AKAP149 is a novel PP1 specifier required to maintain nuclear envelope integrity in G1 phase

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Summary

Reassembly of the nuclear envelope (NE) at the end of mitosis requires targeting of the B-type lamin protein phosphatase, PP1, to the envelope by A-kinase anchoring protein AKAP149. We show here that NE-associated AKAP149 is a novel PP1-specifying subunit involved in maintaining nuclear architecture through G1 phase. PP1 remains associated with NE-bound AKAP149 during G1 but is released from AKAP149 upon S phase entry, as AKAP149 becomes serine-phosphorylated. NE-associated AKAP149 inhibits PP1 activity towards glycogen phosphorylase but enhances PP1 phosphatase activity towards B-type lamins, indicating that AKAP149 is a B-type lamin specifying subunit of PP1. In vivo dissociation of PP1 from NE-bound AKAP149 in G1-phase nuclei triggers phosphorylation and depolymerization of A- and B-type lamins. The lamins solubilize intranuclearly without affecting the inner nuclear membrane or pore complex distribution. This correlates with the induction of a G1 arrest and, ultimately, apoptosis. We propose that AKAP149-regulated PP1 activity at the NE during G1 is required to maintain nuclear integrity and cell survival.

Key words: AKAP149, G1 phase, Nuclear envelope, Protein phosphatase, PP1

Introduction

Intracellular compartmentalization and specificity of cAMP signaling via cAMP-dependent protein kinase (PKA) occurs by association with A-kinase anchoring proteins, or AKAPs, at specific sites (Feliciello et al., 2001). AKAPs bind PKA regulatory subunit dimers through a consensus sequence, while the targeting domain of AKAPs determines localization to discrete compartments including the nuclear envelope (NE). In addition to anchoring PKA, AKAPs can also bind other kinases, protein phosphatases PP1, PP2A or PP2B (Feliciello et al., 2001) or phosphodiesterases (Dodge et al., 2001; Tasken et al., 2001). Consequently, AKAPs have emerged as scaffolding proteins that can integrate multiple signaling pathways (Smith and Scott, 2002).

Growing evidence suggests that the NE serves not only as a barrier separating the nucleus from the cytoplasm but also as a mediator of nuclear functions. The NE consists of two concentric membranes, nuclear pores and the nuclear lamina, a meshwork of intermediate filaments called A- and B-type lamins (Gruenbaum et al., 2000). The inner nuclear membrane (INM) harbors specific integral proteins that provide attachment sites for the lamina, transcriptional regulators, chromatin-associated proteins and DNA (Vlcek et al., 2001). The discovery that mutations in genes coding for lamin A/C and emerin (an integral protein of the INM) cause hereditary disorders affecting skeletal, cardiac and adipose tissues (Vigouroux and Bonne, 2002) suggests a role for the NE in the regulation of gene expression. Lamins also play more than a structural role in the nucleus. Nuclei reassembled in vitro without a lamina (Jenkins et al., 1995) or disorganization of the lamina with dominant negative lamin mutants was shown to alter DNA replication (Ellis et al., 1997; Spann et al., 1997; Moir et al., 2000). Intranuclear lamin foci also co-localize with RNA splicing factors, suggesting that lamins may contribute to organizing the RNA processing machinery (Jagatheesan et al., 1999).

Relatively few AKAPs have been identified at the NE. nAKAP150 (Zhang et al., 1996) and AKAP95 (Coghlan et al., 1994; Collas et al., 1999) have been localized to the nuclear matrix and thus interact with the NE. A 255 kDa AKAP (mAKAP) targets PKA and phosphodiesterase PDE4D3 near the ryanodine receptor at the NE of myocytes and was proposed to modulate activity of the receptor (Kapiloff et al., 2001; Dodge et al., 2001). AKAP149 is a 149 kDa AKAP recently identified as a component of the endoplasmic reticulum-NE system (Steen et al., 2000). AKAP149 also interacts with A- and B-type lamins (Steen and Collas, 2001), suggesting that AKAP149 is associated with both the outer and inner nuclear membranes.

PP1 belongs to the PPP family of protein Ser/Thr phosphatases. PP1 holoenzymes usually consist of a regulatory (R) and a catalytic subunit. Similarly to AKAPs, the R subunits can serve as moieties targeting PP1 to or near its substrate (Bollen, 2001). Most regulators of PP1 harbor a degenerate RV*X*F motif (where *X* is any amino acid) that binds to a hydrophobic pocket of PP1 (Bollen, 2001; Ceulemans et al., 2002a). This does not preclude, however, an association of PP1 with these R subunits via additional binding motifs. PP1 regulates a variety of cellular processes (Bollen, 2001; Cohen, 2002) and is involved in exit from mitosis (Tournebize et al., 1997; Sugiyama et al., 2002; Katayama et al., 2001). Nuclear PP1 has been implicated in the control of transcription,

pre-mRNA splicing and cell-cycle progression by dephosphorylation of key proteins such as RNA polymerase II, SR-splicing factors and the retinoblastoma protein (pRb) (Boudrez et al., 2000; Riedl and Egly, 2000; Rubin et al., 2001). AKAP149 interacts with a fraction of nuclear PP1 via a consensus 'RV*X*F' motif (^{155}KGVLF^{159}) and recruits the phosphatase to the NE upon nuclear reconstitution in vitro (Steen et al., 2000) and at the end of mitosis (Steen and Collas, 2001). Among the late mitotic substrates of PP1 are B-type lamins (Thompson et al., 1997) and recruitment of PP1 to nuclear membranes by AKAP149 is essential for B-type lamin assembly and cell survival (Steen and Collas, 2001).

The role of PP1 at the NE upon nuclear reformation suggests a tight regulation of PP1 activity at the end of mitosis and possibly also in G1 phase. We show here that NE-associated AKAP149 anchors PP1 at the NE throughout G1 and dissociates at the G1/S phase transition upon serine phosphorylation of AKAP149. Premature disruption of the AKAP149-PP1 interaction in G1 results in intranuclear lamina solubilization and G1 arrest. We also demonstrate that AKAP149 is a novel B-type lamin specifying subunit of PP1.

Materials and Methods

Reagents and antibodies

Streptavidin-agarose beads were from Promega (Madison, WI). [γ^{32}P]ATP was from DuPont NEN (Stockholm, Sweden). AKAP149-derived peptides ^{150}SSPKGVLFSS^{159} and ^{150}SSPKGALFSS^{159}, and NIPP1-derived peptides ^{197}KNSRVTFSED^{206} and ^{197}KNSRATASED^{206} ('RA*X*A') were as described previously (Steen et al., 2000; Beullens et al., 1999). Olomoucine was a gift from L. Meijer (CNRS, Roscoff, France). Anti-AKAP149 antibodies were from Transduction Laboratories (Lexington, KY). Anti-PP1 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) recognized all PP1 isoforms. Anti-phosphoserine (anti-pS) antibodies were from Zymed (San Francisco, CA). Antibodies against lamin B receptor (LBR), B-type lamins and lamin A/C were gifts from J.-C. Courvalin (Institut J. Monod, Paris, France) (Chaudhary and Courvalin, 1993; Buendia and Courvalin, 1997). mAb414 (Davis and Blobel, 1986) was a gift from M. Rout (Rockefeller University, New York, NY). Synthetic fragments of human NIPP1 (^{341}PGKKPTPSLLI^{351}), human Sds22 (^{338}QYRRKVMLALPSVR^{350}) and PNUTS (^{384}GPDPNQLTRKGR-KRKTVTWPEEGKL^{407}), coupled to keyhole limpet hemocyanin, were used to generate polyclonal antibodies in rabbits. Antibodies were affinity-purified on respective bovine serum albumin-coupled peptides linked to CNBr-activated Sepharose.

Cells and nuclei

HeLa cells were grown adherent in EMEM (Gibco-BRL) with 10% FCS (Steen and Collas, 2001). Cells were synchronized in M phase with 1 μM nocodazole for 18 hours. To allow cell cycle re-entry, cells were washed and replated at 2.5×10^6 cells per 162 cm^2 flask. 'Time zero' after mitotic release was time of replating. Nuclei were isolated by Dounce homogenization (Steen et al., 2000) at indicated time points after replating. For in vitro nuclear reconstitution assays, nuclei were isolated from confluent HeLa cell cultures by Dounce homogenization (Collas et al., 1999). NEs were prepared from purified nuclei as described previously (Steen et al., 2000).

Nuclear assembly assay

Condensed, membrane-free chromatin masses were prepared from HeLa nuclei disassembled in mitotic extract as described previously (Steen et al., 2000). After sedimentation at 1000 ***g*** through 1 M sucrose, chromosomes (2 μl) were resuspended in 40 μl interphase cytosolic HeLa cell extract (at 5000 chromatin masses/μl), containing 4 μl mitotic membranes, an ATP-regenerating system (1.2 μl) and 100 μM GTP (0.4 μl) (Steen et al., 2000). RV*X*F or RA*X*F peptides (10 μM) were added to the extract and the reaction was placed at 30°C for 2 hours. Nuclear assembly was examined by phase contrast microscopy, membrane labeling with 10 μg/ml $DiOC_6$ and by immunofluorescence.

In vitro replication and quantification of DNA synthesis

DNA replication was assayed in vitro in isolated nuclei by incorporation of [γ^{32}P]dCTP in an S-phase extract using a method derived from those reported previously (Krude et al., 1997; Stoeber et al., 1998). S-phase whole cell extracts were prepared from S-phase HeLa cells collected 15 hours after release from mitotic arrest. Cells were lysed by Dounce homogenization in cell lysis buffer (Martins et al., 2000), and then briefly sonicated on ice to lyse nuclei and release soluble nuclear components. The lysate was sedimented at 15,000 ***g*** for 15 minutes then at 200,000 ***g*** in a Beckman SW41 rotor for 2 hours at 4°C. Protein concentration of the extract was 25-30 mg/ml. G0-phase extracts were prepared as above from confluent HeLa cells serum-starved for five days. Nuclei purified from G1-phase cells (and capable of import; data not shown) were incubated at 30°C for 3 hours at 5000 nuclei/μl in 40 μl S-phase extract containing a mix of buffered dNTPs (40 mM Hepes, pH 7.8, 7 mM $MgCl_2$, 0.1 mM each of dATP, dGTP, dTTP and dCTP; 2 μl) (Krude et al., 1997), 1 μl [γ^{32}P]dCTP (3000 Ci/mmol; Nycomed-Amersham, Piscataway, NJ), the ATP-regenerating system and 100 μM GTP. At the end of incubation, samples were mixed with 1 volume of 20 mM Tris (pH 7.5) and 1 mg/ml proteinase K and digested for 2 hours at 37°C (Gant et al., 1999). Samples were mixed by pipetting and 5 μl aliquots were electrophoresed through 0.8% agarose. Gel loading was assessed by ethidium bromide staining. Samples contained equal numbers of nuclei and sedimentation steps were eliminated to avoid loss of nuclei (Gant et al., 1999). Signals were detected by autoradiography of the gels.

Immunological procedures

Immunofluorescence analysis of methanol-fixed cells or nuclei was performed as described previously (Steen and Collas, 2001). SDS-PAGE and immunoblotting analysis were carried out (Steen et al., 2000) using indicated antibodies. AKAP149 was immunoprecipitated from HeLa NEs after sonication and solubilization in immunoprecipitation buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT and protease inhibitors) (Steen and Collas, 2001). Immune precipitates (IPs) were washed three times in immunoprecipitation buffer and resuspended in 100 mM Tris-HCl (pH 7.5) for phosphatase assays or in SDS-sample buffer for SDS-PAGE. AKAP149, NIPP1, PNUTS and Sds22 were also immunoprecipitated from nuclear lysates as described previously (Steen et al., 2000), then washed and dissolved in SDS-sample buffer. B-type lamins were immunoprecipitated from solubilized NEs (Steen and Collas, 2001), IPs were resuspended in 100 mM Tris-HCl (pH 7.5), phosphorylated in vitro (see below) and used in phosphatase assays. For phosphorylation of immunoprecipitated B-type lamins, phosphatase inhibitors (50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate) were included in the immunoprecipitation buffer.

TUNEL analysis

Cells were fixed with 3% paraformaldehyde. Fragmented DNA was labeled with fluorescein-conjugated dUTP using the Roche

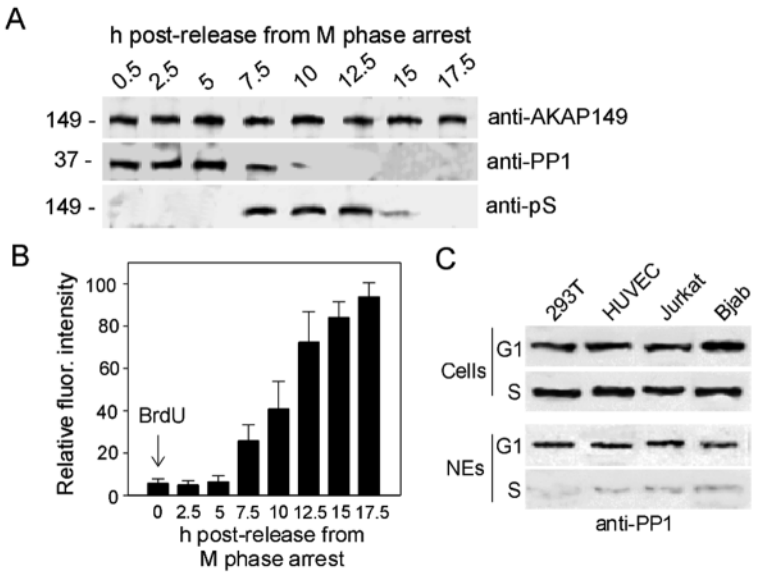

Fig. 1. PP1 transiently associates with the NE. (A) AKAP149 was immunoprecipitated from NEs after release of HeLa cells from mitotic arrest. Dissociation of PP1 from immunoprecipitated AKAP149 was monitored by immunoblotting. AKAP149 was also immunoblotted using anti-pS antibodies. Apparent M_r is shown in kDa. (B) Entry into the cell cycle after release from mitotic arrest was monitored by BrdU incorporation from time of release (arrow). Relative BrdU labeling intensity in two replicates is shown (n=40 cells/time point). (C) 293T, HUVEC, Jurkat and Bjab cells in G1 or S phase (2 and 15 hours post-release from mitotic arrest, respectively) were immunoblotted using anti-PP1 antibodies. NEs were prepared from the G1- and S-phase cells and immunoblotted.

(Indianapolis, IN) In Situ Cell Death Detection Kit. Cells were examined by fluorescence microscopy and images analyzed with the AnalySIS software.

Nuclear microinjection

HeLa cells released from nocodazole-induced mitotic arrest were plated onto coverslips. At indicated time points after replating, nuclei were injected (Collas et al., 1999) with 25 pl PBS containing 10 μg/ml 150 kDa FITC-dextran or 0.1% phenol red, as indicated, to visualize injections, and 100 nM of the indicated peptide. Cells were cultured in EMEM/10% FCS for indicated time periods without or with BrdU and processed for immunofluorescence or BrdU incorporation analysis (see below). S-phase cells were injected 12 hours after release from mitotic arrest. Between 45 and 55 cells were injected per treatment in two to three replicates.

Bromodeoxyuridine labeling

Following release from nocodazole-induced mitotic arrest, cells (injected or non-injected) were labeled with 100 μM BrdU (Sigma, St Louis, MO) for indicated time periods in culture. BrdU incorporation was visualized with FITC-conjugated anti-BrdU antibodies (Sigma) following methanol fixation.

Phosphatase assays

Dephosphorylation of ^{32}P-labeled phosphorylase *a* was done as described previously (Beullens et al., 1998) using as a source of phosphatase an AKAP149-IP from 2×10^6 nuclei per treatment. To assess B-type lamin dephosphorylation, B-type lamins were immunoprecipitated from 10^7 NEs. The IP was incubated for 30 minutes at 23°C in protein kinase C phosphorylation buffer (200 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4, 100 μM $CaCl_2$, 40 μg/ml phosphatidylserine, 20 μM diacylglycerol, 1 mM DTT, 10 μM ATP) containing 1 μCi/ml [γ^{32}P]ATP, 5 ng/μl rat αβγPKC and phosphatase inhibitors (50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate). Phosphorylated B-type lamin-IPs were sedimented, washed twice in 50 mM Tris-HCl/50 mM NaCl/0.01% Tween 20 with phosphatase inhibitors and once without inhibitors. IPs were then incubated with an AKAP149-IP from 10^7 cells in phosphatase assay buffer (25 mM Tris-HCl, pH 7.4, 3 mg/ml BSA, 1 mM DTT) for 15 minutes at 23°C with or without specified inhibitors or peptides. B-type lamin dephosphorylation was measured by scintillation counting of released ^{32}P (Beullens et al., 1998). Percentages of ^{32}P release were compared by Chi-square analysis of duplicate experiments.

Results

PP1 associates with NE-bound AKAP149 in G1 phase

AKAP149 associated with NE recruits a fraction of intranuclear PP1 to the NE upon nuclear reassembly in vitro and after mitosis (Steen et al., 2000). To determine whether PP1 interacts with AKAP149 at the NE during interphase, HeLa cells were released from a nocodazole-induced mitotic arrest, and at regular intervals AKAP149 was immunoprecipitated from solubilized NEs. Association of PP1 with anti-AKAP149 immune precipitates (AKAP149-IPs) was examined by western blot analysis. PP1 co-precipitated with AKAP149 up to 7.5 hours after release from mitotic arrest (Fig. 1A). At this time, PP1 dissociated from AKAP149, a process concomitant with entry into S phase, as shown by BrdU incorporation (Fig. 1B). Co-fractionation of PP1 with the NE in G1 but not in S phase was also shown in 293T fibroblasts, primary human umbilical vein (HUVEC) cells, a Jurkat T cell line and the B cell line Bjab (Fig. 1C). Furthermore, anti-phosphoserine (pS) immunoblots of AKAP149-IPs showed that AKAP149 was serine-phosphorylated at the G1/S phase transition at the time PP1 was released from the AKAP149-IP (Fig. 1A). AKAP149 was dephosphorylated during S phase. No threonine phosphorylation of AKAP149 was detected (data not shown). Therefore, after mitosis PP1 remains associated with the NE-bound AKAP149 complex in G1 phase but is released upon S phase entry.

AKAP149 is the only known binding protein of PP1 at the NE

Although most PP1 holoenzymes are heterodimers of a catalytic and an R subunit, some contain two R subunits (Bollen, 2001). Thus, we explored whether established nuclear R subunits of PP1, i.e. PNUTS, Sds22 and NIPP1 (Bollen and Beullens, 2002), were also part of the AKAP149-PP1 complex.

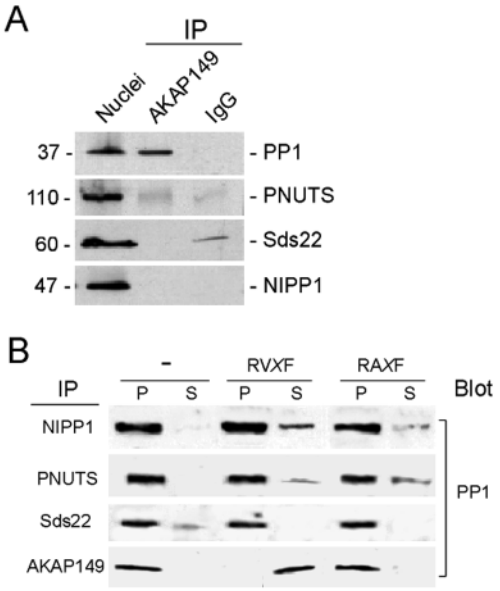

Fig. 2. AKAP149 does not co-precipitate with other known nuclear PP1 regulatory subunits. (A) Isolated nuclei and AKAP149-IPs from NEs were immunoblotted using antibodies against PP1, PNUTS, Sds22 and NIPP1. (B) 10 μM of AKAP149-derived RV*X*F or RA*X*F peptides were co-incubated with NIPP1-, PNUTS-, Sds22- or AKAP149-IPs for 1 hour, IPs were sedimented and dissociation of PP1 from the IPs was examined by western blotting of sedimented (P) and soluble (S) fractions.

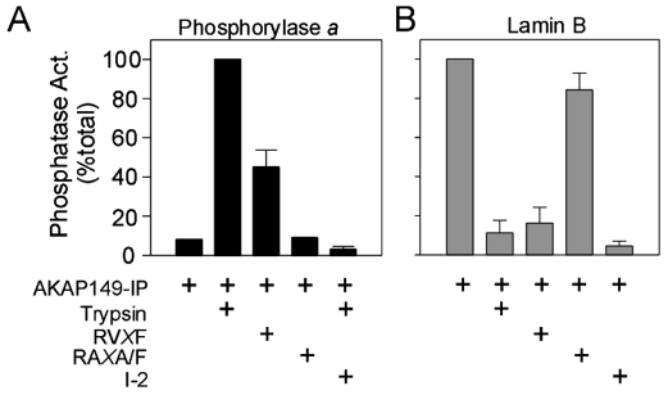

Fig. 3. AKAP149 is as a B-type lamin-specifier of PP1. (A) AKAP149-IPs were incubated with phosphorylase *a* and either 1 μM trypsin, 50 μM KNSRVTFSED ('RV*X*F') peptide, 50 μM KNSRATASED ('RA*X*A') peptide, or 1 μM I-2. Phosphorylase phosphatase activity was evaluated as the release of ^{32}P measured by scintillation counting and expressed as a percentage (±s.d.) of activity after trypsinization in duplicate experiments. (B) AKAP149-IPs were incubated with immunoprecipitated B-type lamins phosphorylated in vitro with rat αβγPKC and [γ^{32}P]ATP. B-type lamin phosphatase activity was measured as in (A) in duplicate experiments. 50 μM SSPKGVLFSS ('RV*X*F') or SSPKGALFSS ('RA*X*F') peptides were used.

PNUTS, Sds22 and NIPP1, present in HeLa nuclei, did not co-immunoprecipitate with AKAP149 from G1-phase nuclear extracts (Fig. 2A). Also, overlay assays of nuclear AKAP149-IPs using recombinant PP1 did not reveal any PP1-binding protein other than AKAP149 (data not shown). This suggests that the AKAP149-PP1 complex of the NE does not contain any other established nuclear PP1-R subunits.

Disruption of RV*X*F-motif-mediated association of PP1 with R subunits often perturbs the activity of the holoenzyme but not always results in the release of the catalytic subunit because most R subunits have multiple phosphatase interaction sites (Egloff et al., 1997). We determined the ability of the RV*X*F-motif-containing AKAP149 peptide ^{150}SSPKGVLFSS^{159} ('RV*X*F') to physically dissociate PP1 from NIPP1, PNUTS or Sds22 in vitro. Ten μM RV*X*F peptide dissociated PP1 from an AKAP149-IP, whereas a mutated version of this peptide (RA*X*F) (Steen et al., 2000) was ineffective (Fig. 2B). However, the RV*X*F peptide did not dislocate the bulk of PP1 from NIPP1- or PNUTS-IPs, despite the existence of RV*X*F motifs in these subunits (Ceulemans et al., 2002a) (Fig. 2B, bottom panel). RV*X*F peptides did not dissociate PP1 from Sds22, consistent with the lack of an RV*X*F sequence in this subunit (Ceulemans et al., 2002b). We concluded that the AKAP149-derived RV*X*F peptide was capable of displacing PP1 from AKAP149-IPs, but not from other known nuclear PP1-R subunits.

AKAP149 functions as a B-type lamin-specifying subunit of PP1

To determine whether AKAP149 regulated PP1 activity, we immunoprecipitated the AKAP149-PP1 complex from NEs (using anti-AKAP149 antibodies) as a source of phosphatase, and took advantage of the resistance of PP1 to trypsin (Beullens et al., 1998) to digest associated AKAP149 (Steen et al., 2000). Phosphatase activity of the complex before and after trypsin digestion was measured using ^{32}P-labeled glycogen phosphorylase *a* as a substrate. Fig. 3A shows that the complex was not capable of dephosphorylating phosphorylase *a*. However, trypsin digestion enhanced the phosphorylase phosphatase activity of the complex by 12-fold (P<0.001), and this activity was blocked by 1 μM inhibitor-2 (I-2), a specific inhibitor of PP1. Co-incubation of the AKAP149-PP1 complex with the peptide KNSRVTFSED, which comprises the PP1-binding RV*X*F motif of NIPP1 and competes with other RVXF-containing regulators for binding to PP1 (Beullens et al., 1999), also increased the phosphorylase phosphatase activity several-fold (P<0.001). On the other hand, an RA*X*F or RA*X*A peptide was without effect (Fig. 3A). This demonstrates that AKAP149 acts as an inhibitor of PP1 when glycogen phosphorylase *a* is used as a substrate.

To provide a physiological NE substrate for AKAP149-bound PP1, phosphatase activity towards immunoprecipitated B-type lamins was measured. Immunoprecipitated B-type lamins were phosphorylated and ^{32}P-labeled with PKC (Goss et al., 1994). The AKAP149-PP1 complex dephosphorylated B-type lamins and this activity was blocked by 1 μM I-2 (Fig. 3B; P<0.001). However, trypsin digestion abolished the B-type lamin phosphatase activity of the complex (P<0.001), suggesting that AKAP149 stimulates dephosphorylation of B-type lamins by PP1 (Fig. 3B). Disruption of the AKAP149-PP1 interaction with RV*X*F peptides also reduced B-type lamin dephosphorylation, whereas non-disrupting RA*X*F peptides had no or little effect (Fig. 3B). These data indicate that AKAP149 functions as a B-type lamin-specifying subunit of PP1.

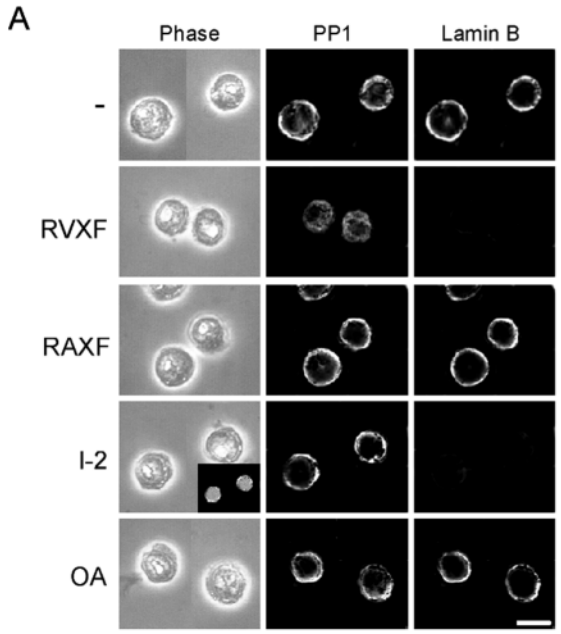

Fig. 4. Targeting of active PP1 to NE-bound AKAP149 is required for B-type lamin assembly in vitro. (A) Nuclei were assembled in a nuclear reconstitution assay containing no peptide (–), 10 μM RV*X*F or 10 μM RA*X*F peptide. Where indicated, 1 μM I-2 or 100 nM okadaic acid (OA) were added one hour after initiation of nuclear reformation in the absence of peptide. PP1 distribution and B-type lamin assembly were analyzed by immunofluorescence two hours after initiation of the assembly reaction. Inset, nuclear membrane staining with 10 μg/ml $DiOC_6$. Bar, 10 μm. (B) Percentages (±s.d.) of nuclei harboring perinuclear B-type lamin and perinuclear PP1 labeling under each condition (n~300 nuclei/treatment in duplicate experiments).

B-type lamin assembly requires AKAP149-mediated targeting of active PP1

Targeting of PP1 to nuclear membranes by AKAP149 upon nuclear reassembly in vitro and in vivo correlates with assembly of B-type lamins into the NE (Steen et al., 2000; Steen and Collas, 2001). We extended these studies to show that AKAP149-induced PP1 activity towards B-type lamins indeed promotes lamin assembly. Nuclear membrane formation, PP1 targeting to the NE, and lamin assembly were elicited in a nuclear reconstitution assay (Fig. 4A, top panels) (Steen et al., 2000). Consistent with our earlier findings, B-type lamin assembly was abolished with the RV*X*F-motif-containing AKAP149 peptide ($P<0.01$; Chi-square test), but not with the RA*X*F peptide (Fig. 4). Moreover, when 1 μM I-2 was added to the assay one hour after initiation of nuclear reformation, that is, after nuclear membranes were assembled but before lamin polymerization occurred (data not shown), B-type lamin assembly was inhibited despite the localization of PP1 at the NE (Fig. 4, I-2; $P<0.01$). In contrast, 100 nM okadaic acid, a PP2A inhibitor at this concentration, did not affect lamin assembly. Thus, sole targeting of PP1 to the NE is not sufficient for B-type lamin assembly into the NE, and PP1 activity is also required. This is consistent with our earlier finding that AKAP149 acts as a B-type lamin-specifier of PP1.

RV*X*F-motif-containing AKAP149 peptides abolish DNA synthesis

To address the significance of the association of PP1 with AKAP149 in G1 phase, we examined the effect of disrupting the AKAP149-PP1 interaction on progression into the cell cycle. AKAP149-derived RV*X*F peptides (100 nM), which dissociate PP1 from AKAP149 (see Fig. 2B), or 100 nM control RA*X*F peptides were injected into nuclei of HeLa cells in early G1, that is, within two hours of release from a nocodazole-induced mitotic arrest. Injections were confirmed by nuclear retention of a 150 kDa FITC-dextran (Fig. 5A). After eight hours of culture, cells injected with RV*X*F retained a round phenotype and remained in doublets resembling the cells in G1 at the time of injection (Fig. 5A). In contrast, mock- or RA*X*F-injected cells were flattened and noticeably larger, indicative of progression into the cell cycle. Moreover, culture of RV*X*F-injected cells with 10 μM BrdU for eight hours indicated that RV*X*F abolished DNA synthesis in >90% of the cells (Fig. 5A). However, 80-90% mock- or RA*X*F-injected cells underwent DNA synthesis, which was inhibited by 50 μM aphidicolin. Notably, RV*X*F injection into S-phase nuclei (12 hours after release from mitotic arrest) did not inhibit DNA replication (Fig. 5A). These data suggest that RV*X*F peptides injected into G1 nuclei inhibit cell cycle progression.

The effect of RV*X*F peptides on initiation of replication in G1-phase nuclei was examined in vitro. RV*X*F or control RA*X*F peptides (100 μM) were incubated for one hour with nuclei purified from G1-phase HeLa cells to allow their uptake into the nuclei. Nuclear uptake of fluorescent RA*X*F peptides was verified by fluorescence microscopy (data not shown). Mock (buffer)- or peptide-loaded G1 nuclei were incubated for three hours at 37°C in S-phase HeLa cell extract containing [γ^{32}P]-dCTP, dNTPs, GTP and an ATP-regenerating system. Synthesized DNA was examined by electrophoresis and autoradiography. G1-phase nuclei underwent replication in the S-phase extract (Fig. 5B, G1→S). Moreover, as initiation of replication requires the cyclin A/Cdk2 complex (Stoeber et al., 1998), we showed that DNA synthesis was inhibited with 10 μM of the Cdk2 inhibitor olomoucine (Stoeber et al., 1998) (Fig. 5C). Thus, DNA synthesis in G1-phase nuclei in our assay was due to initiation of replication. Peptide buffer or RA*X*F peptides did not affect replication (Fig. 5B). In contrast, RV*X*F abolished replication nearly completely (Fig. 5B). We ruled out the hypothesis that replication observed in G1 nuclei represented an elongation phase in already replicating nuclei, as G1 nuclei incubated in a extract from G0-phase HeLa cells did not replicate (Fig. 5B, G1→G0). Moreover, when introduced into S-phase nuclei, none of the peptides impaired DNA replication, indicating that the elongation phase of DNA replication was not affected by the RV*X*F peptide (Fig. 5D).

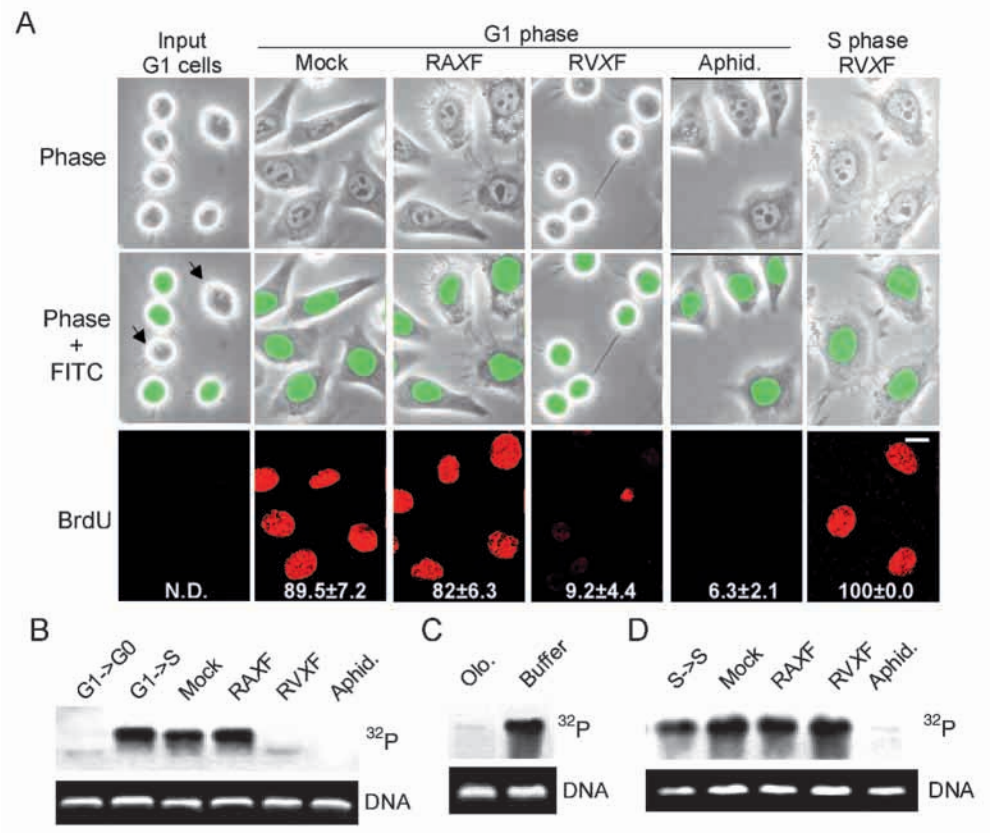

Fig. 5. AKAP149-derived RV*X*F peptides introduced into G1-phase nuclei inhibit DNA replication in vivo and in vitro. (A) Nuclei of early G1-phase HeLa cells were microinjected with peptide buffer (Mock), 10 nM RA*X*F or 10 nM RV*X*F peptide, together with a 150 kDa FITC-dextran (green label). Cells were cultured for eight hours with 100 μM BrdU. Mock-injected cells were also cultured with 100 μM BrdU and 50 μM aphidicolin (Aphid). BrdU incorporation was detected with anti-BrdU antibodies. Nuclei of S-phase cells were also injected with RV*X*F peptides, cultured for five hours and BrdU labeling monitored. Percentages (±s.d.) of cells labeled with BrdU are shown (n=40-45/treatment). Arrows point to non-injected cells. Bar, 10 μM. (B) Isolated G1-phase HeLa nuclei were loaded with RV*X*F or RA*X*F peptides or exposed to peptide buffer (Mock). Nuclei were incubated for three hours in S-phase extract containing [γ^{32}P]dCTP, dNTPs and an ATP-regenerating system. [γ^{32}P]dCTP incorporation was analyzed by autoradiography. G1-phase nuclei were also exposed to extract from G0- or S-phase cells under conditions promoting replication. (C) G1-phase nuclei were incubated in S-phase extract containing 0 mM or 1 mM olomoucine (Olo) and DNA synthesis was assessed as in A. (D) Nuclei purified from S-phase cells were loaded with peptides, incubated in S-phase extract and [γ^{32}P]dCTP incorporation into replicated DNA was evaluated as in B.

These results suggest that the dissociation of PP1 from AKAP149 mediated by RV*X*F peptide in G1 phase affects initiation of replication in vitro.

AKAP149 peptides containing RVXF motif elicit intranuclear lamin phosphorylation and solubilization in G1 phase

To identify possible reasons for RV*X*F peptide-mediated inhibition of DNA replication, immunofluorescence analysis of the NE in G1-phase cells within 30 minutes of intranuclear peptide injection was performed. In order not to interfere with immunofluorescence, peptide injection was monitored with phenol red (Fig. 6A). At the time of injection, A- and B-type lamins, lamin B receptor (LBR) and anti-mAb414-reactive nucleoporins (Nups) showed expected perinuclear distributions (Fig. 6A, Input G1 cells). Absence of soluble (phosphorylated) A- and B-type lamins at the time of peptide injection was demonstrated by the lack of ^{32}P incorporation into the lamins when cells were metabolically labeled between 0.5 and 2 hours after release from mitotic arrest (Fig. 6B, G1). This argued that the lamins were already incorporated into the lamina at the time of peptide injection. Note that cells labeled for 1.5 hours during mitotic block, as a control, contained phosphorylated lamins (Fig. 6B, M). Immunofluorescence labeling patterns of LBR or anti-mAb414-reactive nucleoporins were not altered by buffer (mock), RV*X*F or control RA*X*F peptide injection (Fig. 6A), suggesting that the peptides did not induce gross changes in NE structure. In contrast, lamin distribution was drastically affected by RV*X*F peptides (Fig. 6A). In >90% of RV*X*F-peptide-injected cells, both A- and B-type lamins were detected throughout the nucleoplasm (Fig. 6A). This contrasted with RA*X*F- or mock-injected cells, which displayed perinuclear lamin staining (Fig. 6A). Lamina disruption by RV*X*F peptides appeared to be specific for G1 because the peptides did not alter lamina organization after injection into S-phase nuclei (Fig. 6A).

Disruption of the nuclear lamina by RV*X*F peptides was also shown by western blot analysis. However, in this case, early G1 cells were transfected, rather than injected, with RV*X*F or RA*X*F peptides using the lipophilic reagent, DOTAP (Steen et al., 2000), starting 0.5 hour after release from mitotic arrest. After 1.5 hours of incubation with the DOTAP-peptide mix, nuclei were purified by careful Dounce homogenization (nuclei of RV*X*F-injected cells were fragile) and dissolved in SDS sample buffer or processed for isolation of NEs. Immunoblotting analyses indicate that nuclei contained AKAP149 and PP1 regardless of the peptide transfected (Fig. 7A). However, NEs were depleted of PP1 and both A- and B-type lamins (Fig. 7A). Immunofluorescence analysis of nuclei purified from cells transfected with RV*X*F peptides, but not RA*X*F peptides, indeed revealed strong intranuclear lamin labelling, whereas anti-LBR antibodies decorated the NE (Fig. 7B).

To determine whether nuclei containing RV*X*F peptides

harbored soluble (phosphorylated) lamins, peptides were transfected as described above into 2×10^7 early G1-phase cells that were simultaneously metabolically labeled with ^{32}P (Courvalin et al., 1992). Western blotting and autoradiography analysis of lamins immunoprecipitated from nuclear lysates 1.5-2 hours after start of transfection showed that A- and B-type lamins co-precipitated after RA*X*F peptide transfection (Fig. 7C, Blot) and essentially no lamin phosphorylation was detected, as expected from cells in G1 (Fig. 7C, ^{32}P). In contrast, RV*X*F peptides induced phosphorylation of A- and B-type lamins which, in addition, did not co-immunoprecipitate (Fig. 7C). Similar results were obtained later in G1, when cells were ^{32}P-labeled and transfected with peptides between 5 and 6.5 hours after release from mitotic arrest (data not shown). We concluded that RVXF peptides elicited lamin phosphorylation and released polymerized lamins, rather than prevented assembly of soluble lamins, when introduced into G1-phase nuclei. Nevertheless, the INM remains apparently intact and nuclear pore complex distribution unaffected.

The duration of RV*X*F-induced G1 arrest and whether the cells eventually re-entered the cell cycle were determined by prolonged (17 hour) culture with BrdU following RV*X*F peptide injection. Rather than replicating, the DNA condensed into apoptotic-like structures (Fig. 8A; $P<0.01$ compared to the RA*X*F control; Chi-square test). Apoptosis of all arrested cells was evidenced by TUNEL analysis (Fig. 8). Chromatin condensation and apoptosis were not observed after peptide injection into S-phase nuclei (Fig. 8).

Collectively, our results indicate that RV*X*F peptides, first, trigger dissociation of PP1 from AKAP149 within G1 nuclei, second, induce phosphorylation of A- and B-type lamins, third, cause disassembly of the lamins into the nuclear interior, and fourth, inhibit DNA synthesis in vitro and in vivo. None of these phenotypes were detected upon RV*X*F peptide introduction into S-phase nuclei. This suggests that dissociation of PP1 from NE-bound AKAP149 in G1 phase drastically destabilizes the nuclear lamina and as a result can prevent DNA replication.

Discussion

AKAP149 is a novel substrate-specifier of PP1

AKAP149 has all the hallmarks of an R subunit of PP1 (Bollen, 2001; Cohen, 2002). First, as with most regulators of PP1, AKAP149 contains an RV*X*F motif that mediates interaction with PP1. Second, disruption of the RV*X*F sequence completely abolishes binding to PP1, and a synthetic peptide containing the RV*X*F motif dissociates PP1 from AKAP149, indicating that the RV*X*F motif is the key PP1-binding site of AKAP149. Third, in the nucleus, AKAP149 anchors PP1 in a specific compartment, the NE, in close proximity to the PP1 substrate, B-type lamins. NE-associated AKAP149 also acts as a substrate-specifier of PP1: it increases PP1 activity towards B-type lamins but inhibits dephosphorylation of other substrates.

Other well-studied regulators of PP1 display similar properties. G-subunits anchor PP1 to glycogen particles and

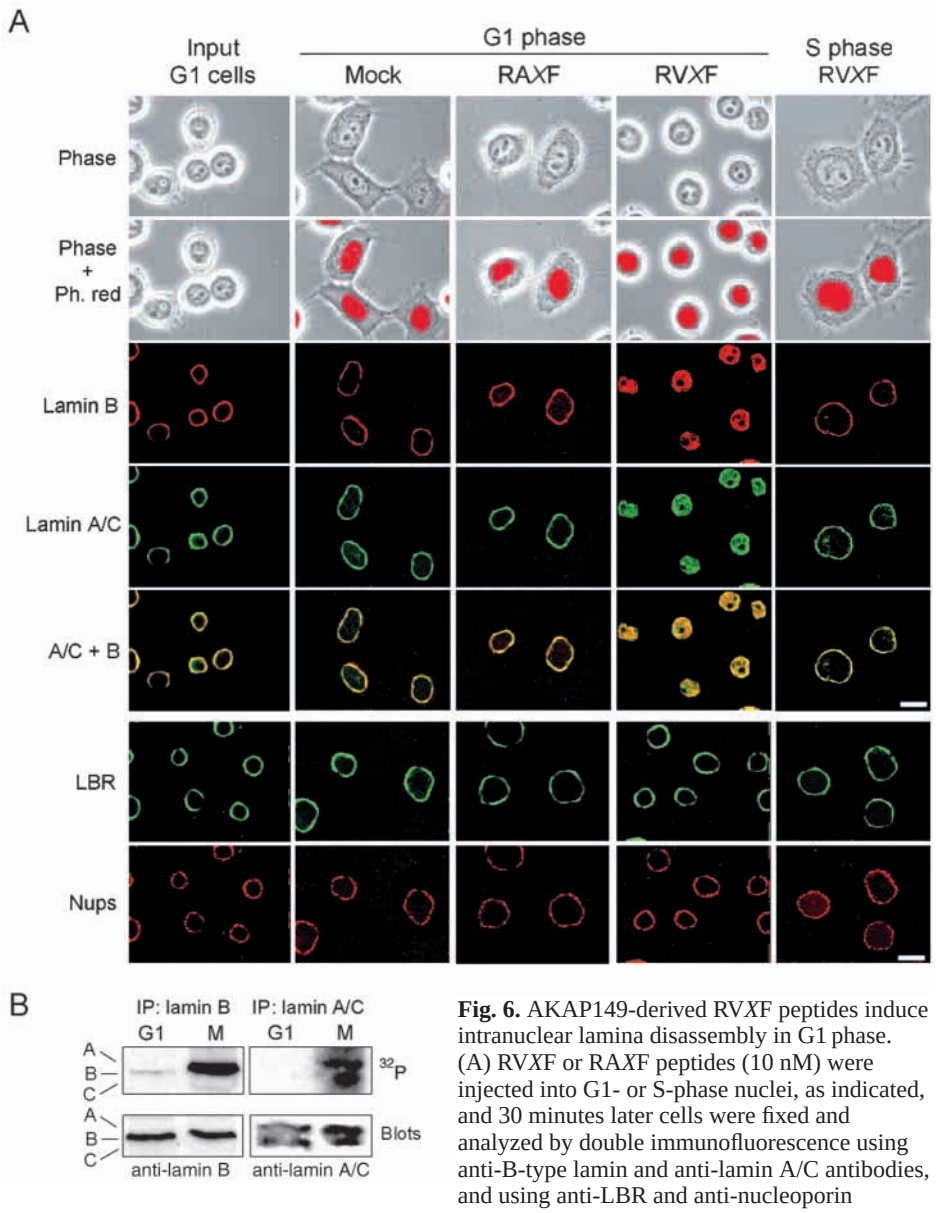

Fig. 6. AKAP149-derived RV*X*F peptides induce intranuclear lamina disassembly in G1 phase. (A) RV*X*F or RA*X*F peptides (10 nM) were injected into G1- or S-phase nuclei, as indicated, and 30 minutes later cells were fixed and analyzed by double immunofluorescence using anti-B-type lamin and anti-lamin A/C antibodies, and using anti-LBR and anti-nucleoporin (mAb414-reactive; Nups) antibodies. The injection solution contained phenol red (Ph, red). Between 45 and 55 cells were injected/treatment. Non-injected (input) G1 cells were also labeled. Bars, 10 μm. (B) Cells were metabolically labeled for 1.5 hours with ^{32}P between 0.5 and 2 hours after release from mitotic arrest (G1) or while under mitotic block (M). B- or A-type lamins were immunoprecipitated and IPs analyzed by autoradiography and immunoblotting. A, B and C point to lamin A, B-type lamins and lamin C, respectively.

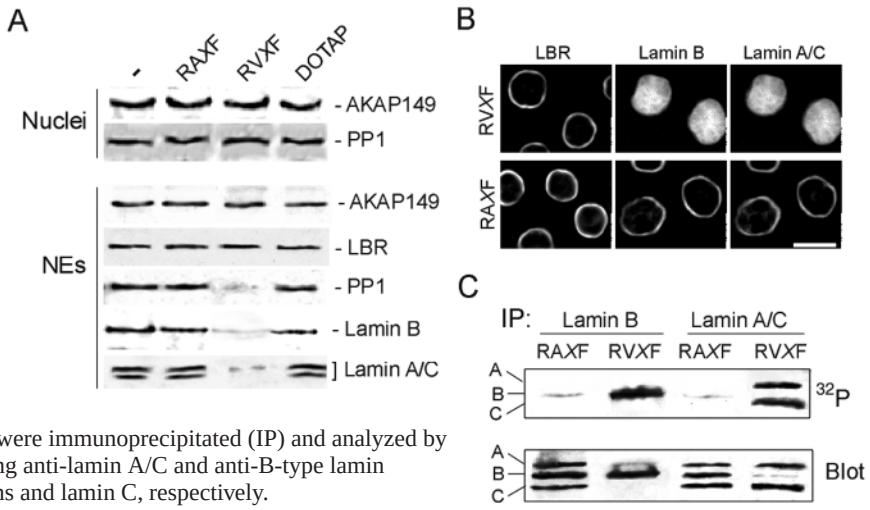

Fig. 7. Transfection of AKAP149-derived RV*X*F peptides in G1 phase elicits lamin phosphorylation and disassembly. (A) RA*X*F or RV*X*F peptides were transfected for 1.5 hours using DOTAP in early G1-phase HeLa cells starting at 0.5 hour after release from mitotic arrest. Nuclei and NEs were purified and proteins immunoblotted using indicated antibodies. – indicates untreated cells; DOTAP indicates cells exposed to DOTAP alone. (B) Nuclei isolated from RV*X*F- or RA*X*F-transfected G1 cells were analyzed by immunofluorescence using anti-B-type lamin and anti-lamin A/C antibodies. Bar, 10 μm. (C) G1-phase cells were transfected with peptides as in A, while under metabolic ^{32}P labeling. Nuclei were isolated, A- and B-type lamins were immunoprecipitated (IP) and analyzed by autoradiography (^{32}P) and immunoblotting (Blot) using anti-lamin A/C and anti-B-type lamin antibodies. A, B and C point to lamin A, B-type lamins and lamin C, respectively.

increase phosphatase activity towards glycogen synthase, but also inhibit dephosphorylation of glycogen phosphorylase (Armstrong et al., 1998; Liu and Brautigan, 2000). Similarly, MYPT-proteins target PP1 to myosin and enhance the myosin-phosphatase activity of PP1 while decreasing PP1 activity towards other substrates (Toth et al., 2000; Johnson et al., 1996). The distinct effects of these R subunits have been explained by their association with different sets of binding sites on PP1 (Bollen, 2001; Cohen, 2002). NE-bound AKAP149 might stimulate B-type lamin dephosphorylation by inducing conformational changes in PP1 to allow a better binding of the lamins. Indeed, NE-associated AKAP149 itself could also bind B-type lamins (it co-immunoprecipitates with lamins in interphase) (Steen and Collas, 2001) and target lamin phosphoserine(s) (Ottaviano and Gerace, 1985) to the catalytic site of PP1. As specifically NE-associated AKAP149-IPs were used in this study, it would be interesting to determine the substrates for the AKAP149-PP1 holoenzyme co-fractionating with the endoplasmic reticulum (R.L.S., M.B., H.B.L. et al., unpublished) and the nature of the regulation of PP1 by AKAP149.

Regulation of the AKAP149-PP1 interaction at the NE

Regulation of PP1 holoenzymes involves modulation of subunit interactions, which are often mediated by phosphorylation of the R subunits. In three unrelated R subunits phosphorylation of serine residues near or within the RV*X*F motif disrupts binding to PP1 (Beullens et al., 1999; Liu and Brautigan, 2000; McAvoy et al., 1999), altering the activity of the holoenzyme or resulting in a release of PP1. Serine phosphorylation of AKAP149 correlates with the release of PP1 from the NE-bound AKAP. Our previous (Steen and Collas, 2001) and current data suggest that the AKAP149-PP1 interaction at the NE is controlled in a cell-cycle- and phosphorylation-dependent manner. The role of AKAP149 phosphorylation on the regulation of its association with PP1 is currently being examined.

At the G1/S phase transition, phosphorylation of NE-associated AKAP149 correlates with the release of PP1 from the AKAP149 complex and downregulates PP1 activity towards assembled lamins. In spite of the down regulation of PP1, B-type lamins remain hypophosphorylated, probably because B-type lamin kinases are also downregulated. Furthermore, as S phase progresses, AKAP149 is dephosphorylated by an unidentified protein Ser/Thr phosphatase and remains dephosphorylated during the rest of interphase (R.L.S., M.B., H.B.L. et al., unpublished). Immunoprecipitation experiments indicate that dephosphorylation of AKAP149 during early S phase is not associated with the reformation of an AKAP149-PP1 complex at the NE, and the AKAP149-PP1 complex at the NE is not reformed until the end of mitosis (R.L.S., M.B., H.B.L. et al., unpublished). This suggests that the AKAP149-PP1 interaction at the NE is not solely controlled by phosphorylation of AKAP149: binding of PP1 could also be regulated by the

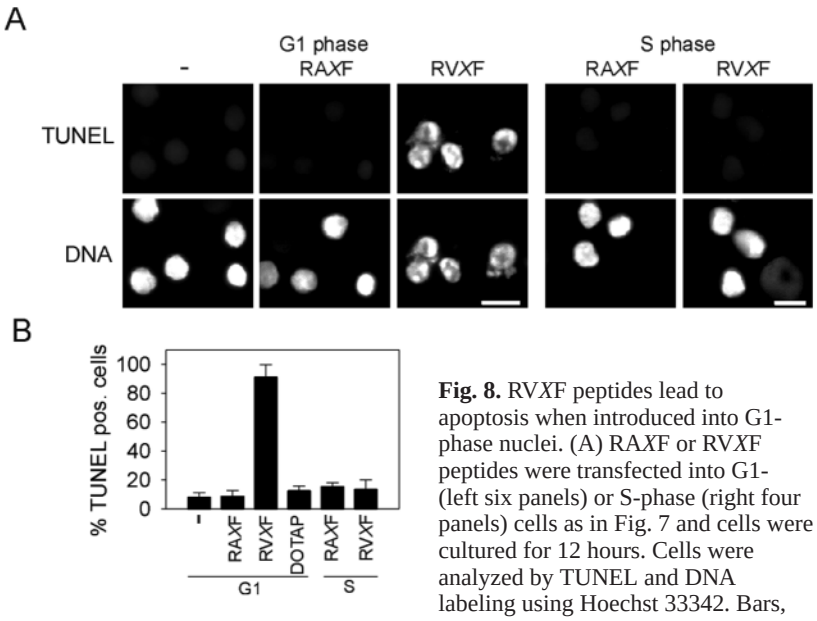

Fig. 8. RV*X*F peptides lead to apoptosis when introduced into G1-phase nuclei. (A) RA*X*F or RV*X*F peptides were transfected into G1- (left six panels) or S-phase (right four panels) cells as in Fig. 7 and cells were cultured for 12 hours. Cells were analyzed by TUNEL and DNA labeling using Hoechst 33342. Bars, 20 μm. (B) Proportions (±s.d.) of TUNEL-positive cells treated as indicated in G1 or S phase (*n*=300 cells/treatment in duplicate experiments).

occupation of PP1 binding sites by another protein, or by allosteric regulators. In this respect, it should be noted that most R subunits form multiple points of interaction with PP1; nevertheless, disruption of a single interaction site can be sufficient to weaken or destroy interaction with PP1 (Bollen, 2001).

AKAP149-mediated PP1 phosphatase activity towards B-type lamins in G1

B-type lamins are likely to be substrates for dephosphorylation by the AKAP149-PP1 holoenzyme, and this is expected to promote lamina polymerization and completion of NE assembly (Thompson et al., 1997; Steen and Collas, 2001). Dephosphorylation of disassembled B-type lamins promotes their polymerization into the reforming lamina during G1 (Gerace and Blobel, 1980). In contrast, B-type lamins that are synthesized de novo in S phase are not phosphorylated and therefore do not require a dephosphorylation step for polymerization. Consequently, the B-type lamin phosphatase activity of PP1 mediated by NE-associated AKAP149 appears to be dispensable beyond the G1/S phase transition. This could account for the lack of effect of RV*X*F peptides on NE organization in S-phase nuclei.

Our results argue that in G1, continuous dephosphorylation of B-type lamins at the NE needs to take place to maintain lamins in a polymerized form. Exogenous RV*X*F peptides have a dramatic effect on lamina structure: they elicit phosphorylation of A- and B-type lamins and their disassembly into the nuclear interior, as shown by ^{32}P incorporation, immunolabeling and lack of co-immunoprecipitation of A- and B-type lamins. Intranuclear, as opposed to cytoplasmic, solubilization of the lamins may be explained by the maintenance of intact nuclear membranes and of nuclear pore distribution, based on immunostaining of LBR and nucleoporins. The detrimental effects of RV*X*F peptides in G1 nuclei on lamina organization appear to be irreversible. Apoptosis takes place several hours after the G1 arrest phenotype is first observed. This contrasts with the rapid apoptosis occurring after nuclear membrane formation at mitosis exit, when B-type lamin assembly is prevented by the RV*X*F peptide (Steen and Collas, 2001). Interestingly, attempts to rescue the cells from G1 arrest simply by elevating intranuclear PP1 concentration by injection of purified PP1 into nuclei of arrested cells failed (R.L.S., M.B., H.B.L. et al., unpublished), suggesting that the amount of intranuclear PP1 activity per se is not a factor hindering cell cycle progression beyond G1.

Rather, we propose that the anchoring of PP1 in defined subnuclear loci is important for proper modulation of PP1 phosphatase activity towards specific substrates in G1-phase nuclei. Once the balance of PP1 activities is perturbed (by RV*X*F peptides) the cell does not enter S phase and commits to apoptosis. Thus, premature dissociation of PP1 from NE-associated AKAP149 in G1, and/or disruption of the intranuclear balance of PP1 activities with RV*X*F peptides are detrimental. It should be mentioned that although RV*X*F peptides do not disrupt the structure of known nuclear PP1 holoenzymes other than the AKAP149-PP1 complex (Fig. 2), mislocalization of AKAP149-bound PP1 and/or alteration of activity of other nuclear PP1-R holoenzymes by RV*X*F peptides may also account for the phenotypes reported.

We cannot at present exclude the possibility that AKAP149-PP1 is also involved in dephosphorylation of key regulatory proteins in addition to lamins, in particular in the late M and early G1 phases, when a burst of protein dephosphorylation occurs (Bollen and Beullens, 2002). These could in principle include lamina-associated proteins of the INM and chromatin. There is considerable evidence for essential functions of PP1 at these stages of the cell cycle in many organisms, although the involved R subunits remain to be identified (Tournebize et al., 1997; Hsu et al., 2000; Sugiyama et al., 2002). For example in G1, PP1 prevents premature entry into S phase by keeping pRb hypophosphorylated (Rubin et al., 2001). AKAP149-PP1 might contribute to modulating pRb phosphorylation and it remains possible that exogenous RV*X*F peptides affect pRb phosphorylation in G1. AKAP149-PP1 might also be involved in the attenuation of PKA-mediated CREB signaling, which results from CREB dephosphorylation by Ser/Thr phosphatases including PP1 (Mayr and Montminy, 2001). These alternatives remain to be examined.

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