe.

Cell culture, transfection and preparation of lysates
Human embryonic kidney (HEK) 293 cells were cultured, transfected
PPP4 interacts with the SMN complex

1907

PPP4 interacts with the SMN complex and lysed as described previously (Hastie et al., 2000). HeLa cells expressing cyan fluorescent protein tagged SMN protein (Sleeman et al., 2003) were cultured and lysed as described previously (Hastie et al., 2000).

Purification of Flag-R2–PPP4c complexes

20-100 10 cm² dishes of 293 cells were transfected with pCMV5-Flag-R2 and cultured for 44 hours prior to lysis in buffer A (50 mM Tris/HCl pH 7.5, 0.03% Brij-35, 2 mM EDTA, 0.1 mM EGTA) plus 5% glycerol, 0.15 M NaCl and ‘Complete’ protease inhibitor cocktail (Roche Diagnostics Ltd, Lewes, UK). The lysate was mixed with 5 ml of anti-Flag agarose (Sigma, Poole, UK) in a tube by end over end rotation for 1 hour at 4°C. The agarose beads were separated by centrifugation and washed in buffer A containing 5% glycerol and 500 mM NaCl several times for 1 hour at 4°C. Bound material was eluted from the anti-Flag agarose column by the addition of 20 ml Flag peptide (100 μg/ml) and collected in 20 x 1 ml fractions. These fractions were analysed for co-elution of Flag-R2 and PPP4c. Peak fractions were then pooled, concentrated and desalted in buffer A using a Vivaspin column (Vivascience, Lincoln, UK) prior to further study. Gel filtration analysis of the purified Flag-R2–PPP4c material on Superose 6 columns was performed as described previously (Hastie et al., 2000). Molecular mass marker proteins used were thyroglobulin (670 kDa), ferritin (450 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa).

Identification of proteins co-eluting with Flag-R2–PPP4c

Purified Flag-R2–PPP4c material was fractionated by SDS-PAGE and stained with Coomassie Blue. Proteins that consistently co-eluted with Flag-R2 and PPP4c were excised from the gel and digested in situ with trypsin as described previously (Jensen et al., 1997). Tryptic peptide masses were analysed using a thin film matrix of 4-hydroxy-α-cyanocinnamic acid/nitrocellulose (2:1) in an Elite STR mass spectrometer (PerSeptive Biosystems, Foster City, CA, USA) in reflectron mode. Spectra were internally calibrated with matrix and trypsin autolysis ions and the peptide masses were used to search databases within the UCSF Protein Prospector program using MS-FIT analysis software with a mass error set at 50 ppm. Flag-R2 and PPP4c were initially identified by immunoblotting but were subsequently verified by identification using mass spectrometry.

Immunological techniques

Immunoblotting was performed following fractionation of proteins by SDS/PAGE, using NuPage 4-12% Bis-Tris gels (Invitrogen, Groningen, Netherlands), and transfer to nitrocellulose membranes (Shleicher and Schull, Dassel, Germany). The blots were probed with affinity-purified antibodies, and antibody binding was detected using anti-sheep IgG antibodies conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (Amersham International, Little Chalfont, UK). Anti-PPP4c antibodies were raised against the N-terminal 57 amino acids of human PPP4c (Brewis et al., 1993). Anti-Gemin4 antibodies were made against the peptide AEGIGPEERRQTLQKMSF (corresponding to amino acids 1039-1059 of Gemin4) and coupled to keyhole limpet haemocyanin. The above antibodies were raised in sheep at the Scottish Antibody Production Unit, Carluke, Penicuik, Midlothian, UK and affinity purified. Murine anti-Gemin3 antibodies were purchased from BD Transduction Laboratories (Lexington, KY, USA) and murine anti-Flag antibodies were purchased from Sigma. Anti-GFP and anti-Xpress antibodies were purchased from Invitrogen (De Schelp, Netherlands). Anti-sheep and anti-mouse secondary antibodies were from Pierce (Chester, UK).

Immunoprecipitation of tagged proteins expressed in mammalian cells was carried out using lysates containing 1 mg of total cell protein for each immunoprecipitation. The lysates were preclared by incubation at 4°C for 50 minutes on a shaking platform with sheep premucous IgG covalently coupled to protein G-Sepharose using dimethylpimelimidate (Harlow and Lane, 1988). Following centrifugation for 1 minute at 16,000 g, the supernatant was removed and incubated for 1 hour as above, with 5 μg antibody coupled to 10


Microinjection of expression plasmids into HeLa cells

Plasmid DNAs were diluted to 15 μg/ml with 100 mM glutamic acid (titrated to pH 7.2 with citric acid), 140 mM KOH, 1 mM MgSO4 and 1 mM DTT and injected into HeLa cells using an Eppendorf 5242 microinjector. Cells were cultured at 37°C in Dulbecco’s modified Eagles’ medium. Two hours after injection, cells were washed in phosphate-buffered saline, fixed for 5 minutes at 37°C in 3.7% (w/v) paraformaldehyde in 60 mM PIPES, 27 mM HEPES, 10 mM EGTA, 4 mM MgSO4 titrated to pH 7.0 with 10 M KOH. Permeabilisation was performed with 1% Triton in PBS for 15 minutes at room temperature.

Mouse anti-Flag and rabbit anti-HA monoclonal antibodies (clone 12CA5) were from Roche Diagnostics Ltd (Lewes, UK). Secondary anti-mouse IgG antibodies conjugated to Cy3 were from Jackson Immuno Research Labs, Inc. (West Grove, PA). Immunofluorescence was detected using a Zeiss DeltaVision Restoration microscope (Applied Precision Inc.) equipped with a 3D motorised stage and a 100× NA 1.4 Plan-Apochromat objective. Optical sections separated by 200 nm were recorded and images were restored using an iterative deconvolution algorithm. For EGFP, EYFP, Cy3 and DAPI excitation wavelengths were 460 nm (bandwidth 20 nm), 523 nm (20 nm) 555 nm (28 nm), 360 nm (40 nm) and emission wavelengths 500 nm (73 nm), 457 nm (73 nm), 457 nm (73 nm), respectively.

Results

PPP4R2 may be a ‘core’ regulatory subunit of PPP4

To determine whether the regulatory subunit, R2, of PPP4 was a variable subunit participating in only some functions of PPP4 or a ‘core’ subunit that formed a complex with PPP4c in virtually all PPP4 complexes (analogous to the PR65 subunit of PP2A), the expression of R2 mRNA was examined in a range of human tissues. Fig. 1 shows that there are two transcripts of 5.6 kb and 3.6 kb for human R2 and that although their relative level differs in some tissues (for example, in testis and ovary), their combined level of expression in different tissues appears to vary coordinately with the level of expression of the 1.8 kb PPP4c mRNA. Expression of R2 mRNA and PPP4c mRNA are both very high in testis in accordance with earlier studies for PPP4c mRNA and protein (Brewis et al., 1993; Kloeker et al., 1997). These data suggest that R2 is more likely to be a ‘core’ regulatory subunit than a variable regulatory subunit that might be expected to show differential expression from the catalytic subunit in some tissues. Previous studies also support this concept. R2 (calculated molecular mass 50.4 kDa, mobility on SDS gels 65 kDa) was isolated from porcine testis in a 1:1 complex as judged by protein staining (Hastie et al., 2000). In addition, purified native PPP4c was shown to interact with purified His-tagged R2 expressed from a baculovirus vector in insect cells. Since the molecular mass of the His-tagged R2 measured by glycerol density-gradient sedimentation was 96 kDa these studies suggest that the R2-PPP4c complex is a tetramer of two R2 subunits and two PPP4c subunits. Interestingly His-tagged R2 and the R2-PPP4c complex elute from gel filtration columns at an apparent size of ~450 kDa. These studies indicate that the R2 dimer is a highly asymmetric molecule.

Five genes encoding R2, located on chromosomes 3 and 5, are found in the NCBI Homo sapiens genomic contig sequences database. The R2 gene located at 3p14-2 is probably the functional gene, as its sequence (NT_005561 / Hs3.3_5683) is virtually identical to that of the cDNA (Accession No. AJ 271448) (Hastie et al., 2000) and other R2 partial cDNA sequences in the human databases. In addition, this R2 gene contains nine introns and is spread over more than 40 kb. Two other R2 genes (NT_022440.4/ Hs3.3_22596 and NT_015983.5/ Hs3.3_16139) with >98% DNA coding sequence identity, each containing only the most 3’ intron are located at 3q28. Two more R2 genes (NT_029228.1/Hs5.7_29447 and NT_006611.5/ Hs5.7_6768) with >96% DNA coding sequence identity, each containing only the most 3’ intron are located at 5p15.5. Each pair of genes at 3q28 and 5p15.5 have very similar 5’ and 3’ UTR sequences, suggesting that they are likely to have arisen by gene duplication. Isolation of a BAC clone carrying R2 complex elutes from gel filtration columns at an apparent size of ~450 kDa. These studies indicate that the R2 dimer is a highly asymmetric molecule.
genes located on chromosome 5 allowed mapping of R2 genes to 3(q27-qter) and 5p15.5 (kindly performed by Mark Sales, Dundee, UK), in accordance with the data in the genome database. No genomic clones encoding R2 were identified in the LLNL human single chromosome 3 library, and the high identity (>90%) of the 3’UTR sequences of all the R2 genes prevented ascertainment of whether the two R2 mRNAs (Fig. 1) are derived from the gene at 3p14-21 as predicted.

Identification of proteins interacting with PPP4c and PPP4R2

Following transfection of cDNA encoding the Flag-tagged regulatory subunit R2 of PPP4c into HEK 293 cells, proteins co-sedimenting with anti-Flag agarose beads in the cell lysate were examined. Fig. 2 shows the co-elution of Flag-R2 and PPP4c from an anti-Flag agarose column. Use of the Flag peptide for elution minimises the detachment of proteins bound non-specifically to agarose. Analysis of the eluted Flag-R2-PPP4c material by gel filtration on a Superose 6 column indicated that the purified Flag-R2-PPP4c material comprises high molecular mass complexes, ranging from 450 kDa to >670 kDa (Fig. 3). A large proportion of this material eluted at 450 kDa, a size identical to that of the complex formed with purified free PPP4c and baculovirus-expressed R2 in previous experiments (Hastie et al., 2000). Therefore the pool of protein eluting at 450 kDa is likely to be a complex of Flag-R2 and PPP4c. There is also further material eluting at a much higher molecular mass (approximately 800 kDa-450 kDa), which is likely to represent Flag-R2-PPP4c complexes with other proteins.

Fig. 4 shows a Coomassie-Blue-stained gel of material eluted from the anti-Flag agarose column. Following excision of protein bands and trypptic digestion, the proteins were identified by mass spectrometry and comparison of the peptide ions generated with sequences of previously characterised proteins in the UCSF Protein Prospector database. Along with Flag-R2 (with 21 peptides matched, giving 60% sequence coverage) and PPP4c (19 peptides matched, giving 65% sequence coverage) other proteins that were identified are Gemin4 (with 35 peptides matched, giving 40% sequence coverage) and Gemin3 (with 20 peptides matched, giving 33% sequence coverage). Both α- and β-tubulins were also identified, which may be relevant to the function of R2-PP4c at the centrosomes (Helps et al., 1998; Sumiyoshi et al., 2002).

Gemin3 and Gemin4 have recently been identified in a number of studies analysing the SMN protein complex (Campbell et al., 2000; Charroux et al., 2000; Charroux et al., 1999). On the basis of amino-acid sequence alignments, Gemin3 is thought to be a member of the DEAD box family of putative ATP-dependent RNA helicases and is also termed DEAd-box protein103 kDa (DP103). The name ‘DEAD’ is derived from the single amino code for a conserved motif, Asp-Glu-Ala-Asp, which confers ATPase activity. Gemin3 (824 amino acids) has been detected in cellular lysates by SDS-PAGE as a 103 kDa protein, although the calculated molecular mass from the cDNA is 92.2 kDa, in agreement with the size of 92.5 kDa determined on SDS-PAGE after in vitro transcription/translation (Charroux et al., 1999; Grundhoff et al., 1999). Gemin4, also termed Gemin3-interacting protein 1 (GIP1) comprises 1058 amino acids and has a calculated molecular mass of 119.9 kDa but has an apparent molecular mass on SDS-PAGE of 97 kDa (Charroux et al., 2000). No functional or conserved domains of Gemin4 have been identified, but it has been shown to interact directly with Gemin3 and is thought to be a component of the SMN complex via this interaction.

Co-elution of Gemin3 and Gemin4 from the anti-Flag agarose column with Flag-R2-PPP4c was readily reproducible; therefore peptide antibodies were raised against these two proteins. The antibodies recognised a major band of Gemin3 at ~94 kDa and a major band of Gemin4 at ~97 kDa on immunoblots of Flag-R2-PPP4c-interacting proteins (data not shown) and HEK 293 cell lysates (Fig. 5), which validates the
PPP4c (D) antibodies. Xpress (right hand side 3 lanes) (A), anti-Gemin3 (B), anti-R2 (C) and anti-

Subsequent immunoblotting with anti-Gemin4 (left hand side 3 lanes), anti-

recovered from 500 µl) fractions were analysed by SDS-PAGE and subsequent immunoblotting with anti-Gemin4 (left hand side 3 lanes), anti-Xpress (right hand side 3 lanes) (A), anti-Gemin3 (B), anti-R2 (C) and anti-PPP4c (D) antibodies.

Identification of these proteins by mass spectrometry (Fig. 4). Although the protein methylase JBP1 was also found co-eluting with Flag-R2–PPP4c and methylation of proteins is known to be important in assembly of SMN complexes (Friesen et al., 2001; Meister et al., 2001), it is presently unclear whether this is a specific interaction, because JBP1 has also been found in the eluates with other Flag-tagged proteins (G.K.C., N.M. and P.T.W.C., unpublished).

The anti-Gemin peptide antibodies did not immunoprecipitate endogenous Gemin3 or Gemin4. Therefore cDNA encoding Gemin4 was obtained by screening a human multi-tissue cDNA panel using PCR. Following ligation of the Gemin4 cDNA into an expression vector and transfection into HEK 293 cells, a reciprocal immunoprecipitation experiment was carried out using lystate from HEK 293 cells expressing epitope-tagged Gemin4. Fig. 6 shows the co-immunoprecipitation of Gemin4 with endogenous Gemin3 (a positive control), R2 and PPP4c. This suggests that the interaction between R2-PPP4c and Gemin4 and/or Gemin3 may be a specific interaction that occurs in vivo.

Co-immunoprecipitation of PPP4c with the SMN complex
Since Gemin3 and Gemin4 have both been recently identified as components of the SMN protein complex, it was important to determine whether R2-PPP4c interacted with these proteins when they were part of the SMN protein complex. Therefore lysates from a HeLa cell line expressing the SMN protein with a cyan fluorescent protein (CFP)-epitope tag (Sleeman et al., 2003) were subjected to immunoadsorption using anti-GFP antibodies (which readily cross react with CFP) and analysed for co-sedimentation of known components of the SMN complex and PPP4c. The results show that the SMN protein fused to the 27 kDa CFP tag forms a complex with Gemin2, Gemin3, Gemin4, as previously described, and in addition with PPP4c (Fig. 7). Control immunoprecipitates from lysates of HeLa cells transfected with GFP showed no Gemin2, Gemin3, Gemin4 or PPP4c. These results indicate that PPP4 interacts with the SMN protein complex and points to a novel role for this phosphatase associated with the function of the SMN complex.

Analysis of snRNP localisation in the presence of the R2-PPP4c complex
Newly formed snRNPs can be detected by injecting plasmids into cultured cells capable of expressing Sm proteins tagged with a fluorescent label, and the maturation of the snRNPs can be monitored by observing temporal changes in the fluorescent pattern (Sleeman and Lamond, 1999). To determine whether the R2-PPP4c complex has an effect on the pathway of entry of new snRNPs into the nucleus and/or their subsequent movements, plasmid DNAs capable of expressing YFP-SmB, GFP-R2 and/or HA-PPP4c were injected into HeLa cells. Of the 200-250 cells injected for each condition approximately one third could be visualised and scored clearly. Two hours after injection, cells expressing the plasmid encoding YFP-SmB alone showed a YFP-SmB signal in Cajal bodies in 92% of cells (Fig. 8E) and a signal in the nucleoli as well as the Cajal bodies in only 8% of cells, in accordance with previous studies (Sleeman and Lamond, 1999). In contrast, cells expressing all three tagged proteins showed marked accumulation of YFP-SmB-labelled snRNPs within the nucleoli and Cajal bodies in 89% of cells (Fig. 8B) and a YFP-SmB signal in Cajal bodies but not the nucleoli in 11% of cells. Nucleolar localisation is not usually seen in the majority of cells on expression of YFP-SmB alone until later time points (3 to 7 hours). Cells expressing YFP-SmB and GFP-R2 in the absence of HA-PPP4c (Fig. 8H) or YFP-SmB and HA-PPP4c in the absence of GFP-R2 (Fig. 8K) showed the normal localisation of YFP-SmB to Cajal bodies at this time point. The experiments gave the same results on three separate occasions. The signal intensities for YFP-SmB and GFP-R2 were similar and the filters used eliminated cross-talk between the YFP and GFP channels. These studies demonstrate that the R2-PPP4c complex modifies the localisation of newly formed snRNPs. Furthermore, HA-PPP4c and GFP-R2 co-localised with YFP-SmB in the nucleolus [Fig. 8A-C and merged images (data not shown)]. Since it is possible snRNPs are continuously cycling through the Cajal bodies and nucleoli and R2-PPP4c blocks their exit from a nucleolar localisation, a late time point was examined, which showed that YFP-SmB exhibited a ‘mature’ localisation, being present in Cajal bodies and interchromatin granule clusters (speckles) within the nucleoplasm in ~80% of cells expressing YFP-SmB, GFP-R2 and HA-PPP4c as seen for those expressing YFP-SmB alone. Thus the R2-PPP4c
PPP4 interacts with the SMN complex

Gemin4 from cell lysates supports the validity of the interaction. However, as Gemin4 interacts with Gemin3 (Charroux et al., 2000; Meister et al., 2000), any one (or both) of these Gemins may be binding directly with R2-PPP4c. The indications that Gemin4 may be a cofactor required for Gemin3 RNA helicase activity (Charroux et al., 2000; Meister et al., 2000) raise the possibility that Gemin4 may directly interact with R2-PPP4c and target this protein phosphatase to Gemin3 and/or its associated proteins.

RNA helicases have been implicated in nearly all processes that are linked to RNA metabolism, such as translation initiation, pre-mRNA splicing, ribosome assembly and mRNA stabilisation and transport (Linder, 2000). Gemin3 was identified as a nuclear phosphoprotein interacting with Epstein-Barr virus encoded antigens EBNA2 and EBNA3C, both of which form a complex with the cellular transcription factor RBP-Jk and thereby modulate the expression of target genes (Grundhoff et al., 1999). Independent studies detected Gemin3 as a component of the SMN protein complex interacting directly with the SMN protein (Campbell et al., 2000; Charroux et al., 1999). Since the Sm proteins bind to the SMN complex, the helicase activity of Gemin3 may be required for the U RNAs to bind to the Sm proteins for the assembly of snRNPs.

Gemin4 does not interact directly with the SMN complex and its presence in the SMN complex is probably the result of its direct and stable interaction with Gemin3 (Charroux et al., 2000). Gemin4, the SMN protein and Gemin3 associate with U1 and U5 RNAs in the cytoplasm of Xenopus oocytes, but not after these RNAs have been assembled into snRNPs and imported into the nucleus. Therefore it is thought that the SMN complex containing Gemin4 dissociates from the spliceosomal snRNPs either immediately before or shortly after nuclear entry. Nevertheless Gemin4 is found in the nucleus, colocalising with SMN in Cajal bodies and is also detected in nucleoli (Carvalho et al., 1999; Charroux et al., 2000; Sleeman et al., 2001). Our studies show that R2 and PPP4c are found predominantly in the nucleus and are also detected in nucleolar accumulations (Fig. 8), localisations that are consistent with interaction of this phosphatase with Gemin4 and association with the SMN complex.

The R2-PPP4c complex influences the temporal localisation of newly formed snRNPs in the nucleus

SnRNAs are transcribed in the nucleus and transported to the cytoplasm where Sm proteins (a group of seven polypeptides termed B/B', D1, D2, D3, E, F and G) bind, and the 5' end of the snRNA is modified to form the characteristic trimethylguanosine cap (Fischer et al., 1993; Hamm et al., 1990; Lührmann et al., 1990; Mattaj, 1986; Nagai and Mattaj, 1994). The newly formed snRNPs are then imported into the nucleus. The fully mature snRNPs also contain snRNP-specific proteins and numerous base and sugar modifications of the snRNA but the location(s) where these modifications occur is

Fig. 7. Co-immunoprecipitation of PPP4c with proteins previously identified in the SMN protein complex. HeLa cells expressing CFP-SMN were used for immunoadsorption of SMN and HeLa cells transfected with vector expressing GFP were used in controls. Supernatant (S) and pellet (P) fractions were obtained by centrifugation following immunoadsorption from cell lysates (L) using anti-GFP-Sepharose (protein G), which readily interacts with both CFP and GFP. Proteins in the lysate (10 µl), supernatant (10 µl) and pellet (recovered from 500 µl) fractions were analysed by SDS-PAGE and subsequent immunoblotting with anti-GFP (A), anti-Gemin2 (B), anti-Gemin3 (C), anti-Gemin4 (D) and anti-PPP4c (E) antibodies. The 50 kDa bands in the pellet fractions of A are caused by the presence of large amounts of the antibody heavy chain.

Discussion
The PPP4c-R2 complex interacts with Gemin4 and/or Gemin3 in the SMN complex
The studies described here show that the Ser/Thr protein phosphatase PPP4c associates with the SMN complex and that PPP4c in complex with its regulatory subunit R2 binds to Gemin4 and/or Gemin3. PPP4c and R2 are likely to form a dimeric ‘core’ complex to which variable regulatory subunits bind, forming holoenzyme structures similar to those of PP2A. The identification of Gemin3 and Gemin4 in R2-PPP4c immune pellets suggest that one or both may be variable subunit(s) interacting directly with the R2-PPP4c dimeric ‘core’ and targeting it to a specific function. Reciprocal precipitation of PPP4c, R2 and Gemin3 with epitope-tagged complex does not block the progress of YFP-SmB and the overall results indicate that R2-PPP4c enhances the movement of newly formed snRNPs through the first stages of their normal maturation pathway.
unclear. Newly assembled snRNPs show a characteristic temporal sequence of localisation patterns on their initial import into the nucleus, which can be followed using fluorescently labelled Sm proteins (Sleeman et al., 2001; Sleeman and Lamond, 1999). At early time points following injection (1-2 hours) of expression plasmids encoding GFP- or YFP-tagged Sm proteins, new snRNPs are detected as a diffuse pool throughout the cell, with accumulation also seen in nuclear Cajal bodies containing SMN. Later (between 3 and 7 hours following injection), new snRNPs show nucleolar accumulation in addition to accumulation in Cajal bodies. Only at later time points (7 hours onwards) do the majority of cells show the mature pattern of snRNP localisation to Cajal bodies and interchromatin granule clusters (speckles). The R2-PPP4c complex alters this temporal sequence of localisation, with the 3-7 hour pattern being observed 2 hours after injection, results that are consistent with the R2-PPP4c complex playing a role in enhancing the movement of snRNPs through the first stages of their normal maturation pathway.

There are many points at which PPP4 might regulate the snRNP maturation pathway (Gall, 2000). Since PPP4c is present at low levels in the cytoplasm, it is possible that PPP4 could regulate its helicase activity by dephosphorylation, which may modulate the assembly of snRNPs. Alternatively, dephosphorylation of Gemin3 or another SMN component may be a necessary prerequisite for the binding of the snRNPs to nuclear importers. The movement and/or processing of snRNPs as they flow through the Cajal bodies and enter the nucleolus may be subject to regulation by PPP4. It is also possible that PPP4 may participate in the nuclear SMN function(s) of recruiting components to the spliceosomes and transcriptosomes and regenerating snRNPs after pre-mRNA splicing. It will be important to ascertain whether PPP4 is associated with a particular SMN complex found either in the cytoplasm and/or the nucleus or, like many protein phosphatases, participates in the regulation of several cellular processes by interacting transiently with several SMN complexes. Although we consistently found Gemin3 and Gemin4 in the R2-PPP4c immunoprecipitates, we did not find SMN or Gemin 2. This data suggest that Gemin3-Gemin4-R2-PPP4c may possess different functions to that interacting with the SMN complex. In this respect it will be interesting to know whether the viral EBNA2 and EBNA3C proteins modulate transcription by binding to Gemin3 within the SMN complex or to a different pool of Gemin3. The nuclear transcriptional activator E2 of papillomavirus has been shown to modulate transcription through an interaction with the SMN complex (Strasswimmer et al., 1999). Further experiments are being undertaken to delineate the precise role(s) of Gemin3-Gemin4-R2-PPP4c complexes in relation to SMN and the assembly of complexes involved in pre-mRNA splicing, pre-rRNA splicing and transcription.

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