Association of PP1 with Its Regulatory Subunit AKAP149 Is Regulated by Serine Phosphorylation Flanking the RV*X*F Motif of AKAP149[†]

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ABSTRACT: Reformation of the nuclear envelope at the end of mitosis involves the recruitment of the B-type lamin phosphatase PP1 to nuclear membranes by A-kinase anchoring protein AKAP149. PP1 remains associated to AKAP149 throughout G1 but dissociates from AKAP149 when AKAP149 is phosphorylated at the G1/S transition. We examine here the role of phosphorylation of serines flanking the RV*X*F PP1-binding motif of AKAP149, on PP1 anchoring. The use of AKAP149 peptides encompassing the RV*X*F motif and five flanking serines, either wild type (wt) or bearing S→A or S→D mutations, specifically shows that phosphorylation of S151 or S159 abolishes PP1 binding to immobilized AKAP149. Peptides with S151 or S159 as the only wt serine residue trigger dissociation of PP1 from immunoprecipitated AKAP149, whereas S151/159D mutants are ineffective. Furthermore, immunoprecipitated AKAP149 from purified G1-phase nuclear envelopes binds PKA and PKC in overlay assays. PKA binding to AKAP149 in vitro is unaffected by the presence of PKC or PP1, and similarly, PKC binding is independent of PKA or PP1. The immunoprecipitated AKAP149 complex contains PKA and PKC activities. Both AKAP149-associated PKA and PKC serine-phosphorylate immunoprecipitated AKAP149 in vitro; however, only PKC-mediated phosphorylation promotes dissociation of PP1 from the AKAP. The results suggest a putative temporally and spatially controlled mechanism promoting release of PP1 from AKAP149. AKAP149 emerges as a scaffolding protein for multiple protein kinases and phosphatases that may be involved in the integration of intracellular signals that converge at the nuclear envelope.

The nuclear envelope segregates chromosomes from the cytoplasm and contributes to nuclear functions such as replication and transcription through interactions of proteins of the inner nuclear membrane with A- and B-type lamins, chromatin, DNA, and transcriptional regulators (*1*, *2*). The nuclear envelope breaks down upon entry into mitosis, reassembles around each set of daughter chromosomes in telophase, and expands to accommodate nuclear growth in G1 and S phases (*1*, *2*). Because hyperphosphorylation of nuclear lamins is associated with lamina disassembly, continuous polymerization of nuclear lamins during G1 necessitates the presence of a lamin dephosphorylase activity at of the near the nuclear envelope (*3*).

Nuclear dynamics is controlled by an array of protein phosphatases and kinases, suggesting that several signaling pathways intersect at the nuclear envelope. One class of adaptor proteins tethering multiple signaling molecules includes A-kinase anchoring proteins, or AKAPs[1] (*4*). AKAPs mediate intracellular compartmentalization and temporal specificity of cAMP signaling by cAMP-dependent protein kinase (PKA) (*5*). AKAPs bind a PKA regulatory subunit dimer through a consensus sequence, while a targeting domain specifies subcellular localization. AKAPs also interact with other signaling molecules such as protein kinase C (PKC), phosphodiesterases, and protein phosphatases in a space- and time-regulated fashion (*4*, *6*).

Protein phosphatase 1 (PP1) is a Ser/Thr phosphatase consisting of a catalytic and a regulatory subunit. The latter serves as targeting module for the PP1 catalytic subunit to, or in vicinity of, its substrate. Most PP1 regulators contain a degenerate RV*X*F motif which interacts with the hydrophobic pocket of PP1; this, however, does not preclude an interaction of PP1 with other binding motifs (*7*, *8*). The regulatory subunit may also modulate the phosphatase activity of PP1 through inhibition or activation mechanisms. Interestingly, some regulatory subunits act as substrate-specifiers of PP1 and repress or stimulate PP1 activity in a substrate-dependent manner (*9*−*13*).

AKAP149 (*14*), also designated AKAP1, is a human 149-kDa anchoring protein homologous to mouse AKAP121 identified in mitochondria (*15*−*17*) and in the endoplasmic reticulum-nuclear envelope network (*18*). AKAP149 also harbors the hallmarks of a PP1 regulatory subunit (*13*, *18*). It contains an RV*X*F motif ($K^{153}GVLF^{157}$) that mediates interaction with PP1. Disruption of this sequence completely abolishes PP1 binding, indicating that the KGVLF motif is a key determinant in tethering PP1 to AKAP149 (*18*). AKAP149 targets a fraction of nuclear PP1 to the nuclear

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[1] Abbreviations: AKAP, A-kinase anchoring protein; $\alpha\beta\gamma$PKC, mix of α, β, and γ PKC isoforms; CDK, cyclin-dependent kinase; DTT, dithiothreitol; IP, immune precipitate; MYPT1, myosin phosphatase target subunit 1; *n*-OG, *n*-octyl glucoside; PKA, cAMP-dependent protein kinase; PKA-C, PKA catalytic subunit; PKC, protein kinase C.; PKI, inhibitor of PKA.; PP1, protein phosphatase 1; PDE, phosphodiesterase; pSer, phosphoserine.

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envelope and enhances PP1 phosphatase activity toward B-type lamins upon nuclear envelope reformation at mitosis exit, to promote lamin dephosphorylation and polymerization (*13*, *19*). In contrast, AKAP149 inhibits PP1 activity toward phosphorylase *a* in vitro; as such, AKAP149 qualifies as a substrate-specifying PP1 subunit (*13*).

Association of PP1 with AKAP149 at the nuclear envelope is maintained throughout G1 (*13*). The importance of this interaction to preserve nuclear integrity during G1 has been underscored by inhibition of cell cycle progression and depolymerization of the nuclear lamina after disrupting this association, thereby displacing a pool of nuclear PP1, specifically in G1 (*13*). However, PP1 is released from nuclear envelope-associated AKAP149 at the G1/S phase transition, concomitantly with Ser-phosphorylation of the AKAP (*13*). On the basis of these observations, we set out to determine the extent to which association of PP1 with nuclear envelope-bound AKAP149 was regulated by phosphorylation of AKAP149 in vitro. Using AKAP149-derived peptides encompassing the PP1-binding motif and bearing S→A or S→D (single letter amino acid code) substitutions, we show that phosphorylation of S151 or S159, on either side of the RV*X*F motif, is sufficient to inhibit PP1 binding to AKAP149. In addition, nuclear envelope-bound AKAP149 coimmunoprecipitates PKA and PKC activities. Both AKAP149-associated PKA and PKC can phosphorylate AKAP149; however, only PKC-mediated phosphorylation of AKAP149 promotes dissociation of PP1 from AKAP149 in vitro. Our results suggest a temporally and spatially controlled mechanism promoting release of PP1 from AKAP149 and provide insights on the nature of signaling pathways integrated by AKAP149 and that converge to the nuclear envelope.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies. Chelerythrine chloride, protein kinase A inhibitor (PKI), *n*-octyl glucoside (*n*-OG), and peroxidase-conjugated ExtrAvidin were from Sigma-Aldrich (St. Louis, MO). The PKC pseudosubstrate peptide PKC-(19−31), a cocktail of purified rat brain α, *β*, and *γ* isoforms of PKC (referred here to as α*β*γPKC), and recombinant PP1 were from Upstate Biotechnology (Lake Placid, NY). Purified human *β*II PKC was a gift from A. Fields (Mayo Clinic Comprehensive Cancer Center, Jacksonville, FL). Streptavidin-agarose beads were from Promega (Madison, WI). Autocamtide 3 was from Life Technologies (Bethesda, MD). [*γ*-^{32}P]ATP was from DuPont NEN (Berverly, MA). Cyclin-dependent kinase (CDK) inhibitors olomoucine and roscovitine were from L. Meijer (CNRS, Roscoff, France). Synthetic AKAP149 peptides, wild type (wt) and with S→A substitutions (Table 1), were biotinylated at the NH_2-terminal end. The AKAP149-derived RV*X*F peptide SSPKGVLFSS was as described (*18*). The Ht31 peptide, which binds the RII subunit of PKA and is commonly used as an AKAP−RII disruptor, and the control nondisruptor Ht31-P mutant peptide, were as described (*20*−*22*). Anti-*γ*-tubulin monoclonal antibody (mAb; clone GTU-88) was from Sigma-Aldrich. Anti-AKAP149 and anti-RIIα mAbs, and antibodies against various PKC isoforms (sampler kit), were from BD Biosciences (Santa Fe, CA). Recombinant human RIIα, rabbit polyclonal anti-RIIα antibodies and rabbit polyclonal antibodies against the PKA catalytic subunit (PKA-C) were gifts from K. Taskén (University of Oslo) (*23*, *24*). Antibodies against PKC (clone MC-5) and PP1 (rabbit polyclonal FL-18, mAb E−19) were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PP1 mAb recognized all PP1 catalytic subunits. Anti-phosphoserine (anti-pSer) antibodies were from Zymed (San Francisco, CA). Rabbit polyclonal antibodies against B-type lamins were a gift from B. Buendia (Institut J. Monod, Paris, France) (*25*).

Table 1. Mutational Analysis of AKAP149 Peptide Binding to PP1 with or without a PKC/CDK1 Kinase Mix[a]

	peptide sequence	PP1 binding	
		− kinase	+ kinase
P1	PLECPLSSP*KGVLF*SSKSAEVC	+	−
P2	PLECPLSSP*KGA*LFSSKSAEVC	−	−
P3	PLECPLAAP*KGVLF*AAKAAEVC	+	+
P4	PLECPLAAP*KGVLF*SSKSAEVC	+	−
P5	PLECPLAAP*KGVLF*AAKSAEVC	+	+
P6	PLECPLSSP*KGVLF*AAKAAEVC	+	−
P7	PLECPLASP*KGVLF*AAKAAEVC	+	−
P8	PLECPLSAP*KGVLF*AAKAAEVC	+	+
P9	PLECPLAAP*KGVLF*SSKAAEVC	+	−
P10	PLECPLAAP*KGVLF*ASKAAEVC	+	−
P11	PLECPLAAP*KGVLF*SAKAAEVC	+	+
P12	PLECPLSAP*KGVLF*SAKAAEVC	+	+
P13	PLECPLSAP*KGVLF*SSKAAEVC	+	−
P14	PLECPLSSP*KGVLF*SAKAAEVC	+	−
P15	PLECPLSDP*KGVLF*SDKSAEVC	−	−
P16	PLECPLSDP*KGVLF*SSKSAEVC	−	−
P17	PLECPLSSP*KGVLF*SDKSAEVC	−	−
P18	PLECPLDSP*KGVLF*SSKSAEVC	+	−
P19	PLECPLSSP*KGVLF*DSKSAEVC	+	−
P20	PLECPLSSP*KGVLF*SSKDAEVC	+	−

[a] Biotinylated AKAP149(144−165) peptides were used in overlays of immobilized PP1 with or without a PKC/CDK1 protein kinase mix. Binding was detected with peroxidase-conjugated streptavidin. Amino acids in italics denote the PP1 binding ('RV*X*F') domain or AKAP149. Underlinings denote mutated amino acids. Results from two to three replicates. A corresponding autoradiogram and overlay is shown in Figure 1B.

Cells, Nuclei and Nuclear Envelopes. HeLa cells were grown adherent in DMEM (Life Technologies) with 10% fetal calf serum. For G1 phase synchronization, cells were first arrested in M phase with 1 *μ*M nocodazole for 16 h. To allow cell cycle re-entry, cells were washed and replated at 2.5×10^6 cells per 175 cm^2 flask. Cells in G1 phase were harvested within 3 h of replating (*13*). Nuclei were isolated from G1 cells (or from confluent cell cultures, as indicated) by Dounce homogenization using a tight-fitting glass pestle, and nuclear envelopes were prepared as described (*18*). Purified nuclei and nuclear envelopes were either analyzed by SDS−PAGE or solubilized as described below for immunoprecipitations.

AKAP149 Peptide Phosphorylation. Phosphorylation of biotinylated AKAP149 peptides was carried out using a PKC and CDK1 protein kinase mix. Peptides (10 *μ*M) were phosphorylated by 5 ng/*μ*L rat α*β*γPKC and 25 ng purified CDK1 for 30 min in 200 mM NaCl, 50 mM Tris-HCl (pH 7.4), 80 *μ*M *β*-glycerophosphate, 10 mM $MgSO_4$, 100 *μ*M $CaCl_2$, 40 *μ*g/mL phosphatidylserine, 20 *μ*M diacyglycerol, 80 *μ*M *β*-glycerophosphate, 1 mM dithiothreitol (DTT), 100 *μ*M ATP, 0.01% Tween 20, and phosphatase inhibitors (*26*). [*γ*-^{32}P]ATP (1 *μ*Ci/mL) was added for autoradiography analyses, in which case 10 *μ*M cold ATP was used. Phosphorylated peptides were sedimented using Streptavidin-

agarose beads, washed in 200 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.01% Tween 20, and phosphatase inhibitors, and analyzed by autoradiography after spotting onto nitrocellulose or by scintillation counting.

N-Octyl Glucoside Extraction of Nuclear Envelopes. Nuclear envelopes isolated from 50 000 nuclei purified from G1 phase HeLa cells were digested for 1 h with 100 μg/mL proteinase K in 10 mM Tris-HCl (pH 7.2)/100 mM NaCl containing 250 mM $MgCl_2$, sedimented at 15 000*g* and washed by suspension and sedimentation in 10 mM Tris/100 mM NaCl (*27*). This removed proteins, including endogenous PKC. Membrane lipids were extracted for 1 h at room temperature with 2% *n*-OG in 250 μL of extraction buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 0.1% β-mercaptoethanol) (*27*). The detergent was removed by overnight dialysis against extraction buffer at 4 °C. One-half of the dialyzed fraction ('*n*-OG extract 1') was used as a competitor for PKC binding in a single PKC overlay. A control, noncompeting fraction was also prepared by leaving *n*-OG in the fraction, thus preventing lipid reconstitution and PKC binding ('*n*-OG extract 2'). This fraction was dialyzed as above against extraction buffer containing 2% *n*-OG.

Protein Kinase and Phosphatase Assays. PKC activity associated with AKAP149-IPs was assessed by phosphorylation of immunoprecipitated B-type lamins. AKAP149-IPs from nuclear envelopes purified from 10^7 HeLa cells in G1 phase were incubated for 30 min with immunoprecipitated B-type lamins (see below) in PKC phosphorylation buffer containing 5 ng/μL rat $\alpha\beta\gamma$PKC and 10 μCi/mL [γ-^{32}P]ATP (*26*), and lamin phosphorylation was assessed by autoradiography. In vitro phosphorylation of immunoprecipitated AKAP149 (from $\sim 5 \times 10^7$ nuclear envelopes) by 5 ng/μL rat $\alpha\beta\gamma$PKC was performed in PKC phosphorylation buffer containing 10 μCi/mL [γ-^{32}P]ATP. Phosphorylated substrates were washed in the presence of phosphatase inhibitors before analysis by autoradiography. When indicated, 10 μM chelerythrine chloride was added to the reaction. Phosphatase activity associated with AKAP149-IPs was determined using as a substrate, immunoprecipitated and in vitro phosphorylated B-type lamins, as described previously (*13*). PKA phosphorylation reactions were carried out using 1.5 ng/μL recombinant PKA catalytic subunit in PKA phosphorylation buffer containing 10 μCi/mL [γ-^{32}P]ATP (*26*).

Immunological Procedures. SDS−PAGE and immunoblotting analysis were performed as described (*18*) using indicated antibodies. AKAP149 was immunoprecipitated from G1 phase purified HeLa cell nuclei or nuclear envelopes, as indicated, after 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) (*18*). B-type lamins were immunoprecipitated from solubilized nuclear envelopes isolated from confluent HeLa cell cultures (*13*) and used as substrate for phosphorylation studies. When required, phosphatase inhibitors (50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate) were included in the immunoprecipitation buffer. RIIα, PKC, and PP1 were immunoprecipitated from nuclear envelopes purified from G1 phase nuclei as described (*18*) using 1:40 antibody dilutions.

Microcystin−Sepharose Affinity Purification of PP1. Purification of PP1 on microcystin conjugated to sepharose beads was done as described (*18*, *28*). In short, nuclei purified from G1-phase HeLa cells were harvested and lysed in immunoprecipitation buffer. Lysates (250 μL; 4 mg protein) were cleared with protein A-sepharose beads for 30 min at 4 °C and incubated with 25 μL of a 50% slurry of microcystin-sepharose beads (Upstate Biotechnology) for 1 h. Beads were washed with buffer M (50 mM triethanolamine, pH 7.5, 0.1 mM EGTA, 5% glycerol, 0.5 M NaCl, 0.1% β-mercaptoethanol, and protease inhibitors) to remove unbound proteins. Affinity-purified PP1 and bound proteins were eluted with 3 M NaSCN in buffer M. Input nuclear lysates, unbound fractions and bound eluates were subjected to immunoblotting. Control nuclear lysates samples were incubated with protein A-sepharose beads and processed as above.

In Vitro Binding Assays. For RIIα and PKC overlays, AKAP149-IPs were resolved by SDS PAGE and immobilized on nitrocellulose (*18*). RII binding was detected using recombinant ^{32}P-labeled human RIIα as a probe as described earlier (*29*). For PKC overlays, the nitrocellulose was incubated with 100 μM rat $\alpha\beta\gamma$PKC or 100 μM human βII-PKC for 1 h in Tris-buffered saline/0.01% Tween 20 (TBST) and washed in TBST, and PKC binding was detected using anti-PKC mAbs (1:500 dilution) and peroxidase-conjugated anti-mouse antibodies. For AKAP149 peptide overlays, 5 U recombinant PP1 were spotted onto nitrocellulose and overlaid for 1 h with 10 μM biotinylated AKAP149 peptides. Peptides were used following a 30-min phosphorylation reaction with a PKC/CDK1 kinase mix as described above. Peptide binding was detected using peroxidase-conjugated ExtrAvidin. Control peptides were incubated in phosphorylation buffer without kinases. Membranes were washed in 50 mM Tris-HCl (pH 7.4)/50 mM NaCl/0.01% Tween 20 containing phosphatase inhibitors and binding was detected using peroxidase-conjugated ExtrAvidin.

RESULTS

We have previously shown that a fraction of nuclear PP1 is recruited to nuclear envelope-associated AKAP149 in late mitosis-early G1, a step necessary for lamin polymerization (*19*). This interaction is essential for maintaining nuclear integrity during G1 (*13*). PP1 dissociates from AKAP149 at the G1/S phase transition concomitantly with Ser-phosphorylation of AKAP149 (*13*). On the basis of these observations, we addressed the extent to which interaction of PP1 with nuclear envelope-bound AKAP149 is, in vitro, regulated by phosphorylation of AKAP149.

Phosphorylation of Serines Flanking the RVXF Motif of AKAP149 Peptide Inhibits Interaction with PP1 In Vitro. Many PP1 holoenzymes are controlled by reversible phosphorylation of their regulatory subunits (*7*). Most regulators of PP1, including AKAP149, contain a degenerate RV*X*F sequence that binds PP1 (*7*, *8*). For a number of these regulatory subunits, phosphorylation of serine residues within or close to the RV*X*F motif disrupts binding of this motif to PP1. The RV*X*F sequence of AKAP149 is flanked by five serines ($S^{150}S^{151}$PKGVLF$S^{158}S^{159}$KS^{161}) that qualify as potential phosphorylation sites for PKC, caseine kinases, and CDKs (Table 1).

To determine whether binding to PP1 of AKAP149 peptides harboring the RV*X*F motif and flanking residues

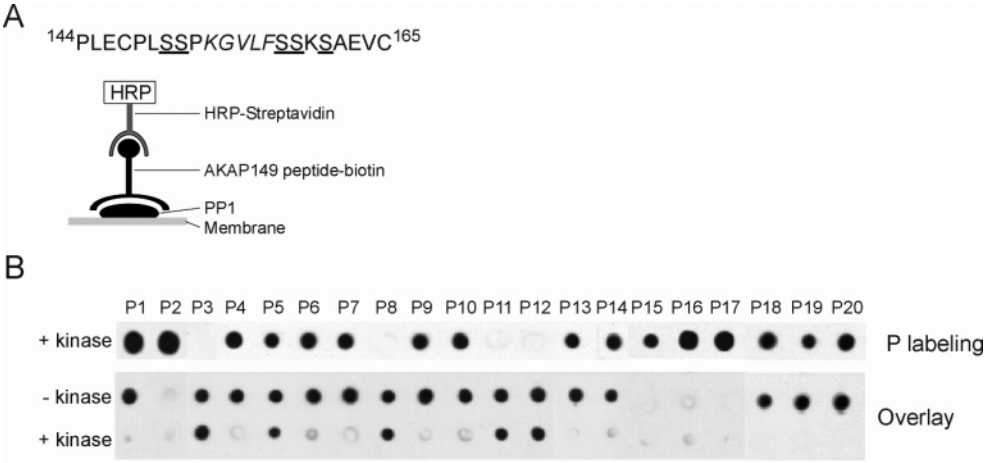

FIGURE 1: Phosphorylation of AKAP149 peptides on S151 or S159 inhibits binding to PP1. (A) Principle of AKAP149 peptide overlay of immobilized PP1. (B) Biotinylated AKAP149(144−165) peptides (see Table 1) were phosphorylated with a PKC/CDK1 protein kinase mix and [γ-^{32}P]ATP in vitro. Peptide phosphorylation was assessed by autoradiography of dot-blotted peptides (^{32}P labeling). Note that a mix of, presumably, phosphorylated and nonphosphorylated peptides was used here, as no separation of phosphorylated from potentially unphosphorylated peptides was done. Immobilized recombinant PP1 was overlaid with AKAP149 peptides (P1−P20) after incubation of the peptides with PKC/CDK1 kinase mix under phosphorylation conditions (+kinase) or in kinase buffer alone (−kinase). Peptide binding was detected with peroxidase-conjugated streptavidin (overlay).

was affected by serine phosphorylation, biotinylated AKAP149(144−165) peptides (designated peptides P1−P20) were synthesized. The peptides were either wild type or contained single or multiple combinations of S→A mutations to destroy a phosphorylation site, or S→D mutations to constitutively mimic a phosphorylated Ser (Table 1). Binding to PP1 was examined in an overlay assay in which immobilized recombinant PP1 was overlaid with biotinylated AKAP149 peptides. Peptide binding was detected using HRP-conjugated streptavidin (Figure 1A). AKAP149 peptides were phosphorylated in vitro by a mix of purified PKC and CDK1. Phosphorylation was assessed by scintillation counting (data not shown) and demonstrated qualitatively by autoradiography of dot-blotted peptides (most likely representing a mixture of phosphopeptides and nonphosphopeptides; Figure 1B, upper panel). Filters containing immobilized PP1 were overlaid with each peptide following preincubation of the peptides without or with the kinase mix under phosphorylation conditions. Binding results are shown in Figure 1B (lower panel) and summarized in Table 1. Phosphorylation of wild type (wt) peptide P1 abolished binding to PP1, whereas preincubation of P3 (in which all five serines were mutated to alanines) with the kinase mix did not affect its ability to bind PP1. Thus, binding of AKAP149 peptides to immobilized PP1 is regulated by serine phosphorylation near the RV*X*F motif. Figure 1B also shows that S151, S159, and S161 were phosphorylated by the kinase mix (peptides P3 to P14; Table 1). Of these three sites, only S151 or S159 phosphorylation disrupted the peptide-PP1 interaction (Figure 1B). Phosphorylation of S161 did not affect the interaction. Therefore, S151 or S159 is implicated in protein kinase-mediated control of PP1 binding to AKAP149 peptides. S150 and S158 are not phosphorylated by the kinase mix (Figure 1B). Moreover, a kinase-mediated modulation of binding could be confirmed by S→D mutations on S151 or S159 (peptides P16, P17 and P15), which mimics constitutive phosphorylation of these sites by introducing a negative charge while maintaining the mass of a phosphorylated Ser (Figure 1B). Last, S→D substitutions on S150, S158 and S161 did not abolish binding to PP1 in the absence of protein kinase mix (Figure 1B; peptides P18−P20), ruling out a role of phosphorylation of these residues as a modulator of binding to PP1.

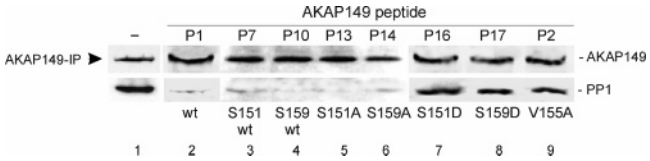

FIGURE 2: A G1-phase HeLa cell nuclear extract was incubated for 1 h without peptide (lane 1) or with indicated AKAP149(144−155) peptides (lanes 2−9). Nonphosphorylated peptides were used. Endogenous AKAP149 was immunoprecipitated and association of PP1 was monitored by immunoblotting of the immune precipitates using rabbit polyclonal anti-PP1 antibodies. Mutations carried by the peptides are shown below the blots. Wt, wild type.

The role of S151 and S159 phosphorylation in the disruption of the AKAP149-PP1 complex was examined in a binding competition assay. Nuclear extracts prepared from G1-phase HeLa cells (to ensure an association of PP1 with nuclear envelope-bound AKAP149) (*13*) were incubated for 1 h with 10 μM AKAP149 peptides. Endogenous AKAP149 was immunoprecipitated and coprecipitation of PP1 was monitored by immunoblotting. As expected, AKAP149 coimmunoprecipitated PP1 (Figure 2, lane 1). However, PP1 no longer coprecipitated with AKAP149 after preincubation with P1 (wt peptide), P7 or P10 (containing only S151wt or S159wt, respectively), and with peptides bearing the S151A (P13) or S159A (P14) mutations in combination with S161A (Figure 2). In contrast, peptides that harbored S151D (P16), S159D (P17) or V155A (a mutation in the RV*X*F PP1-binding motif that abolishes interaction with PP1; peptide P2) mutations did not affect coprecipitation of PP1 with AKAP149 (Figure 2). We concluded that binding of AKAP149(144−165) peptides to PP1 is modulated by phosphorylation of the peptide on S151 or S159.

The AKAP149 Complex of the Nuclear Envelope Contains PKA Activity. In the identification of a putative kinase associated with AKAP149, that might mediate AKAP149 phosphorylation, we first examined PKA. Indeed, AKAP149 has been shown to bind the RIIα subunit of PKA with a nM

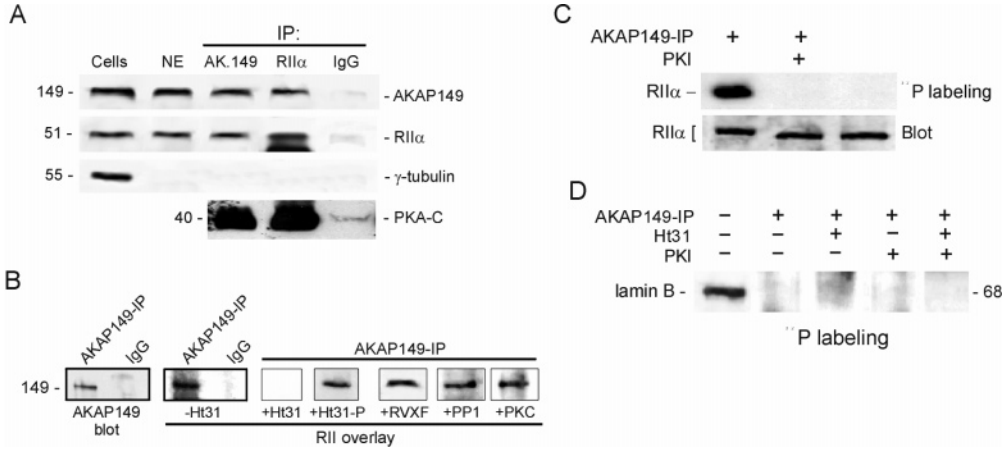

FIGURE 3: The nuclear envelope-associated AKAP149 complex harbors PKA activity. (A) AKAP149 or PKA-RIIα was immunoprecipitated (IP) from nuclear envelopes of G1-phase nuclei using anti-AKAP149 mAbs and anti-RIIα polyclonal antibodies, respectively. Immune precipitates were immunoblotted using anti-AKAP149 mAbs, anti-RIIα mAbs, anti-PKA-C antibodies or anti-γ-tubulin antibodies. IgG, control immunoprecipitation with nonimmune mouse IgGs. NE, nuclear envelopes. (B) AKAP149-IPs or control precipitates (IgG) immobilized on nitrocellulose were incubated with ^{32}P-labeled RIIα alone or together with 10 μM Ht31, 10 μM Ht31-P, 10 μM AKAP149-derived RV*X*F peptide, 5 U recombinant PP1 or 10 μM purified human βII PKC. RII binding was detected by autoradiography. (C) An AKAP149-IP was incubated with [γ-^{32}P]ATP with or without 100 μM PKI under PKA phosphorylation conditions. RIIα autophosphorylation was detected by autoradiography (^{32}P labeling). RIIα was also detected by immunoblotting (anti-RIIα mAbs; Blot) after sedimentation of the AKAP149-IP. (D) PKA does not affect the phosphatase activity of PP1 toward B-type lamins. An AKAP149-IP and immunoprecipitated B-type lamins phosphorylated by purified PKC in vitro were mixed in buffer containing 10 μM chelerythrine chloride to inhibit AKAP149-associated PKC, and 10 μM Ht31 or 100 μM PKI. Dephosphorylation of B-type lamins was visualized by autoradiography.

affinity in vitro (*30*). AKAP149 also binds RI isoforms (*31*). Further evidence argues that AKAP149 targets at least some RIIα to the nuclear envelope. First, AKAP149 and RIIα coimmunoprecipitated from purified nuclear envelopes using antibodies to either protein (Figure 3A). Blotting immune precipitates with an antibody against γ-tubulin indicated that nuclear envelope-associated RIIα did not originate from centrosomes (*32*−*34*). Second, as expected, immunoprecipitation of AKAP149 or RIIα coprecipitated the PKA catalytic subunit PKA-C (Figure 3A). Third, ^{32}P-labeled RIIα bound to immunoprecipitated AKAP149 in an overlay assay and binding was inhibited by 10 μM of the AKAP−RII anchoring competitor peptide Ht31, but not by the Ht31-P mutant peptide (Figure 3B). Binding of RIIα was not affected by addition of 10 μM of an AKAP149-derived PP1-binding RV*X*F peptide (SSPKGVLFSS) or of 5 U recombinant PP1 in the assay (Figure 3B). Fourth, anti-AKAP149 antibodies immunoprecipitated PKA activity, as shown by autophosphorylation of AKAP149-bound RIIα following stimulation of the complex with cAMP (Figure 3C). Phosphorylation of AKAP149 and RIIα was inhibited by 100 μM of the PKA-specific inhibitor PKI (Figure 3C).

AKAP149-bound PKA did not affect the PP1 phosphatase activity previously found to be associated with AKAP149 (*18*). AKAP149-IPs can dephosphorylate immunoprecipitated, in vitro phosphorylated, B-type lamins, an activity mediated by PP1 (*13*). This observation was confirmed here (Figure 3D, lanes 1, 2). Furthermore, dephosphorylation of B-type lamins by the AKAP149-PP1 holoenzyme was not impaired by 10 μM of the AKAP−RII anchoring disruptor peptide Ht31 and/or by 100 μM PKI in the assay (Figure 3D). Collectively, these results indicate that nuclear envelope-associated AKAP149 anchors PKA, and this association is compatible with the phosphatase activity of PP1 toward B-type lamins.

Nuclear Envelope-Associated AKAP149 Anchors PKC Activity. PKC is also a known interphase B-type lamin kinase (*35*), although the role of interphase lamin phosphorylation remains unclear. We examined by immunoprecipitation whether PKC was associated with the AKAP149 complex of the nuclear envelope. Figure 4A shows that PKC cofractionated with nuclei and nuclear envelopes isolated from confluent HeLa cells and coimmunoprecipitated with nuclear envelope-associated AKAP149. Furthermore, an immunoblot of purified HeLa cell nuclear envelopes using antibodies against various PKC isoforms showed that PKCα and the atypical PKC isoforms PKCι and PKCλ were detected at the nuclear envelope (Figure 4B). In addition, PKCα coimmunoprecipitated with AKAP149 from a purified nuclear envelope preparation (Figure 4C). Whether PKC directly bound to AKAP149 was determined in a PKC overlay assay of AKAP149 using two forms of purified PKC. Both rat αβγPKC and human βII PKC were found to bind to immunoprecipitated and immobilized AKAP149 (Figure 4D, upper and lower panels, respectively). Binding of these PKC isoforms to AKAP149 was competed in the assay by a 2% *n*-octyl glucoside (*n*-OG)-soluble and reconstituted nuclear membrane lipid fraction previously shown to anchor and bind PKC (*36*) (Figure 4D, *n*-OG extract 1). However, a control (*n*-OG-soluble, nonreconstituted) fraction not binding PKC had no effect (Figure 4D, *n*-OG extract 2). Last, PKC binding was not affected by adding 10 μM RV*X*F peptide, 10 μM Ht31 peptide, 10 μM recombinant RIIα, or 1 μM recombinant PP1 to the assay (Figure 4D).

We next determined whether AKAP149 immunoprecipitated from nuclear envelopes harbored PKC activity. Incubation of AKAP149-IPs with Ca^{2+} and diacylglycerol caused phosphorylation of an immunoprecipitated B-type lamin substrate (Figure 4E). Scanning densitometry of autoradiograms (Figure 4F) showed that this kinase activity was inhibited by ~80% with 10 μM chelerythrine chloride (a specific PKC inhibitor) or 50 μM of the pseudosubstrate PKC inhibitor peptide PKC(19−31) (Figure 4E,F). Under these conditions, 1 mM of the CDK inhibitor roscovitine, 50 μM

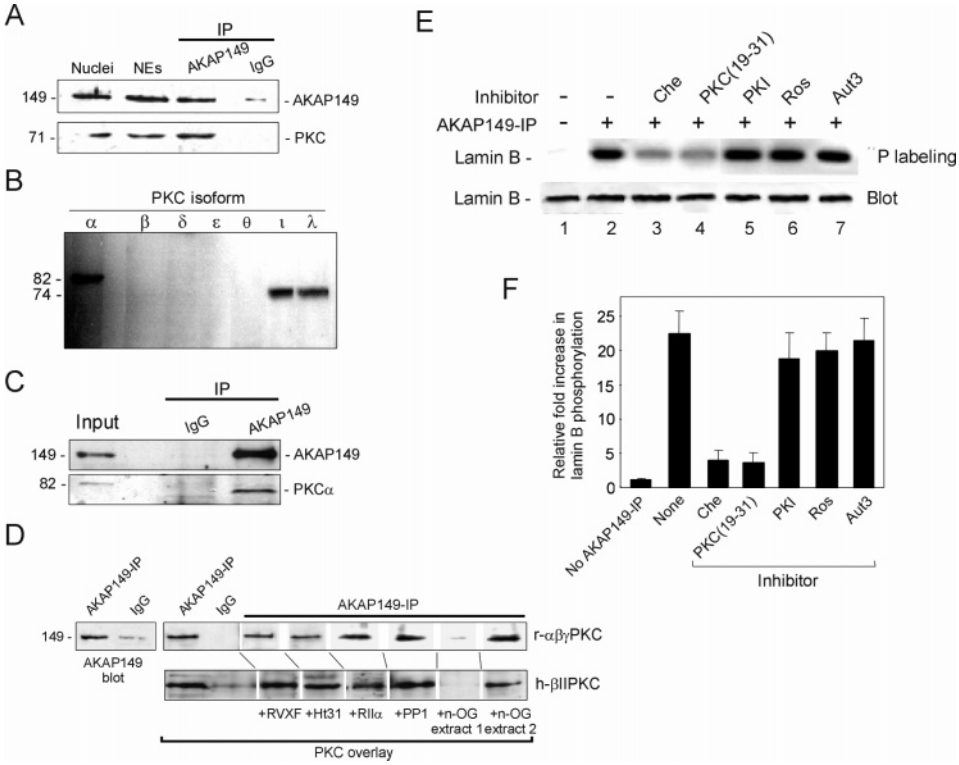

FIGURE 4: The AKAP149 complex contains PKC and harbors PKC activity. (A) AKAP149 was immunoprecipitated (IP) from nuclear envelopes, and immune precipitates were immunoblotted using anti-AKAP149 or (pan) PKC antibodies. IgG, control immunoprecipitation with nonimmune mouse IgGs. NE, nuclear envelopes. (B) Nuclear envelopes purified from HeLa cells in G1 phase were analyzed by immunoblotting using a panel of antibodies against indicated isoforms of PKC. (C) Purified nuclear envelopes were solubilized (Input) and AKAP149 immunoprecipitated. Immune precipitates were immunoblotted using anti-AKAP149 or anti-PKCα antibodies. IgG, control immunoprecipitation with nonimmune IgGs. (D) PKC overlay of immobilized AKAP149-IPs. Immobilized AKAP149 and control (IgG) IPs were incubated with 5 ng/μL rat $\alpha\beta\gamma$PKC. Where indicated, 10 μM of the AKAP149-derived RV*X*F peptide, 10 μM Ht31, 10 μM RIIα, 5 U PP1, or competitor (*n*-OG extract 1) and noncompetitor (*n*-OG extract 2) *n*-OG extracts of nuclear envelopes were added. PKC-binding to AKAP149 was detected using anti-PKC antibodies. (E) B-type lamin phosphorylation by AKAP149 complex-associated PKC in vitro. Immunoprecipitated B-type lamins, as a substrate for PKC, were incubated with the AKAP149-IP in the presence of [γ-^{32}P]-ATP and indicated protein kinase inhibitors (Che, 10 μM; PKC(19−31), 50 μM; Ros, 1 mM; PKI, 100 μM; Aut3, 50 μM). Lamin B phosphorylation was evaluated by autoradiography (^{32}P labeling). B-type lamins were also immunoblotted to assess gel loading (Blot). (F) Densitometric analysis of B-type lamin phosphorylation from duplicate autoradiograms such as that shown in (C) (mean±SD).

of the MEK inhibitor autocamtide 3, or 100 μM PKI had no significant inhibitory effect on the B-type lamin kinase activity of the AKAP149-IP (Figure 4E,F). We concluded from these experiments that immunoprecipitated AKAP149 harbors PKC activity.

PKC and PKA Can Phosphorylate AKAP149 in Vitro but Only PKC Abolishes Interaction with PP1. To determine whether nuclear envelope-associated AKAP149 was a substrate for PKA and PKC phosphorylation in vitro, AKAP149-IPs from purified nuclear envelopes were incubated with [γ-^{32}P]ATP and rat $\alpha\beta\gamma$PKC under PKC phosphorylation conditions. Alternatively, AKAP149-IPs were incubated with [γ-^{32}P]ATP and purified PKA catalytic subunit under PKA phosphorylation conditions. Phosphorylation of AKAP149 was examined by autoradiography. AKAP149 was phosphorylated by both PKC and PKA, and this was inhibited by 10 μM chelerythrine chloride or 100 μM PKI, respectively (Figure 5A). Further, serine phosphorylation of AKAP149 by either kinase was shown by an anti-pSer immunoblot (Figure 5A). Thus, immunoprecipitated AKAP149 is a substrate for PKC or PKA phosphorylation in vitro.

Phosphorylation of immunoprecipitated AKAP149 by exogenous PKC elicited the release of coimmunoprecipitated PP1 from the complex, and this release was inhibited by 10 μM chelerythrine chloride (Figure 5B, lanes 1, 2). In contrast,

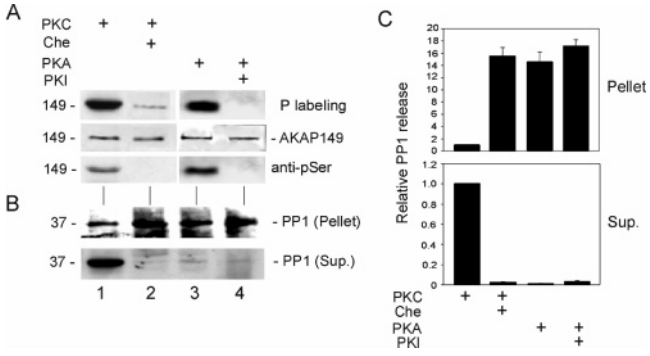

FIGURE 5: Exogenous PKC and PKA phosphorylate AKAP149 but only PKC-mediated phosphorylation promotes dissociation of PP1 from the AKAP149 complex in vitro. (A) AKAP149 IPs were incubated with 5 ng/μL rat $\alpha\beta\gamma$PKC with or without chelerythrine chloride, or alternatively, with 1.5 ng/μL PKA catalytic subunit, under PKC or PKA phosphorylation conditions, respectively. AKAP149 phosphorylation was assessed by autoradiography (^{32}P labeling) and by anti-pSer immunoblotting. (B) Dissociation of PP1 from the AKAP149 complex was monitored by immunoblotting of the AKAP149-IP (pellet) and reaction supernatant (sup) using rabbit anti-PP1 antibodies. (C) Densitometric analysis of relative PP1 release from the AKAP149-IP under conditions described above. Duplicates of pellet and supernatant fractions were examined.

PKA-mediated phosphorylation of AKAP149 did not promote dissociation of PP1 (Figure 5B, lanes 3, 4). Relative

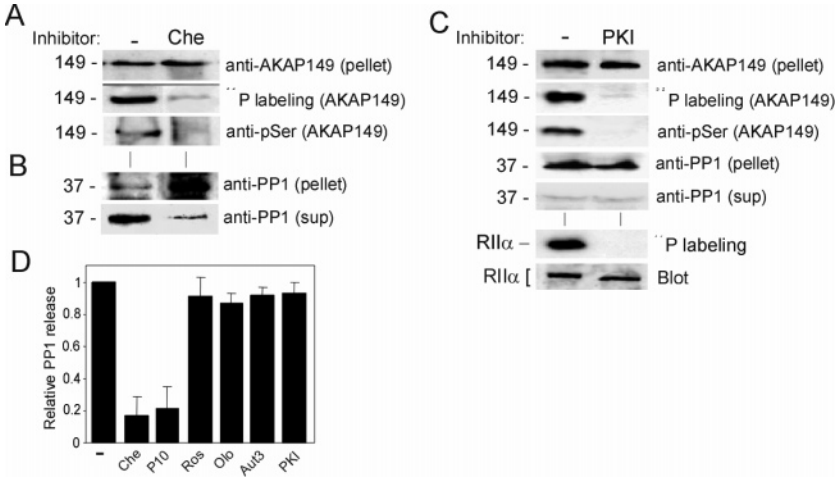

FIGURE 6: AKAP149-bound PKC phosphorylates AKAP149 and causes release of PP1 from the AKAP149 complex. (A) An AKAP149-IP from purified nuclear envelopes was incubated for 30 min in PKC phosphorylation buffer containing phosphatase inhibitors, [γ-^{32}P]ATP, and either 0 (−) or 10 μM chelerythrine chloride (Che). AKAP149 phosphorylation was assessed by autoradiography (^{32}P labeling). The AKAP149-IP was immunoblotted with anti-AKAP149 and anti-pSer antibodies. (B) The AKAP149-IP and the phosphorylation reaction supernatant (sup) were also immunoblotted using rabbit anti-PP1 antibodies to monitor PP1 dissociation from the AKAP149 complex. (C) AKAP149-IPs from purified nuclear envelopes were incubated for 30 min in PKA phosphorylation buffer containing [γ-^{32}P]ATP and either 0 (−) or 100 μM PKI. AKAP149 phosphorylation was assessed by autoradiography (^{32}P labeling), and AKAP149-IPs were immunoblotted with anti-AKAP149, anti-pSer and rabbit anti-PP1 antibodies as in A and B. The reaction supernatant (*sup*) was also probed with anti-PP1 antibodies as in (B). Lower two panels, autophosphorylation of PKA-RIIα bound to AKAP149 under PKA phosphorylation conditions, monitored by autoradiography and electrophoretic shift by SDS−PAGE and immunoblotting. (D) Relative release of PP1 from the AKAP149 complex was assessed by densitometric analysis of triplicate blots of reaction supernatants. The phosphorylation reaction contained chelerythrine chloride (10 μM), PKI (100 μM), as shown in (B) and (C), but also AKAP149 peptide P10, and the protein kinase inhibitors roscovitine (1 mM), olomoucine (1 mM), and autocamtide 3 (50 μM).

amounts of PP1 released from AKAP149-IP are indicated in Figure 5C. Moreover, PP1 release was abrogated by 10 μM of the competing AKAP149-derived peptide substrate P10 (Table 1), which contained S159 as only serine residue (data not shown). We concluded that both PKC and PKA can phosphorylate immunoprecipitated AKAP149 in vitro. However, phosphorylation by PKC, but not by PKA, promotes dissociation of PP1 from the AKAP149 complex.

Activation of AKAP149-Associated PKC Phosphorylates AKAP149 and Releases PP1 from the AKAP149 Complex. The PKC activity found to be associated with immunoprecipitated AKAP149 prompted the question of whether AKAP149-bound PKC would phosphorylate AKAP149. AKAP149-IPs from nuclear envelopes purified from G1 phase cells were incubated with [γ-^{32}P]ATP under PKC phosphorylation conditions, without any exogenous kinase. AKAP149 was phosphorylated and phosphorylation was inhibited by 10 μM chelerythrine chloride (Figure 6A; Che) or 10 μM AKAP149 competitor peptide P10 (data not shown). An anti-pSer blot confirmed that AKAP149 was serine-phosphorylated under these conditions (Figure 6A). Immunoblotting of the AKAP149-IP with anti-PP1 antibodies showed that AKAP149 phosphorylation by bound PKC in vitro promoted the release of PP1 from the AKAP149 complex into a supernatant fraction (Figure 6B,D). PP1 dissociation was prevented with chelerythrine chloride and peptide P10 (Figure 6B,D). The CDK inhibitors roscovitine and olomoucine, as well as autocamtide 3, did not inhibit PP1 release from AKAP149 (Figure 6D), arguing for a PKC-specific effect.

We next determined whether PKA activity associated with immunoprecipitated AKAP149 could phosphorylate the AKAP. When PKA activity was stimulated with cAMP (assayed by RIIα serine-autophosphorylation; Figure 6C, lower two panels), AKAP149 phosphorylation was detected (Figure 6C). However in this instance, PP1 remained associated with the AKAP149-IP (Figure 6C) and PKI logically did not affect PP1 release (Figure 6C,D). Taken together, these results argue that, although both PKA and PKC associated with the AKAP149 complex are able to phosphorylate AKAP149 in vitro, dissociation of PP1 from AKAP149 is elicited by PKC-mediated phosphorylation of AKAP149.

DISCUSSION

In most cases, the phosphatase activity of PP1 is regulated through a controlled interaction of PP1 with its regulatory subunit (*7*, *8*). This interaction is often mediated by reversible phosphorylation of the regulatory subunit on serine residues flanking the RV*X*F motif, which may alter or disrupt binding of the motif to PP1 (*11*, *37*, *38*). We show here that S151 or S159 phosphorylation of AKAP149, on either side of the PP1-binding RV*X*F motif, promotes release of PP1 from AKAP149 in vitro. Dissociation is promoted by the activity of AKAP149-associated PKC. Associated PKA does not promote PP1 release although it is also able to phosphorylate AKAP149. Interestingly, PKC-mediated phosphorylation of the PP1 regulatory subunit myosin phosphatase target subunit 1 (MYPT1) on T34, which immediately precedes the KVKF PP1-binding motif of MYPT1, does not seem to affect PP1 binding to MYPT1, although MYPT1 interaction with target myosin is impaired (*39*). However, phosphorylation of a second site of MYPT1 not only attenuates PP1 binding to the regulatory subunit, but also affects the phosphatase activity toward myosin light chain (*39*).

AKAP149 is only one of several AKAPs that bind and regulate protein phosphatases. The neuronal AKAP79 has been shown to anchor PKA, PKC and PP2B/calcineurin modules in membranes (*40*), and acts as an inhibitor of calcineurin (*41*). AKAP220 has also been shown to bind and regulate PP1 (*42*). Interestingly, the ability of AKAP220 to inhibit PP1 activity toward glycogen phosphorylase *a* is enhanced in vitro by anchoring of the RII subunit of PKA (*42*). Other phosphatase-interacting AKAPs are the centrosomal/Golgi AKAP350/AKAP450 (*43*) and the NMDA receptor-associated protein Yotiao, which regulates NMDA receptor phosphorylation by targeting of PKA and PP1 (*44*). However, in contrast to AKAP220 and AKAP149, Yotiao anchors PP1 in a constitutively active state toward its known substrate (*44*). Last, mAKAP anchors PKA, PP1 and PP2A, together with phosphodiesterase (PDE) PDE4D3 at the nuclear envelope of cardiomyocytes (*45*−*47*). PKA may phosphorylate the ryanodine receptor RyR2 and PDE4D3 at the nuclear envelope to promote ion channel activity, whereas PP2A reverses RyR2 phosphorylation (*45*).

It has recently been reported that AKAP149 coimmunoprecipitates with PDE4A in Jurkat T cell lysates (*48*); thus, it would be of interest to determine whether nuclear envelope-associated AKAP149 also harbors a phosphodiesterase, and if so, which isoform. PDEs control cAMP concentrations by hydrolyzing cAMP formed by adenylyl cyclases and are key players in the formation of local cyclic nucleotides gradient, thereby contributing to the spatio-temporal regulation of cyclic nucleotide signaling (*6*).

We have shown here that AKAP149 also associates with PKA and PKC. The role of PKA at the nuclear envelope remains unclear at present. A possibility is that a local elevated concentration of PKA at the nuclear envelope is important for eliciting CREB signaling upon a rise in cAMP (*49*). One function of the AKAP149-associated PKC might be to down-regulate PP1 activity toward B-type lamins by phosphorylating AKAP149, thus dissociating PP1 from AKAP149 and removing it from the vicinity of the nuclear lamina. This role would be particularly interesting in light of results showing that PKC is also implicated in the interphase phosphorylation of B-type lamins (*35*). PKC could therefore control the phosphorylation state of B-type lamins in interphase, both directly and by modulating the presence and activity of PP1 in the vicinity of the lamina through phosphorylation of AKAP149. To support this hypothesis, AKAP149 has been reported to coimmunoprecipitate with B-type lamins in HeLa cells (*19*) and to directly bind lamins in myotubes (*50*). Despite its anchoring of PKC, AKAP149 is not phosphorylated until the end of G1, suggesting that PKC activity during G1 is specific for, at least, B-type lamins. This implies the existence of additional spatial regulators of PKC activity at or near the nuclear envelope during interphase, or the presence of active phosphatases opposing PKC activity toward AKAP149.

Other roles for AKAP149 may include enhancement of PKC targeting to the nuclear envelope upon mitogenic stimulation, perhaps to facilitate the activation of PKC at the nuclear membrane (*36*). It is known that the nucleus-targeted βII isoform of PKC is required for the G2-M phase transition (*51*) and has been identified as a mitotic nuclear lamin kinase (*52*) promoting, together with other kinases, nuclear lamina disassembly. Another role for AKAP149-bound PKC at the nuclear envelope might be in apoptosis, as human leukemia HL60 cells undergoing apoptosis display lamin B phosphorylation by PKCα and proteolysis before DNA fragmentation (*53*).

AKAP149 emerges from our studies as a scaffolding protein for PKA, PKC, and PP1. It would be interesting to determine whether all these signaling molecules are anchored at the nuclear envelope by AKAP149 at the same time and in the same complex. Given the highly dynamic picture that has emerged from the studies of the assembly of AKAP complexes, it is very tempting to speculate that the nuclear envelope-associated AKAP149 complex shows great diversity in its composition. This diversity might result from differences in tissue, bound substrate and in response to cell signaling events. We suggest that the AKAP149-associated complex is involved in the integration of signaling pathways that converge at or pass through the nuclear envelope, and that a tight spatio-temporal control of the composition of the AKAP149-associated complex in the nuclear envelope allows for a dynamic and flexible response to extracellular and intracellular signals.

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