Nuclear PtdIns(4,5)P_2 assembles in a mitotically regulated particle involved in pre-mRNA splicing

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SUMMARY

Phosphoinositide turnover regulates multiple cellular processes. Compared with their well-known cytosolic roles, limited information is available on the functions of nuclear phosphoinositides. Here, we show that phosphatidylinositol(4,5)-bisphosphate (PtdIns(4,5)P_2) stably associates with electron-dense particles within the nucleus that resemble interchromatin granule clusters. These PtdIns(4,5)P_2-containing structures have a distribution which is cell-cycle dependent and contain components of both the transcriptional and pre-mRNA processing machinery, including RNA polymerase II and the splicing factor SC-35. Immunodepletion and add-back experiments demonstrate that PtdIns(4,5)P_2 and associated factors are necessary but not sufficient for pre-mRNA splicing in vitro, indicating a crucial role for PtdIns(4,5)P_2-containing complexes in nuclear pre-mRNA processing.

Key words: Mitosis, Phosphatidylinositol(4,5)-bisphosphate, RNA polymerase II

INTRODUCTION

Phosphorylated products of phosphatidylinositol play a pivotal role in several physiological processes in the cytoplasm, ranging from signalling via the generation of the second messengers inositol(1,4,5)-trisphosphate (InsP_3) and diacylglycerol (DAG), to the regulation of membrane trafficking and the homeostasis of intracellular compartments (Toker, 1998). Phosphoinositides and their biosynthetic machinery are also present in the nucleus (Boronenkov et al., 1998; D'Santos et al., 1998; Maraldi et al., 1999; Irvine, 2000). The regulation of these two phosphoinositide pools is largely independent, suggesting that the nucleus constitutes a functionally distinct compartment for inositol phospholipid metabolism (D'Santos et al., 1998; Irvine, 2000).

Several roles have been ascribed to nuclear phosphoinositides. Nuclear PtdIns(4,5)P_2, like cytoplasmic PtdIns(4,5)P_2, has been suggested to be the target of PtdIns(4,5)P_2-specific phospholipases. The resultant production of DAG is in turn required for the activation of a sub-set of protein kinase C (PKC) isoforms with a nuclear localisation (D'Santos et al., 1998). Nuclear targets whose functions are modulated by PKC-mediated phosphorylation include DNA polymerases, topoisomerases, histones and nuclear envelope proteins (D'Santos et al., 1998; Irvine, 2000). The concomitant production of the second messenger InsP_3 appears to participate in nuclear calcium homeostasis, which has been implicated in several physiological processes such as DNA synthesis, modulation of gene transcription, apoptosis and chromatin condensation.

A more direct link has recently been demonstrated between PtdIns(4,5)P_2 and the process of chromatin remodelling. PtdIns(4,5)P_2 is able to stabilise the association of the SWI/SNF-like BAF complex with chromatin and the nuclear matrix (Zhao et al., 1998). PtdIns(4,5)P_2 could also influence DNA template availability via the inhibition of histone-mediated repression on RNA polymerase II activity (Yu et al., 1998). In support of this, chromatin has been shown to bind phospholipids via histones and non-histone chromosomal-associated proteins (Manzoli et al., 1977).

PtdIns(4,5)P_2 and some enzymes involved in its synthesis have been co-localised in the nucleus with components of small nuclear ribonucleoprotein particles (snRNPs) (Boronenkov et al., 1998), which are involved in pre-mRNA processing. Interestingly, genetic evidence has implicated nuclear phosphoinositides and their hydrolysis products in the export of mRNA via the nuclear pore complex (York et al., 1999). This novel regulatory pathway involves the generation of several inositol polyphosphates, which appear to have distinct functions (York et al., 1999; Odom et al., 2000; Saiardi et al., 2000). In addition, inositol hexakisphosphate has recently been demonstrated to act as an essential cofactor in DNA repair by non-homologous end joining (Hanakahi et al., 2000).

Here, we demonstrate that detergent-resistant nuclear PtdIns(4,5)P_2 is associated with electron-dense structures, whose morphology and distribution are cell-cycle dependent and resemble that of interchromatin granule clusters (IGCs). Elements of the transcriptional and pre-mRNA processing machinery interact with this pool of nuclear PtdIns(4,5)P_2, and PtdIns(4,5)P_2 immunoprecipitates contain intermediates and products of the splicing reaction. Immunodepletion and add-back experiments demonstrate that PtdIns(4,5)P_2 and interacting factors are essential, but not sufficient, for pre-mRNA splicing. These findings suggest that PtdIns(4,5)P_2 is a component of the pre-mRNA processing machinery.

MATERIALS AND METHODS

Liposome and dot blot binding assays

Liposomes containing either 99% (mole/mole) phosphatidylcholine (PC) and 1% phosphatidylinositol (PtdIns), or 98% PC, 1% PtdIns and 1% PtdIns(4,5)P_2 together with 30 nCi [^{14}C]PC (Amersham-Pharmacia Biotech) were prepared by resuspending the dry lipid mixtures in 20 mM Hepes-KOH, pH 7.5, 250 mM KCl, 0.1 mM DTT, followed by sonication. The liposomes were spun to eliminate aggregates and incubated for 1 hour at room temperature with 2-5 μg of immobilised 2C11 antibody (Thomas et al., 1999). Briefly, protein G-sepharose beads (Amersham Pharmacia Biotech) were incubated with anti-mouse IgM (Dako) as a bridging antibody for 2 hours at 4°C and subsequently incubated with 2C11 for 1 hour. Beads were pre-incubated with 0.5 mg/ml ovalbumin to block non-specific binding sites. After incubation with liposomes, beads were washed three times in 20 mM Hepes-KOH, pH 7.5, 0.1 mM DTT and the radioactivity quantified by scintillation counting.

Salmon sperm DNA or total cellular RNA were spotted on a nylon Hybond N plus membrane (Amersham Pharmacia Biotech) and PtdIns(4,5)P_2 on nitrocellulose (Schleicher and Schuell). Nucleic acids were crosslinked to the membrane by heating for 2 hours at 80°C. Filters were blocked for 1 hour at room temperature with 1% ovalbumin, 1% polyvinylpyrrolidone in PBS and then incubated with 2C11 (1:500) in the same buffer. HRP-conjugated anti-mouse secondary antibodies (1:2000, Dako) were applied in 3% polyvinylpyrrolidone in PBS. Blots were developed using ECL Plus (Amersham Pharmacia Biotech).

Immunofluorescence and electron microscopy analysis

HeLa and NIH-3T3 were synchronised by treatment with nocodazole (100 ng/ml) overnight, tapped off and after washing, plated on poly-L-lysine coated coverslips. After paraformaldehyde fixation (3.7% in PBS) for 10 minutes, coverslips were incubated with 50 mM NH_4Cl for 15 minutes and then blocked using PBS containing 2% bovine serum albumin (BSA), 0.25% gelatin, 0.2% glycine and 0.2% Triton X-100 for 1 hour. The primary antibody was appropriately diluted (2C11 1:200; anti-Sm 1:2000; anti-SC35 1:2000; H5 1:1000) in PBS with 1% BSA, 0.25% gelatin and 0.2% Triton X-100 and incubated for 1 hour. Cells were washed with 0.2% gelatin in PBS and the fluorescent secondary antibody (1:200, Molecular Probes) applied for 20 minutes in the same buffer as the primary antibody. Cy3-2C11 was prepared by incubating 2C11 with N-hydroxysuccinimidyl-Cy3 ester (Amersham-Pharmacia Biotech) in 100 mM Hepes-NaOH, pH 8.0. The ratio between dye and 2C11 was optimised for each reaction. For co-localisation experiments using two monoclonal antibodies, an additional blocking step with an excess of unlabelled primary antibody (30-fold) was performed following incubation with the secondary antibody and prior to application of Cy3-2C11.

In competition experiments, 2C11 was pre-incubated with liposomes containing 95% (mole/mole) PC and 5% mole/mole of different phosphoinosidites (Echelon) in PBS for 1 hour at room temperature. Where indicated, neomycin (1 mM) was added to the blocking solution. RNase A (1 mg/ml; 15 minutes) and DNase I (100 μg/ml; 2 hours) treatments were carried out post-fixation in PBS containing 5 mM $MgCl_2$, 4% Tween-20 prior to blocking.

Cryosections of HeLa cells and extruded liposomes (Duzgunes and Wilschut, 1993) containing 90% PC plus 10% PtdIns, or 94% PC, 2% PtdIns(4,5)P_2 and 4% PtdIns in 20 mM Hepes-KOH, pH 7.4, 0.1 mM DTT were labelled as previously described (Slot and Geuze, 1985). 2C11 antibody was used at 1:10 dilution and followed by 10 nm gold-conjugated rabbit anti-mouse IgM (1:100, British Biocell). Sections were examined and photographed with a JEOL 1010 TEM.

Immunoprecipitation

HeLa nuclear extracts (Dignam et al., 1983) were pre-cleared by incubation with 20 μl protein G-sepharose beads for 1 hour at 4°C, before the addition of either 20 μl of anti-IgM conjugated or 20 μl 2C11-conjugated protein G-sepharose beads. Samples were incubated for 2 hours at 4°C and beads were collected by centrifugation for 1 minute at 1200 ***g*** at 4°C. Immunoprecipitates were washed four times in 20 mM Hepes-NaOH pH 7.9, 100 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.25% NP-40 and then prepared for SDS-PAGE. Proteins were either stained with Coomassie blue or transferred to nitrocellulose and analysed by western blotting with appropriate antibodies. For analysis of associated RNAs, HeLa nuclear extracts were prepared using the Dignam method with modifications (Abmayr et al., 1988) from cells labelled overnight with 1 mCi [γ-^{32}P]-orthophosphate per 150 mm dish. Associated [^{32}P]-labelled RNAs were phenol/chloroform extracted following proteinase K treatment and separated on a 6% acrylamide/7 M urea denaturing gel. snRNAs were identified according to molecular weight and by comparison with parallel immunoprecipitations using the Y12 anti-snRNP antibody (Lerner et al., 1981).

Splicing assays

Splicing assays were carried out in a final volume of 20 μl, containing 30% HeLa nuclear extract, 0.8 U/ml RNasin, 0.4 mM ATP, 20 mM creatine phosphate, 3 mM $MgCl_2$, 0.6% polyvinyl alcohol and 3 ng RNA probe. Uniformly radiolabelled β-globin (Krainer et al., 1984), Ad-2 (Pellizzoni et al., 1998) or δ-crystallin (sp14-15) (Pellizzoni et al., 1998) transcripts were prepared using [α-^{32}P]-CTP (Amersham Pharmacia Biotech) and the Riboprobe in vitro transcription system (Promega). After incubation for 3 hours (β-globin) or 1 hour at 30°C (δ-crystallin, Ad-2), the RNA was purified by phenol/chloroform extraction and ethanol precipitation and analysed by gel electrophoresis on a 6% (β-globin, δ-crystallin) or 10% (Ad-2) acrylamide, 7 M urea denaturing gel. For immunodepleted samples, the reaction mix was incubated for 1 hour at 4°C with protein G-sepharose beads alone or conjugated with anti-IgM or 2C11 prior to the addition of the RNA probe. For the antibody competition, 2C11 beads were pre-incubated with 250 μM GroPIns or GroPIns(4,5)P_2 in PBS for 30 minutes at room temperature. Beads were washed once with splicing buffer prior to use. Quantitation was performed using a Phoshorimager (Molecular Dynamics) for splicing reactions and using NIH Image for western blots.

For the elution and add-back experiments, immunoprecipitations were carried out in 19 μl splicing reaction containing 40% nuclear extract for 90 minutes at 4°C. Immunoprecipitated material was eluted by incubating beads for 15 minutes at 4°C with 5 μl elution buffer (14 mM Hepes-NaOH, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 0.4 mM ATP, 20 mM creatine phosphate, 0.6% polyvinyl alcohol) containing 300 μM PtdIns, di-butyl PtdIns(4,5)P_2 or GroPIns(4,5)P_2. The supernatant or 5 μl of the lipids alone was added to the depleted reaction mix to give a final concentration of 30% nuclear extract. 3 ng δ-crystallin RNA was used per reaction.

RESULTS

The monoclonal antibody 2C11 recognises PtdIns(4,5)P_2 inserted in a lipid bilayer

To investigate the cellular distribution of phosphoinositides, we recently developed monoclonal antibodies that recognise PtdIns(4,5)P_2 (Thomas et al., 1999). The monoclonal antibody 2C11 detected PtdIns(4,5)P_2 in a dot-blot assay, but did not recognise DNA or total RNA (Fig. 1A), nor did it significantly crossreact with any proteins present in HeLa cell extracts by western blot. The ability of 2C11 to recognise the head group of PtdIns(4,5)P_2 inserted in a lipid bilayer was tested using a liposome-binding assay. Phosphatidylcholine (PC) liposomes containing either 1% phosphatidylinositol (PtdIns) or 1%

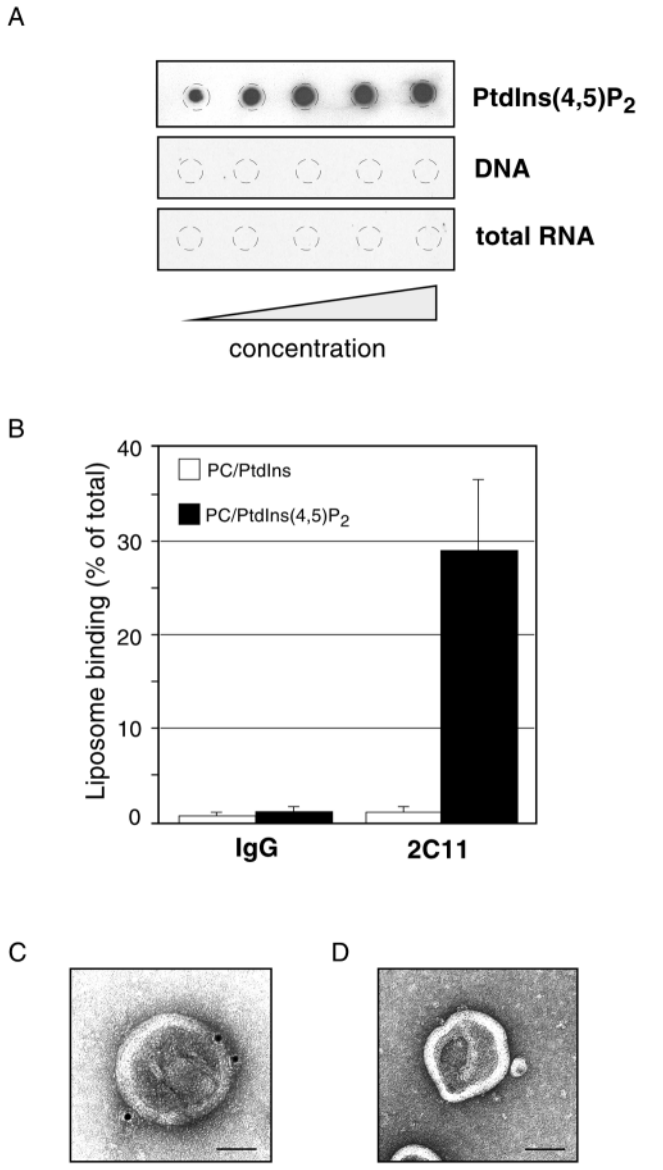

Fig. 1. Monoclonal antibody 2C11 recognises PtdIns(4,5)P_2 inserted in a membrane bilayer. (A) 2C11 recognises PtdIns(4,5)P_2 but not DNA or RNA on dot-blot. Serial dilutions of PtdIns(4,5)P_2 (0.0125-0.4 μg), DNA and total PC12 cell RNA (0.03-1 μg) were spotted on membranes and probed with 2C11 antibody. (B) 2C11 specifically interacts with PtdIns(4,5)P_2-containing liposomes. Liposomes containing radioactive PC as a tracer and either PtdIns (empty bars) or PtdIns(4,5)P_2 (filled bars) were incubated with immobilised anti-IgM or 2C11. Radioactivity associated with beads was measured and results expressed as a percentage of the total. Error bars represent s.d. based on three experiments. (C,D) Cryosections of extruded liposomes containing PtdIns(4,5)P_2 (C) and PtdIns (D) were probed with 2C11 followed by gold-conjugated secondary antibodies. 34% of the total PtdIns(4,5)P_2-containing liposomes (n=170) were labelled with one or more gold particles compared with 0% (n=96) in the case of PtdIns-containing liposomes. Bars, 50 nm.

PtdIns(4,5)P_2 were incubated with immobilised 2C11 antibody. 2C11 efficiently recovered PtdIns(4,5)P_2 but not PtdIns-containing vesicles (Fig. 1B), demonstrating that the presence of negatively charged lipids alone is not sufficient for 2C11-mediated recovery. The interaction of 2C11 with PtdIns(4,5)P_2-containing liposomes was visualised directly by immuno-electron microscopy. Gold particles were seen in close proximity to the lipid bilayer surface when PtdIns(4,5)P_2 (Fig. 1C) but not PtdIns was present (Fig. 1D).

PtdIns(4,5)P_2-specific antibodies stain interphase nuclei

Immunofluorescence labelling of several cell lines with anti-PtdIns(4,5)P_2 antibodies revealed distinct staining patterns depending on the permeabilisation procedure. Protocols that permit access of the antibody to the intracellular space while allowing preservation of cellular membranes, such as permeabilisation with streptolysin O or cytoplasmic microinjection of Cy3-labelled 2C11, revealed a discontinuous staining of the plasma membrane (S.L.O. and G.S., unpublished). By contrast, a characteristic nuclear staining is observed upon paraformaldehyde fixation in the presence of detergents, such as Tween-20 or Triton X-100 (Fig. 2A). PtdIns(4,5)P_2 immunoreactivity is restricted to discrete areas inside the nucleus that do not contact the nuclear envelope. This staining is reduced by methanol fixation, a treatment expected to extract lipid components from proteolipid complexes. The nuclear distribution of PtdIns(4,5)P_2 is seen in different cell types, including HeLa (Fig. 2A), NIH-3T3 (Fig. 3), PC12, Vero and primary fibroblasts. These results have been confirmed with another anti-PtdIns(4,5)P_2 antibody previously described (Thomas et al., 1999).

Cryo-immuno-electron microscopy on HeLa cells in the absence of detergent revealed a large number of gold particles localised to nuclear electron-dense structures with an average diameter of 0.4 μm (Fig. 2B). In addition, PtdIns(4,5)P_2 shows a sparse nucleoplasmic distribution. PtdIns(4,5)P_2 immunoreactivity is also associated with fibrillar centres and the dense fibrillar component of the nucleolus (Olson et al., 2000) (Fig. 2C). The same distribution is observed by cryo-immuno-electron microscopy in the presence of detergent (S.G. and G.S., unpublished), indicating that the nuclear localisation seen by immunofluorescence is not due to a detergent-induced redistribution of PtdIns(4,5)P_2. The absence of nucleolar staining in immunofluorescence may be due to a lack of accessibility of the PtdIns(4,5)P_2 in this compartment.

Pre-incubation of the 2C11 antibody with an excess of liposomes containing different phosphoinositides showed that the nuclear staining is abolished by PtdIns(4,5)P_2, but not by any other lipid, including PtdIns(3,4,5)P_3 and the PtdIns(4,5)P_2 isomers PtdIns(3,4)P_2 and PtdIns(3,5)P_2 (Fig. 2D-G). PtdIns(4,5)P_2-treated samples present a cytoplasmic dotted staining (Fig. 2G). This was never observed in control preparations and could be due to the non-specific binding of antibody-PtdIns(4,5)P_2 liposome aggregates to cytoplasmic structures. Similar competition experiments were therefore performed using soluble phosphoinositide head-groups. L-α-glycerophospho-D-myo-inositol(4,5)bisphosphate (GroPIns(4,5)P_2) (Fig. 2H) but not the unphosphorylated GroPIns, totally abolished the nuclear signal and did not result in the additional cytoplasmic staining. InsP_3 can compete 2C11 binding (S.L.O. and G.S., unpublished). However neomycin, an aminoglycoside antibiotic that binds to several phosphoinositides (Gabev et al., 1989) but not InsP_3 (Arbuzova et al., 2000), abolished 2C11 immunolabelling (Fig. 2I).

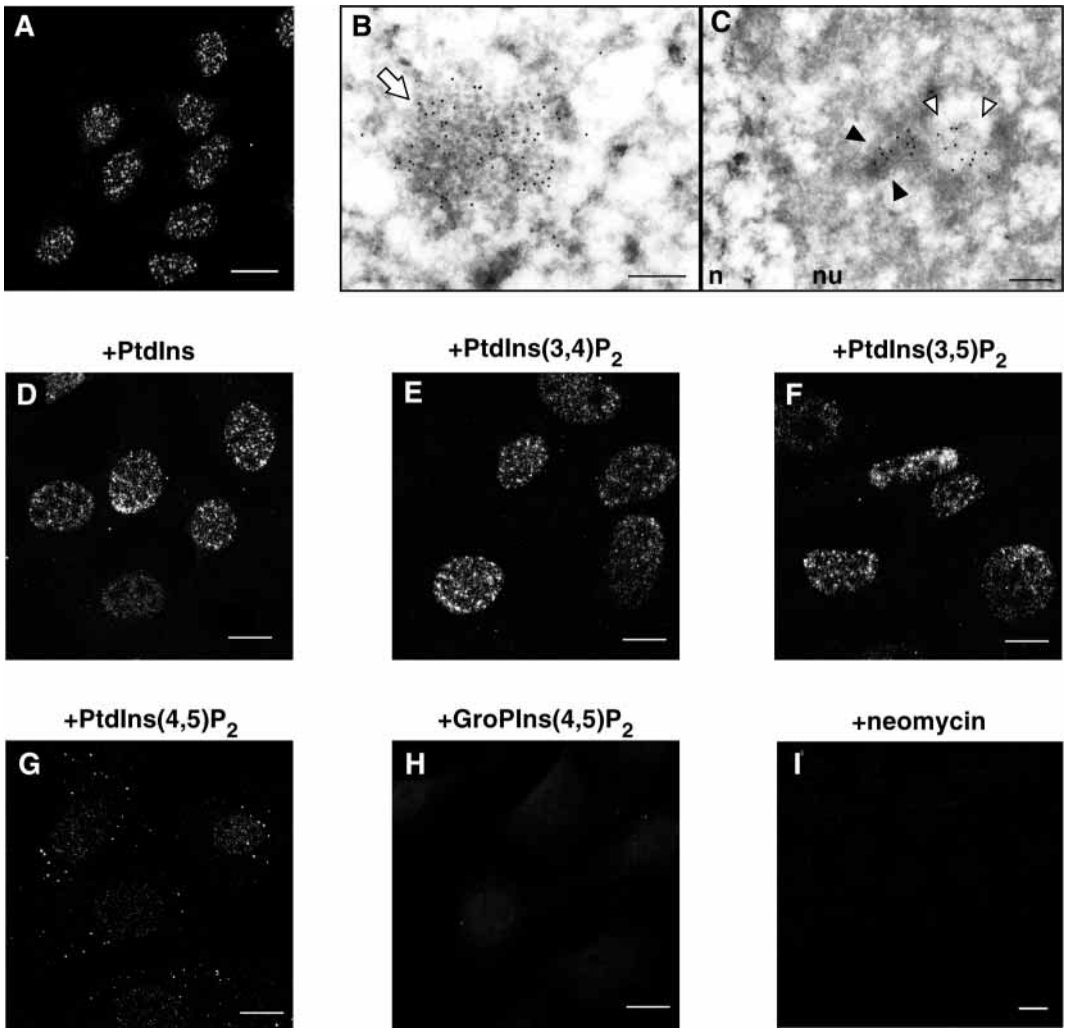

Fig. 2. PtdIns(4,5)P_2 is localised in electron-dense particles in HeLa cell nuclei. Detergent-permeabilised (A,D-I) or unpermeabilised (B,C) HeLa cells were incubated with 2C11 followed by a fluorescent (A and D-I) or a 10 nm gold (B,C)-conjugated secondary antibody. PtdIns(4,5)P_2 signal was associated with electron dense areas in the nucleus (B), resembling IGCs (arrow), and in the nucleolus (C), where both the fibrillar centres (open triangles) and portions of the dense granular components (filled triangles) are labelled. n, nucleus; nu, nucleolus. Fluorescent images were acquired with a CCD camera-equipped Zeiss microscope. Pre-incubation of 2C11 with liposomes containing PtdIns(4,5)P_2 (G) or with an excess of the soluble headgroup of PtdIns(4,5)P_2 (GroPIns(4,5)P_2, (H) abolished the nuclear staining, while PtdIns (D) or isomers of PtdIns(4,5)P_2 (E,F) had no effect. Pre-incubation of cells with neomycin, which binds to several phosphoinositides, also abolished staining (I). Bars, 10 μm (A,D-I); 200 nm (B,C).

Together, these results strongly suggest that the nuclear antigen recognised by 2C11 is PtdIns(4,5)P_2.

The localisation of detergent-resistant PtdIns(4,5)P_2 is cell-cycle dependent

Biochemical studies investigating the cell-cycle regulation of phosphoinositides have suggested that they vary during S-phase and are important for the progression of mitosis (Uno et al., 1988; Imoto et al., 1994; York and Majerus, 1994). As shown in Fig. 3, the distribution of the detergent-resistant PtdIns(4,5)P_2 changes dramatically during mitosis. Upon nuclear membrane disassembly, detergent-resistant PtdIns(4,5)P_2 immunoreactivity shifts to the cytoplasm, where it remains during chromosome partitioning (Fig. 3I,L). During the later stages of mitosis, PtdIns(4,5)P_2 staining undergoes a remarkable concentration into a limited number of very bright structures that remain cytoplasmic even when the DNA has re-localised to the newly formed nuclei of the two daughter cells (Fig. 3M-O). As shown by immuno-electron microscopy (Fig. 3P), these mitotic structures appear morphologically indistinguishable from those observed in interphase, lacking any apparent lipid bilayer morphology and with no visible connection to the plasma membrane. PtdIns(4,5)P_2 immunoreactivity does not overlap with the DNA. This is particularly evident during chromosome condensation, when PtdIns(4,5)P_2 staining is clearly excluded from the area occupied by the genetic material (Fig. 3D-F)

Nuclear PtdIns(4,5)P_2 is associated with a sub-class of nuclear bodies

To identify the nature of the PtdIns(4,5)P_2-containing compartment, an immunofluorescence screen was performed

using several nuclear markers. Only a limited overlap was observed between the interphase distribution of PtdIns(4,5)P_2 and that of a large number of antigens tested, including various transcription factors, such as the polycomb family member Ring 1 and PML (Lamond and Earnshaw, 1998; Matera, 1999). The 2C11 staining pattern is reminiscent of that seen for a number of nuclear antigens involved in pre-mRNA processing and associated with interchromatin granule clusters (IGCs) and perichromatin fibrils (PFs) (Spector, 1993b; Lewis and Tollervey, 2000). IGCs lack active transcription foci, are resistant to nuclease treatment and have been suggested to act as storage compartments for splicing factors (Spector, 1993a; Fakan, 1994; Mintz et al., 1999). By contrast, PFs, which are often associated with the periphery of IGCs, contain nascent transcripts and are sensitive to RNase degradation (Spector, 1993a; Fakan, 1994).

We tested the co-localisation of nuclear PtdIns(4,5)P_2 with the splicing factor SC-35, a classic marker of IGCs (Spector et al., 1991) and PFs, and the common snRNP components, Sm proteins. SC-35 and PtdIns(4,5)P_2 signals co-localise in interphase cells (Fig. 4A-C), whereas Sm antigens only partially overlap with PtdIns(4,5)P_2 immunoreactivity as expected from their wider distribution not only in IGCs and PFs but also in the nucleoplasm and coiled bodies (Spector et al., 1983) (Fig. 4D-F). A partial co-localisation was also observed with the hyperphosphorylated form of the largest subunit of RNA polymerase II (Kim et al., 1997) (H5, Fig. 4G-I). The C-terminal domain of this subunit is present as an unphosphorylated (RNA Pol IIa) or as a number of hyperphosphorylated forms (RNA Pol IIo) (Corden and Patturajan, 1997; Bentley, 1999; Hirose and Manley, 2000). Recent work has shown that RNA Pol IIo associates with IGCs and shuttles between these structures and sites of active transcription (Bregman et al., 1995). PtdIns(4,5)P_2 is not present in coiled bodies as demonstrated by the lack of co-localisation with p80-coilin (S.L.O. et al., unpublished).

Although the antibody against PtdIns(4,5)P_2 does not crossreact with DNA or RNA (Fig. 1), pre-treatment of samples with RNase substantially reduced nuclear staining. By

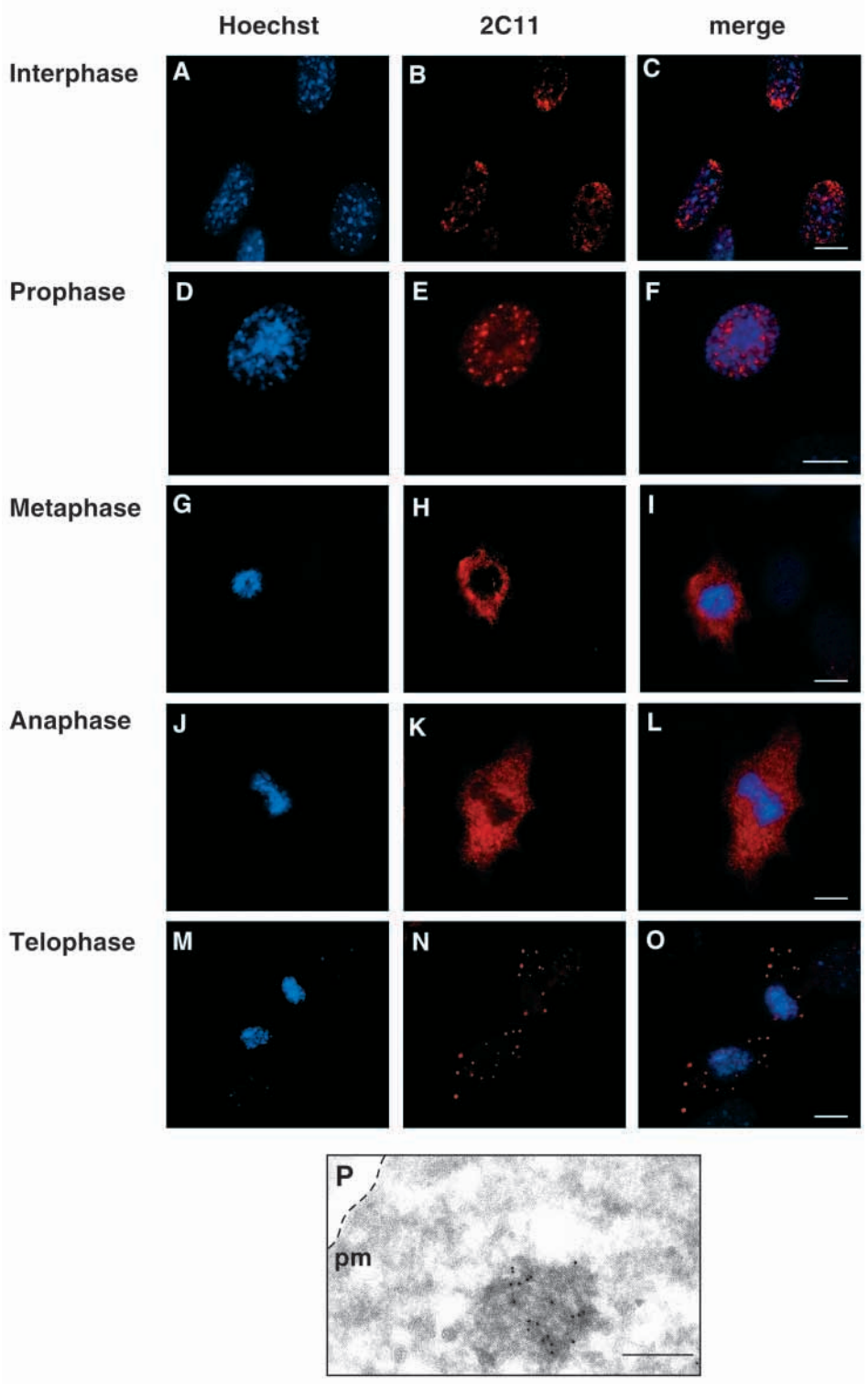

Fig. 3. The localisation of the nuclear structures containing PtdIns(4,5)P_2 is cell-cycle dependent. Synchronised NIH-3T3 cells were fixed at different stages of mitosis and the distributions of DNA and PtdIns(4,5)P_2 were visualised with Hoechst 33342 (blue, A,D,G,J,M), and 2C11 antibody (red, B,E,H,K,N), respectively. Pseudo-colour merged images emphasise the lack of co-localisation between PtdIns(4,5)P_2 and DNA (merge, C,F,I,L,O). Fluorescent images were acquired with a CCD camera-equipped Zeiss microscope. Bars, 10 μm. Cells in telophase were prepared for electron microscopy and labelled with 2C11 (P). The dotted line in (P) outlines the plasma membrane (pm). Bar, 200 nm.

contrast, incubation with DNase using conditions that cause a loss of Hoechst 33342 staining, had no effect (Fig. 4J-K). This result implies that RNA but not DNA is essential for the association of PtdIns(4,5)P_2 with these nuclear structures.

The distribution of PtdIns(4,5)P_2 and RNA Pol IIo exactly overlap during mitosis, this being particularly evident in late telophase (Fig. 5A-C). These PtdIns(4,5)P_2-containing particles also contain the majority of SC-35 (Fig. 5D-F), a feature that identifies them as mitotic interchromatin granules (Spector et al., 1991; Ferreira et al., 1994). However, only a minor fraction of Sm proteins are associated with these structures, the majority localise to an area occupied by the newly formed nuclei of the two daughter cells (Ferreira et al., 1994) (Fig. 5G-I). These results suggest that PtdIns(4,5)P_2-containing particles undergo dynamic changes in composition during the cell-cycle.

PtdIns(4,5)P_2 associates with the pre-mRNA processing machinery

An immunoprecipitation approach was chosen to define the components associated with PtdIns(4,5)P_2 in interphase nuclei. Experiments performed with nuclear extracts from [^{32}P]-labelled HeLa cells demonstrated that 2C11 beads are able to recover PtdIns(4,5)P_2, whose identity was confirmed by deacylation and HPLC analysis of the lipid head group. Under the same conditions, several proteins associate with 2C11, but not with control beads (Fig. 6A). This interaction was PtdIns(4,5)P_2-dependent, as shown by the competition observed by pre-incubation of the 2C11 beads with GroPIns(4,5)P_2. Pre-incubation with GroPIns was ineffective (Fig. 6A), thus demonstrating that these proteins are immunoprecipitated indirectly via their association with PtdIns(4,5)P_2.

To confirm the identity of nuclear PtdIns(4,5)P_2-interacting species, the immunoprecipitated material was probed by western blotting with antibodies directed against different nuclear proteins. RNA Pol IIa and RNA Pol IIo can be separated by SDS-PAGE and detected in western blot using antibodies that recognise RNA Pol IIo (H5; Kim et al., 1997) or RNA Pol IIa (8WG16; Kim et al., 1997). As shown in Fig. 6B, the anti-PtdIns(4,5)P_2 immunoprecipitate contains RNA Pol IIo, but not RNA Pol IIa, suggesting that this lipid is predominantly associated with hyperphosphorylated form. Increasing the stringency of the immunoprecipitation by the addition of 0.5% NP-40 or 150 mM KCl to the wash did not alter the immunoprecipitation pattern. Sm proteins are also associated with the immunoprecipitate (Fig. 6B), as expected from the partial co-localisation seen by immunofluorescence in interphase (Fig. 4D-F). By contrast, hnRNP A1, an abundant nuclear protein, is not recovered by the 2C11 beads, further indicating the selectivity of the PtdIns(4,5)P_2 immunoprecipitation (Fig. 6B).

Extraction of RNA present in 2C11 immunoprecipitates from [^{32}P]-labelled HeLa cell nuclear extracts, revealed radioactive bands corresponding to the U1-U6 small nuclear RNAs (snRNAs) in the anti-PtdIns(4,5)P_2, but not in the control immunoprecipitate (Fig. 6C). The extent of tRNA recovery in the 2C11 immunoprecipitate varied between

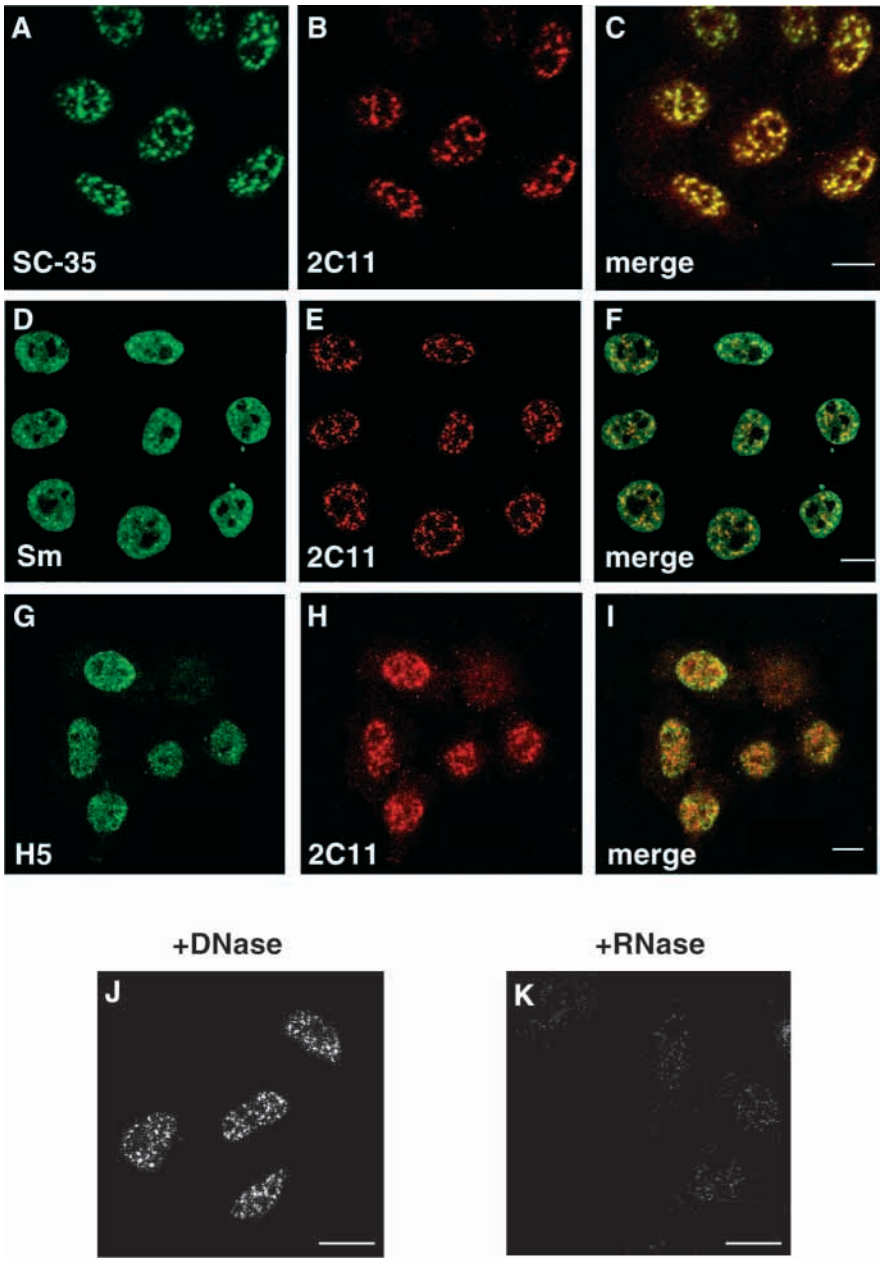

Fig. 4. PtdIns(4,5)P_2 co-localises with SC-35 in interphase cells. Detergent-permeabilised HeLa cells were co-stained with Cy3-2C11 (B,E,H,J,K) and either anti-SC-35 (A), anti-Sm (D) or anti- RNA Pol IIo (H5, G) antibodies. The yellow pseudo-colour (merge; C,F,I) shows the extent of co-localisation between the two antigens. RNase (K), but not DNase I (J) treatment abolishes PtdIns(4,5)P_2 staining. Confocal microscopy images corresponding to the projection of a series of 0.4 μm sections are shown. Bars, 10 μm.

experiments and was not competed by pre-incubation of 2C11 with GroPIns(4,5)P_2.

Together, these results demonstrate that a pool of nuclear PtdIns(4,5)P_2 is associated in a detergent resistant manner with a multi-subunit complex comprising both protein and nucleic acid components of the transcriptional and pre-mRNA splicing machinery.

PtdIns(4,5)P_2-containing nuclear structures are essential for pre-mRNA splicing

Based on its composition, we asked whether the pool of proteins associated with nuclear PtdIns(4,5)P_2 have an active involvement in pre-mRNA splicing. We tested this hypothesis using an in vitro splicing assay combined with an immunodepletion approach. Three different RNA probes were tested: β-globin (Krainer et al., 1984) (Fig. 7), δ-crystallin (Pellizzoni et al., 1998) (Fig. 8) and adenovirus 2 major late pre-mRNA (Ad-2) (Pellizzoni et al., 1998). In all cases, immunodepletion of HeLa cell nuclear extract with anti-PtdIns(4,5)P_2 antibody beads inhibited the splicing reaction, whereas immunodepletion with protein G beads or anti-IgM beads had no significant effect. In Fig. 7A, this inhibition is seen as a decrease in the amount of product and splicing intermediate. Pre-incubation of the antibody with the GroPIns(4,5)P_2, but not GroPIns (Fig. 7A,D) prevents the 2C11-mediated inhibition. Parallel western blotting of the immunoprecipitates with anti-RNA Pol IIo (H5, Fig. 7B) show that RNA Pol IIo is associated with the 2C11 immunoprecipitate only and that this association is competed by pre-incubation of the beads with GroPIns(4,5)P_2 to an extent similar to that seen in the splicing reaction (Fig. 7, compare B,D). The incomplete rescue observed by pre-incubation of the antibody with the soluble headgroup of PtdIns(4,5)P_2 (Fig. 7D) could be explained by the absence of an excess of free competitor during the immunodepletion.

Immunodepletion of HeLa nuclear extracts with 2C11 beads also inhibits the splicing of δ-crystallin RNA and, again, this inhibition is competed by pre-incubation of the beads with GroPIns(4,5)P_2 but not GroPIns (Fig. 8A). Immunoprecipitated material can be eluted from the 2C11 beads by the addition of an excess of short chain PtdIns(4,5)P_2 or GroPIns(4,5)P_2, but not PtdIns. Western blotting with anti-RNA Pol IIo antibody (H5) has been used to follow the elution efficiency (Fig. 8B). Re-addition of this eluted material to the depleted nuclear extract is able to partially restore the splicing activity. Addition of PtdIns, short chain PtdIns(4,5)P_2 or GroPIns(4,5)P_2 alone to the depleted nuclear extract has no effect on the splicing activity (Fig. 8B) and the addition of the eluted material alone to the δ-crystallin mRNA does not support splicing (S.L.O. and G.S., unpublished). We therefore conclude that the PtdIns(4,5)P_2 and associated factors, but not PtdIns(4,5)P_2 by itself, are necessary but not sufficient for splicing to occur in vitro.

The above immunodepletion experiments are carried out in the absence of an RNA substrate. If the splicing reaction is performed prior to immunoprecipitation and the associated RNA analysed, we find that splicing intermediates and the spliced product specifically associate with 2C11-conjugated beads to a similar extent as they do with anti-snRNP antibody beads (Y12, Fig. 9). Pre-incubation of the 2C11 beads with GroPIns(4,5)P_2 but not GroPIns is again able to compete the immunoprecipitation.

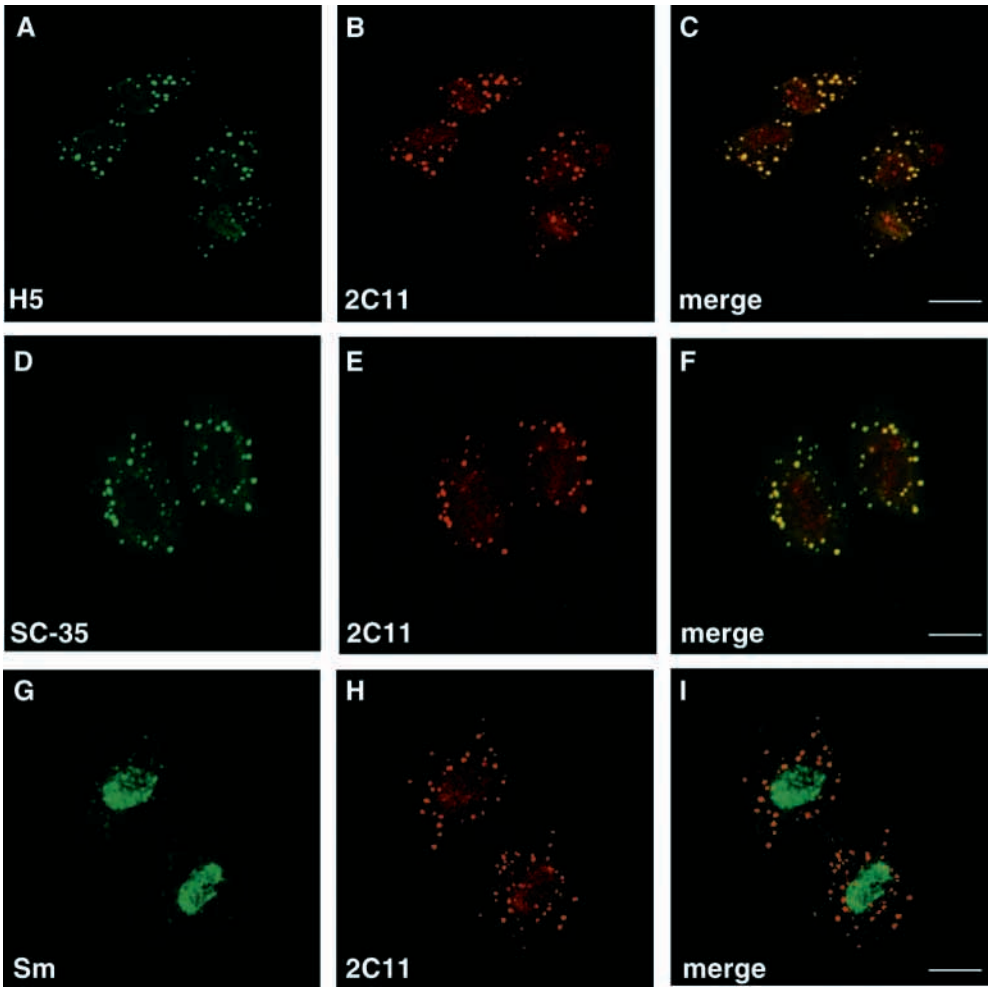

Fig. 5. PtdIns(4,5)P_2 co-localises with RNA Pol IIo and SC-35 in mitotic cells. Synchronised HeLa cells in late telophase were co-stained with Cy3-2C11 (B,E) or 2C11 (H) and either anti-RNA Pol IIo (H5, A), anti-SC-35 (D) or anti-Sm (G). The yellow pseudocolour (merge; C,F,I) shows the extent of co-localisation between the antigens. Confocal microscopy images corresponding to the projection of a selected series of 0.4 μm sections are shown. Bars, 10 μm.

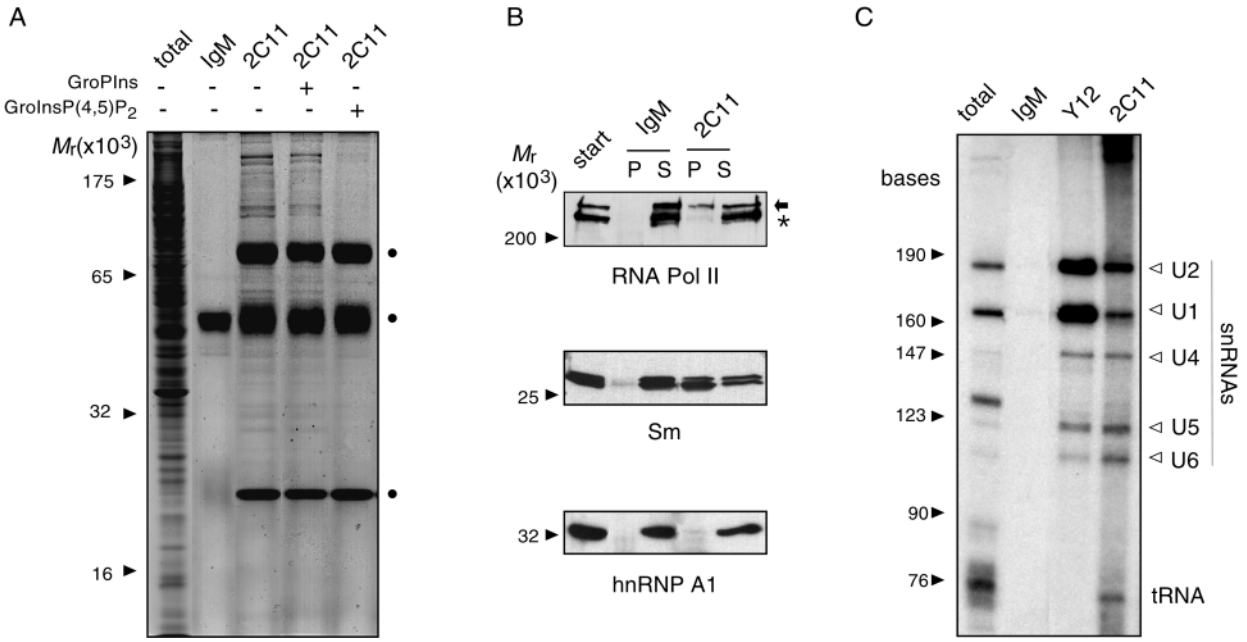

Fig. 6. Nuclear PtdIns(4,5)P_2 associates with RNA Pol IIo and snRNP components. (A) Immunoprecipitates from HeLa nuclear extracts were analysed by SDS-PAGE. Coomassie blue staining shows that several proteins are recovered by 2C11, but not IgM beads. This association is attenuated by pre-incubation with an excess of GroPIns(4,5)P_2 but not GroPIns. Closed circles indicate the position of the antibody bands. Total refers to one tenth of the starting material. (B) Samples were analysed by western blotting with antibodies against the hyperphosphorylated (RNA Pol IIo, arrow) or the unphosphorylated (RNA Pol IIa, *) forms of the large subunit of RNA Pol II (Kim et al., 1997), Sm proteins or hnRNP A1. IgM and 2C11 immunoprecipitates (P) were loaded together with half of the input (start) and half of the supernatant from the immunoprecipitation (S). RNA Pol IIo and Sm proteins are found in the 2C11 but not in the IgM immunoprecipitate, whilst RNA Pol IIa and hnRNP A1 remain in the supernatant. (C) Analysis of [^{32}P]-labelled RNAs associated with the PtdIns(4,5)P_2 immunoprecipitate. Bands corresponding to the U1-U6 snRNAs are recovered by 2C11, but not by the control anti-IgM antibody. For comparison, an immunoprecipitation with the antibody Y12 (anti-snRNP) are included. Total refers to one tenth of the input RNA.

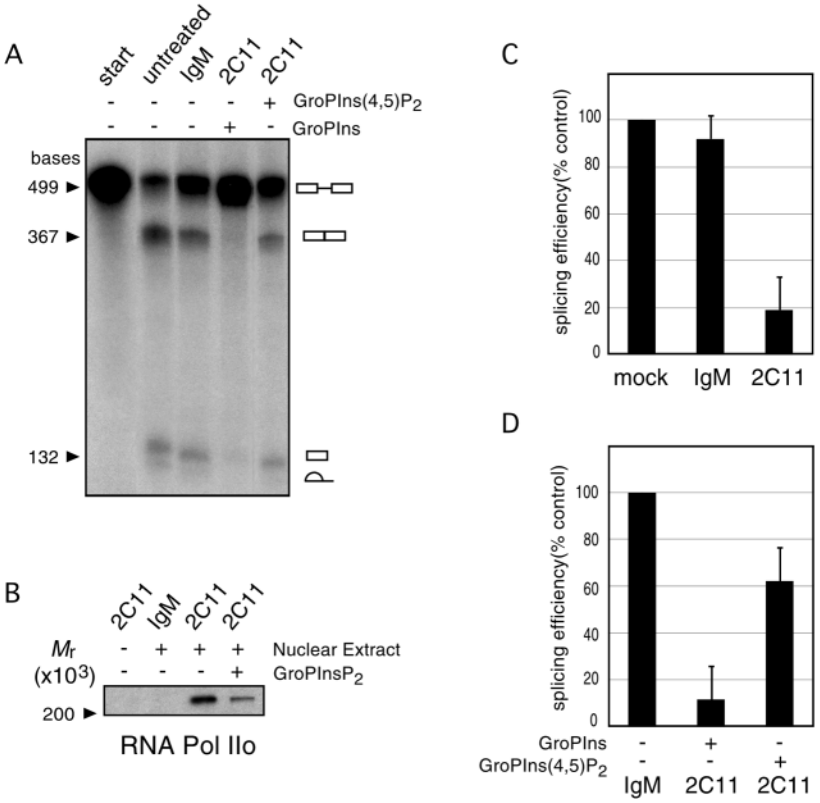

Fig. 7. Immunodepletion of HeLa nuclear extract with 2C11 causes a specific inhibition of pre-mRNA splicing. (A) In vitro splicing reaction with a β-globin RNA probe. Splicing is inhibited by pre-incubation of nuclear extract with 2C11 beads. This effect is blocked by pre-treatment of the antibody with GroPIns(4,5)P_2 but not GroPIns. A schematic representation of the starting probe, intermediates and product of the splicing reaction is shown on the right. (B) The beads used in the immunodepletion experiment presented in (A) and an equivalent amount of untreated 2C11 beads were analysed by western blotting using anti-RNA Pol IIo (H5). (C) Splicing efficiency was quantified and expressed as the percentage of processed RNA in the samples versus the total, taking the mock-treated sample as 100%. Bars represent the s.e. of six experiments. (D) The inhibition of splicing seen on treatment of the nuclear extract with immobilised 2C11 is partially rescued by pre-treatment of 2C11 beads with GroPIns(4,5)P_2 but not GroPIns. Results are expressed as in (C) and bars represent the s.e. of four experiments.

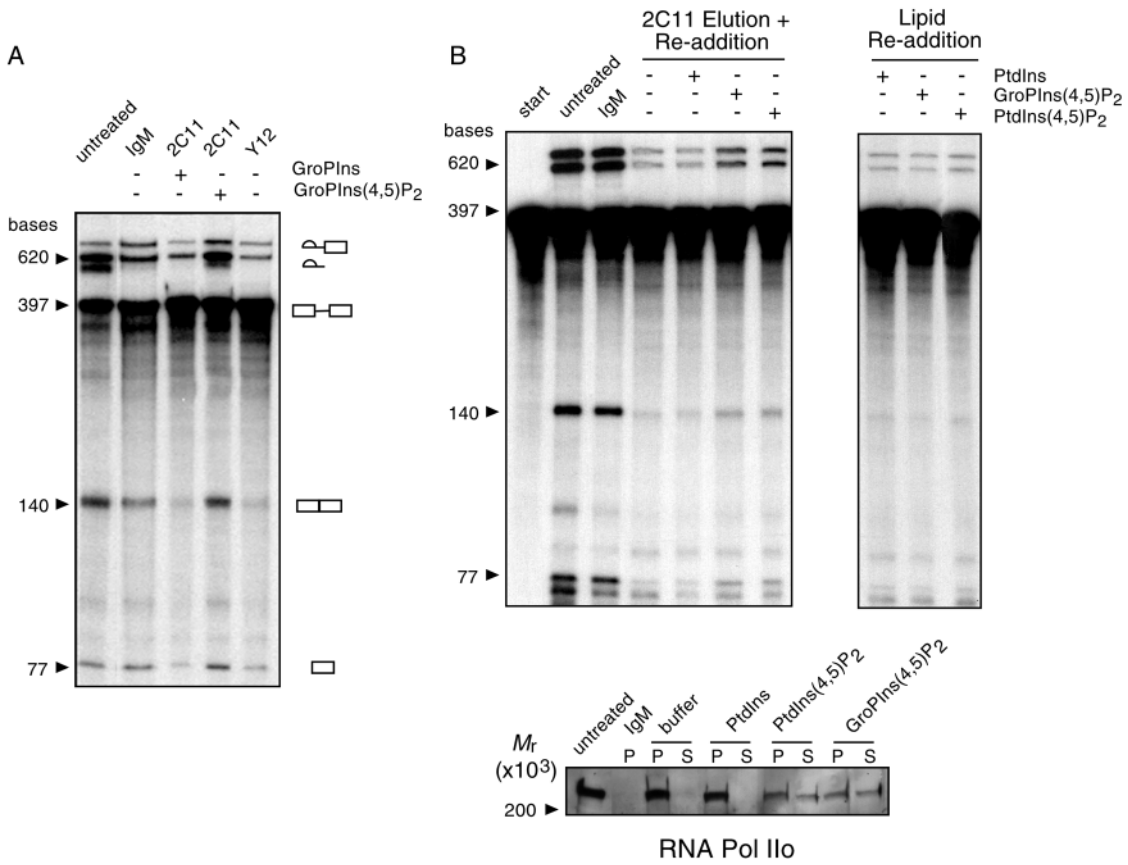

Fig. 8. PtdIns(4,5)P_2-associated factors are required for splicing. (A) 2C11 but not IgM depleted HeLa nuclear extracts are impaired in their ability to support the splicing of δ-crystallin mRNA. The inhibition is blocked by pre-incubation of the 2C11 beads with GroPIns(4,5)P_2 but not GroPIns. The inhibition of splicing seen is comparable to that observed using an anti-snRNP antibody (Y12). A schematic representation of the start, intermediates and product of the splicing reaction is shown on the right. (B) Immunoprecipitated material can be eluted from 2C11 beads by incubation with an excess of PtdIns(4,5)P_2 or GroPIns(4,5)P_2, as seen by western blotting using anti-RNA Pol IIo (H5; lower panel; P, pellet; S, supernatant). Re-addition of these eluates to the depleted splicing reaction partially restores the splicing activity, whereas re-addition of control eluates or the lipids alone have no effect.

DISCUSSION

Growing evidence suggests that compartmentalised pools of phosphoinositides and their biosynthetic machinery are present in the nucleus (D'Santos et al., 1998; Maraldi et al., 1999). The resistance of a pool of nuclear PtdIns(4,5)P_2 to detergent extraction has allowed us to characterise it using a combination of immunofluorescence, immuno-electron microscopy and classic biochemical techniques. Together, our results provide evidence that PtdIns(4,5)P_2 exists within the context of a tripartite complex constituted of protein, lipid and nucleic acids. The presence of PtdIns(4,5)P_2 in such complexes would explain its resistance to detergent extraction and its stability in the absence of a membrane bilayer structure (Mazzotti et al.,

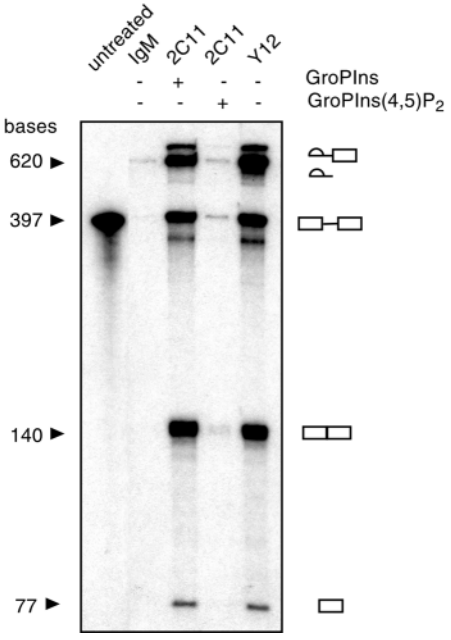

Fig. 9. PtdIns(4,5)P_2 associates with splicing complexes. Splicing reactions were carried out prior to immunoprecipitation. In these conditions, intermediates and products of the splicing reaction associate with anti-PtdIns(4,5)P_2, but not control beads. Pre-incubation of the antibody with GroPIns(4,5)P_2 but not GroPIns inhibits the recovery. A positive control immunoprecipitation with Y12 is shown for comparison.

1995). Associated protein and RNA elements include splicing factors and snRNAs. Depletion of these complexes from HeLa nuclear extracts using an antibody directed against PtdIns(4,5)P_2 inhibits the splicing of pre-mRNA probes in vitro. Addition of PtdIns(4,5)P_2 to untreated nuclear extracts does not alter the apparent rate of splicing in vitro, nor can it recover the splicing activity of the nuclear extract after 2C11 immunodepletion. These findings suggest that PtdIns(4,5)P_2 plays an indirect role in this process.

A hyperphosphorylated form of the largest subunit of RNA Pol II also associates with PtdIns(4,5)P_2. The C-terminal domain of this subunit is essential for the assembly of the spliceosome (Misteli and Spector, 1999), truncation inhibits splicing in vivo (McCracken et al., 1997) and its hyperphosphorylation activates splicing in vitro (Hirose et al., 1999), in keeping with the idea that RNA Pol II plays an active role in coupling transcription and pre-mRNA processing (Hirose and Manley, 2000). Although we cannot exclude a role for PtdIns(4,5)P_2 in the former process, we find that the nuclear localisation of PtdIns(4,5)P_2 and its turnover are independent of active transcription. Inhibiting transcription with the drugs α-amanitin (Haaf and Ward, 1996) or DRB (Zandomeni et al., 1986) causes a re-distribution of PtdIns(4,5)P_2 into larger, rounder nuclear foci. This phenomenon has been previously described for Sm proteins, SC-35 (Spector et al., 1983) and a subset of nuclear phosphatidylinositol phosphate kinases (Boronenkov et al., 1998). Importantly, in these conditions the nuclear levels of PtdIns(4,5)P_2 do not differ between treated and untreated cells (S.L.O. and G.S., unpublished).

During mitosis, when cells are transcriptionally inactive, PtdIns(4,5)P_2 assumes a peripheral distribution similar to that observed for RNA Pol II and certain splicing factors (Spector et al., 1991; Ferreira et al., 1994; Kim et al., 1997). In the late stages of telophase and cytokinesis, PtdIns(4,5)P_2 concentrates in discrete structures that remain peripheral despite the reformation of the daughter cell nuclei. These structures also contain RNA Pol IIo and SC-35 but not Sm proteins. These PtdIns(4,5)P_2-containing complexes thus appear to undergo dynamic changes in composition through the cell-cycle. At the completion of mitosis, it is not clear whether these proteolipid complexes are disassembled before retrieval via the nuclear pore (Nakielny and Dreyfuss, 1999) or if they are transported back through fenestrations still present in the partially assembled nuclear envelope. Direct analysis of PtdIns(4,5)P_2-containing structures in living mitotic cells will provide insights into this transport mechanism.

Bearing in mind the multiple functions of RNA Pol II and that, in addition to their role in splicing, Sm and Sm-like proteins have been implicated in other aspects of mRNA processing such as decapping and decay (Bouveret et al., 2000; Tharun et al., 2000), these PtdIns(4,5)P_2-containing structures might therefore function as central stations for the maturation and quality control of newly formed RNA. Two alternative, but not mutually exclusive roles can be proposed for the phosphoinositide moiety in these complexes. The first possibility is that PtdIns(4,5)P_2 binds nuclear cytoskeletal proteins (Pederson, 2000; Rando et al., 2000). This hypothesis envisages PtdIns(4,5)P_2 as a structural interface between the enzymatic core of the spliceosome and cytoskeletal components, such as protein 4.1 which has been described to functionally interact with the splicing apparatus (Lallena et al., 1998).

Recently, PtdIns(4,5)P_2 has been shown to block the exit of the SWI/SNF-like BAF chromatin remodelling complex from digitonin-permeabilised nuclei (Zhao et al., 1998). This effect is likely to be mediated via interactions with β-actin and actin-related proteins that are intrinsic components of this complex (Zhao et al., 1998; Rando et al., 2000). This finding highlights possible similarities between the chromatin remodelling and splicing machineries and suggests an underlying mechanism whereby PtdIns(4,5)P_2 functions as a direct modulator of various nuclear multi-subunit protein complexes by coupling them to the actin treadmill. The balance between monomeric and polymeric forms of actin appears to act as a regulator of several nuclear functions, as recently demonstrated for serum response factor-dependent gene transcription (Sotiropoulos et al., 1999).

Alternatively, PtdIns(4,5)P_2 could serve as a substrate for nuclear phosphoinositide-modifying enzymes. Hydrolysis of nuclear PtdIns(4,5)P_2 by phospholipase C might provide a localised release of DAG and InsP_3 (D'Santos et al., 1998; Irvine, 2000). Accordingly, the phosphatidylinositol-specific phospholipase C isoforms β_{1b} and δ_4 have been localised to the nucleus (Irvine, 2000). In addition to the regulation of Ca^{2+} release from internal stores, InsP_3 is the precursor of inositol polyphosphates, which have been demonstrated to be essential for RNA transport (York et al., 1999; Odom et al., 2000; Saiardi et al., 2000) and DNA double-strand break repair by non-homologous end joining (Hanakahi et al., 2000). Inositol polyphosphates may therefore act as a high turnover switch of the activity of these molecular machineries, whose activation would be restricted to specific nuclear sub-domains and dependent upon the phosphorylation state of the inositol ring.

This work provides the first direct evidence that the splicing machinery is engaged in a proteolipid complex with PtdIns(4,5)P_2 that is responsible for the majority of splicing activity in vitro. These findings constitute the basis for future investigations into the molecular mechanisms responsible for the assembly, mitotic trafficking and dynamics of these nuclear PtdIns(4,5)P_2-containing complexes and highlight the emerging role of phosphoinositides in nuclear physiology.

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