In Vitro Modulation of the Interaction between HA95 and LAP2β by cAMP Signaling[†]

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ABSTRACT: The nuclear envelope mediates key functions by interacting with chromatin. We recently reported an interaction between the chromatin- and nuclear matrix-associated protein HA95 and the inner nuclear membrane integral protein LAP2β, implicated in initiation of DNA replication (Martins et al. (2003) *J. Cell Biol. 160*, 177−188). Here, we show that in vitro, interaction between HA95 and LAP2β is modulated by cAMP signaling via PKA. Exposure of an anti-HA95 immune precipitate from interphase HeLa cells to a mitotic extract promotes ATP-dependent release of LAP2β from the HA95 complex. This coincides with Ser and Thr phosphorylation of HA95 and LAP2β. Inhibition of PKA with PKI abolishes phosphorylation of HA95 and dissociation of LAP2β from HA95, although LAPβ remains phosphorylated. Antagonizing cAMP signaling in mitotic extract also abolishes the release of LAP2β from HA95; however, disrupting PKA anchoring to A-kinase anchoring proteins has no effect. Inhibition of CDK activity in the extract greatly reduces LAP2β phosphorylation but does not prevent LAP2β release from HA95. Inhibition of PKC, MAP kinase, or CaM kinase II does not affect mitotic extract-induced dissociation of LAP2β from HA95. PKA phosphorylates HA95 but not LAP2β in vitro and elicits a release of LAP2β from HA95. CDK1 or PKC phosphorylates LAP2β within the HA95 complex, but neither kinase induces LAP2β release. Our results indicate that in vitro, the interaction between HA95 and LAP2β is influenced by a PKA-mediated phosphorylation of HA95 rather than by CDK1- or PKC-mediated phosphorylation of LAP2β. This suggests an additional level of regulation of a chromatin−nuclear envelope interaction in dividing cells.

The nuclear envelope (NE)[1] is a highly dynamic structure that reversibly breaks down at mitosis. The NE consists of an outer nuclear membrane continuous with the endoplasmic reticulum and an inner nuclear membrane (INM) attached to the nuclear lamina on its nucleoplasmic face. An increasing number of direct interactions between chromatin and NE are being identified (*1*). The INM contains integral proteins interacting with DNA and/or chromatin. These proteins are believed to be involved in anchoring the nuclear membranes to chromatin upon or after NE reconstitution at the end of mitosis (*2*). Phosphorylation of proteins of the INM, lamina, and chromatin is a likely mechanism regulating interactions between these nuclear compartments (*3*−*5*).

Lamina-associated polypeptide (LAP)2β is a 55 kDa integral protein of the INM (*4*, *6*). It is a member of the LAP2 family of proteins with a common 187-amino acid NH_2-terminal region (*7*) that contains a so-called LEM domain (residues 111−152) shared with emerin and MAN1 (*8*). The LEM domain binds DNA (*9*) and chromatin via the small DNA-bridging component, barrier-to-autointegration factor (*10*, *11*). Association of LAP2β with chromatin is phosphorylation-dependent (*4*, *6*), and interphase phosphorylation of LAP2β (*12*) may also modulate subtle interactions between the INM and nucleoplasmic substrates.

A recently discovered binding partner for LAP2β is HA95 (ref *13*), also called NAKAP95/HAP95 (refs *14* and *15*). HA95 is a 95 kDa protein with high homology to A-kinase anchoring protein (AKAP)95 (ref *16*) but does not harbor the PKA-RII subunit-binding domain of AKAP95 and thus is not an AKAP. HA95 cofractionates with chromatin as well as with a detergent-, high salt-, and nuclease-resistant nuclear matrix compartment (*17*). Binding of LAP2β to HA95 involves two binding regions of LAP2β, one between amino acids 137 and 242 (partially overlapping with the nonmembrane bound intranuclear LAP2 isoforms) (*7*) and a second coinciding with the B-type lamin binding domain of LAP2β at residues 299−373 (refs *18* and *19*). Interaction between HA95 and LAP2β can be disrupted in vitro with a LAP2β peptide containing both HA95-binding regions under conditions not involving protein phosphorylation (*19*). Whereas the HA95-LAP2β interaction does not seem to be required for NE assembly in vitro (*17*, *19*), it has been shown to play a role in the initiation of DNA replication (*19*).

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[1] Abbreviations: AKAP, A-kinase anchoring protein; CDK, cyclin-dependent kinase; HA95, homologous to AKAP95; IP, immune precipitate; IS200, interphase 200 000*g* supernatant; LAP2β, lamina-associated polypeptide 2β; MS200, mitotic 200 000*g* supernatant; PKA, cAMP-dependent protein kinase; PKA-C, PKA catalytic subunit; PKC, protein kinase C; PKI, inhibitor of PKA; pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; Rp-8-Br-cAMPS, Rp isomer of 8-bromo-adenosine-3′,5′-monophosphorothioate; 8-CPT-cAMP, 8-chlorophenylthio-cAMP.

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This paper investigates conditions promoting the dissociation of LAP2β from the HA95 complex in vitro. We report a somatic cell-free system that supports the disassembly of multiprotein complexes. Mitotic cytosol promotes phosphorylation of LAP2β and HA95 and dissociation of LAP2β from the HA95 complex. Kinase inhibition and in vitro phosphorylation data suggest an involvement of cAMP-dependent protein kinase (PKA)-mediated phosphorylation of HA95 in the release of LAP2β from HA95. The results suggest an additional level of control of interactions between NE and chromatin.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents. Affinity-purified polyclonal antibodies against LAP2β (gift from Dr. J.-C. Courvalin, Institut Jacques Monod, Paris, France) and HA95 were described elsewhere (*13*, *20*). Anti-phosphoserine (anti-pS) antibodies were from Zymed (San Francisco, CA), anti-phosphothreonine antibodies (anti-pT) from New England Biolabs (Beverly, MA), and anti-phosphotyrosine (anti-pY) antibodies from Upstate (Lake Placid, NY). Purified rat $\alpha\beta\gamma$PKC was from Upstate. The PKC inhibitor chelerythrine and PKA inhibitor PKI were from Sigma (St. Louis, MO). Active PKA catalytic subunit, cAMP antagonist Rp isomer of 8-bromo-adenosine-3′,5′-monophosphorothiate (Rp-8-Br-cAMPS), and cAMP agonist 8-chlorophenylthio-cAMP (8-CPT-cAMP) were gifts from Dr. K. Taskén (University of Oslo, Norway). The cyclin-dependent kinase (CDK) inhibitor roscovitine was from Dr. L. Meijer (CNRS, Roscoff, France). The CaM kinase II inhibitor autocamtide 3 was from Life Technologies (Bethesda, MD), and the MAP kinase inhibitor PD98059 was from Calbiochem (San Diego, CA). Calf alkaline phosphatase was from Promega (Madison, WI). Ht31 peptides were as described (*21*).

Cells and Nuclei. HeLa cells were cultured in EMEM and 10% fetal calf serum (*16*). Nuclei were isolated from confluent HeLa cells by Dounce homogenization in buffer N (10 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 250 mM sucrose, 25 mM KCl, 1 mM DTT, 1 mM PMSF, and a cocktail of protease inhibitors) (*17*, *22*). Nuclei were sedimented, washed twice in buffer N, and resuspended in immunoprecipitation buffer.

Mitotic and Interphase Extracts. To prepare mitotic extracts, HeLa cells were synchronized in M phase with 1 μM nocodazole for 18 h, and mitotic cells were harvested by shake-off (*16*). Cells were washed in PBS and then in ice-cold lysis buffer (20 mM Hepes, pH 8.2, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT, and protease inhibitors) and sedimented at 800*g*. Cells were resuspended in one volume of lysis buffer and incubated for ~60 min on ice before Dounce homogenization and centrifugation of the lysate at 10 000*g* for 15 min at 4 °C. The supernatant was cleared at 200 000*g* for 3 h at 4 °C in a Beckman SW55 rotor. The clear mitotic extract (MS200; 15−18 mg/mL protein; Bradford method) was aliquoted and stored at −80 °C. Interphase 200 000*g* extracts (IS200) were prepared as above from confluent HeLa cells except that EDTA was omitted from the lysis buffer.

Immunological Procedures. Immunoblotting analysis was performed using relevant antibodies as described (*17*). HA95 and LAP2β were immunoprecipitated from HeLa cells or purified HeLa nuclei, as indicated. Cells or nuclei were sonicated in IP buffer (10 mM Hepes, pH 7.5, 10 mM KCl, 2 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, and protease inhibitors), the lysates were sedimented at 15 000*g* for 15 min, and immunoprecipitations were performed from the supernatants after preclearing with Protein A/G-agarose (*17*). Immune precipitates (IPs) were eluted in SDS sample buffer for Western blot analysis or resuspended in 100 mM Tris (pH 7.5) and 50 mM NaCl for use in the cell-free protein complex disassembly assay.

In Vitro Disassembly of HA95 Protein Complexes. Anti-HA95 immune precipitates (HA95-IPs; 10 μL) from 10^7 interphase cells were incubated for 1 h at 30 °C in 100 μL of MS200 containing an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, 25 μg/mL creatine kinase). When indicated, the MS200 contained [γ-^{32}P]ATP. At the end of incubation, the whole extract was analyzed, or HA95-IPs were sedimented at 4000*g* after a 2-fold dilution of the extract with cell lysis buffer. Sedimented HA95-IPs were washed twice in cell lysis buffer containing 0.1% Triton X-100, and proteins were dissolved in SDS sample buffer. The supernatant was recentrifuged to eliminate any residual HA95-IP, and proteins were precipitated with 10% trichloracetic acid and dissolved in SDS sample buffer. Similar proportions of pelleted and supernatant fractions were analyzed by SDS−PAGE.

Protein Phosphorylation. Metabolic ^{32}P labeling of proteins in interphase and mitosis was performed as described (*4*). Incorporation of ^{32}P into HA95-IPs in MS200 was performed as described (*23*). Briefly, HA95-IPs from 10^7 cells were incubated for 60 min in 100 μL of MS200 containing 1 μCi/mL [γ-^{32}P]ATP. After sedimentation, HA95-IPs and supernatants were subjected to SDS−PAGE and autoradiography. When relevant, protein kinase inhibitors were added to the MS200 20 min prior to adding HA95-IPs. HA95-IPs were also phosphorylated for 30 min with 1.5 ng/μL PKA, 5 ng/μL rat $\alpha\beta\gamma$PKC, or 1.3 ng/μL CDK1 in PKA, PKC, or CDK1 phosphorylation buffers containing 0.75 μCi/μL [γ-^{32}P]ATP (*23*). HA95-IPs were sedimented at 4000*g* and washed, and IPs and supernatant fractions were analyzed by SDS−PAGE, immunoblotting, and autoradiography.

RESULTS

HA95 Is Serine- and Threonine-Phosphorylated at Mitosis. Phosphorylation of proteins of the inner nuclear membrane, including LAP2β, at mitosis correlates with the dissociation of nuclear membranes from chromatin (*4*). Metabolic ^{32}P labeling of HeLa cells verified that LAP2β was hyperphosphorylated at mitosis (*4*, *12*) (Figure 1A). HA95 was also phosphorylated at mitosis but not in interphase (Figure 1A). Immunoblotting analysis of immunoprecipitated HA95 and LAP2β using anti-pS, anti-pT, and anti-pY antibodies revealed that both proteins were serine- and threonine-phosphorylated, but not tyrosine-phosphorylated, at mitosis (Figure 1B). A control blot of p34^{cdc2}, the kinase component of the cyclin B-CDK1 complex, immunoprecipitated from interphase HeLa cells using p13^{suc1}-agarose beads showed that anti-pY antibodies did react with the phosphorylated tyrosine of p34^{cdc2} (Figure 1B, p34) (*24*). The results indicate that HA95 is serine- and threonine-phosphorylated at mitosis.

Phosphorylation of HA95 Promotes Dissociation of LAP2β from HA95 in Mitotic Extract. To examine the significance

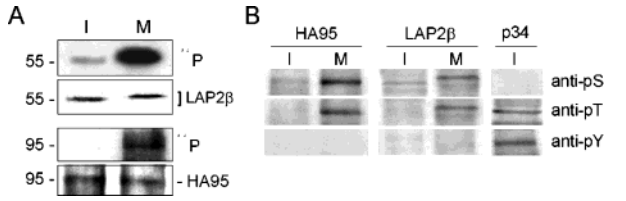

FIGURE 1: In vivo phosphorylation of LAP2β and HA95. (A) Interphase (I) and mitotic (M) HeLa cells were metabolically labeled with $^{32}P_i$. LAP2β (upper two panels) and HA95 (lower two panels) were immunoprecipitated, and precipitates were analyzed by autoradiography (^{32}P) and Western blotting. (B) Immune precipitates were immunoblotted using anti-pS, -pT, and -pY antibodies. Anti-p34^{cdc2} kinase (p34) IPs from interphase HeLa cells were also immunoblotted as a positive control for anti-pY antibody reactivity. Apparent M_r is shown in kDa.

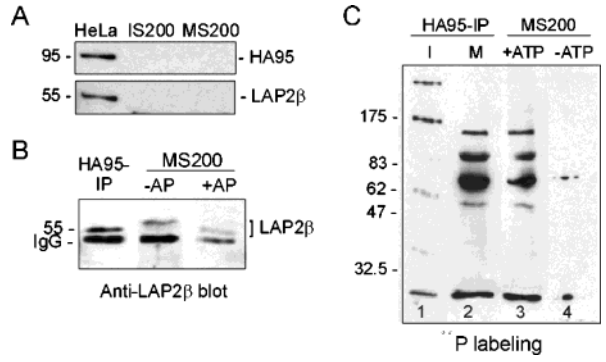

FIGURE 2: Mitotic extract mimics mitotic phosphorylation of HA95-associated proteins. (A) Anti-HA95 and LAP2β immunoblots of HeLa cells (HeLa) and 200 000*g* supernatants of interphase (IS200) and mitotic (MS200) cell lysates. (B) HA95-IPs from interphase HeLa cells were incubated in MS200 containing an ATP-regenerating system. Total extract was immunoblotted using anti-LAP2β antibodies before (−AP) and after (+AP) alkaline phosphatase treatment of the extract. (C) Autoradiogram of HA95-IPs from interphase (I) or mitotic (M) ^{32}P-labeled HeLa cells (lanes 1 and 2) and of interphase HA95-IPs incubated in MS200 containing [γ-^{32}P]ATP with or without the ATP-regenerating system (lanes 3 and 4). Apparent M_r is shown in kDa.

of mitotic HA95 and LAP2β phosphorylation, we prepared cytosolic extracts from mitotic and interphase HeLa cells. Mitotic extracts (MS200s) were previously shown to support the disassembly of exogenous HeLa nuclei, including NE breakdown and chromosome condensation (*22*). Extracts were not apoptotic, as judged by the absence of proteolytic degradation of poly(ADP)ribosyl polymerase and DNA fragmentation characteristic of apoptotic cells (data not shown) (*22*). Neither IS200 nor MS200 contained detectable HA95 or LAP2β (Figure 2A). Incubation of an anti-HA95 immune precipitate (HA95-IP) from interphase HeLa cells in MS200 containing an ATP-regenerating system elicited a mobility shift of LAP2β similar to that detected in mitotic cells (Figure 2B, −AP). The shift was reversed by alkaline phosphatase treatment (100 U/mL, 37 °C, 1 h) of the MS200 containing the HA95-IP before electrophoresis (Figure 2B, +AP), suggesting that it was due to phosphorylation of LAP2β.

Incubation of an HA95-IP in MS200 containing [γ-^{32}P]-ATP induced phosphorylation of proteins associated with HA95, of same mobility in SDS−PAGE as those phosphorylated and immunoprecipitated from mitotic cells with anti-HA95 antibodies (Figure 2C, lanes 2 and 3). Phosphorylation was dependent on an ATP-regenerating system (Figure 2C, lanes 3 and 4). For comparison, the pattern of phosphorylated proteins in an HA95-IP from ^{32}P-labeled interphase HeLa cells is shown (Figure 2C, lane 1). Note that since only HA95-IPs were analyzed (Figure 2C), HA95-associated proteins that are phosphorylated at mitosis (lane 2) or in MS200 (lane 3), and as a result dissociate from the HA95 complex, are not shown.

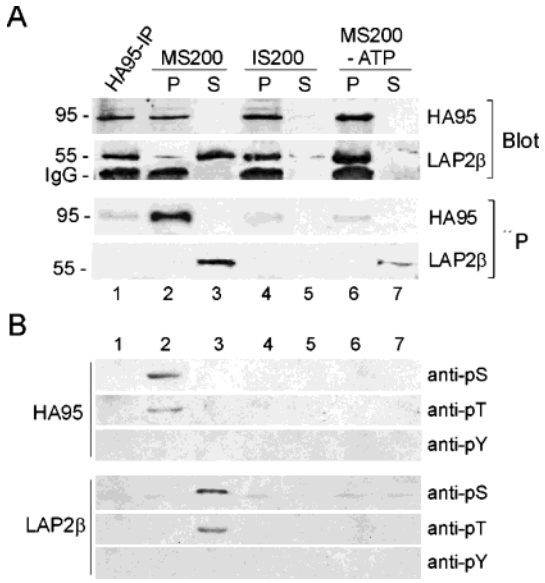

FIGURE 3: Dissociation of LAP2β from HA95-IP in mitotic extract. (A) HA95-IPs from interphase cells (lane 1) were incubated in MS200, IS200, or MS200 without an ATP-regenerating system, each containing [γ-^{32}P]ATP. HA95-IPs were sedimented and washed, and HA95 and LAP2β were analyzed by autoradiography (^{32}P) and immunoblotting (Blot) of sedimented HA95-IP (P) and supernatant (S) fractions. (B) At the end of incubation in IS200 or MS200 as in A, sedimented HA95-IPs (upper three panels) and LAP2β immunoprecipitated from the supernatant fractions (lower three panels) were immunoblotted using anti-pS, -pT, and -pY antibodies. Apparent M_r is shown in kDa.

To determine whether the mitotic extract was capable of dissociating LAP2β from the HA95 complex, HA95-IPs were incubated in MS200 containing [γ-^{32}P]ATP. Immunoblots of sedimented (P) and supernatant (S) fractions at the end of incubation showed that LAP2β was released from the HA95-IP (Figure 3A, Blot, lanes 2 and 3). Release did not occur in IS200 (Figure 3A, Blot, lanes 4 and 5) or in MS200 without the ATP-regenerating system (Figure 3A, Blot, lanes 6 and 7). Dissociation of LAP2β from HA95-IP correlated with phosphorylation of both HA95 and LAP2β as shown by autoradiography (Figure 3A, ^{32}P, lanes 2 and 3) and anti-pS and anti-pT immunoblots (Figure 3B, lanes 2 and 3). Collectively, the data indicate that the mitotic extract induces phosphorylation of HA95-associated proteins and dissociation of LAP2β from the HA95 complex.

Inhibition of PKA Blocks HA95 Phosphorylation and LAP2β Solubilization in Mitotic Extract. To address the nature of the protein kinase(s) eliciting HA95 and LAP2β phosphorylation in MS200, HA95-IPs were incubated in MS200 containing the CDK inhibitor roscovitine (1 mM), the PKC inhibitor chelerythrine (10 μM), the PKA inhibitor PKI (100 μM), the CaM kinase II inhibitor autocamtide 3 (50 μM), or the MAP kinase inhibitor PD98059 (50 μM). At the end of incubation, sedimented HA95-IPs and supernatant fractions were examined by autoradiography, densitometry, and immunoblotting (Figure 4A,B). Without in-

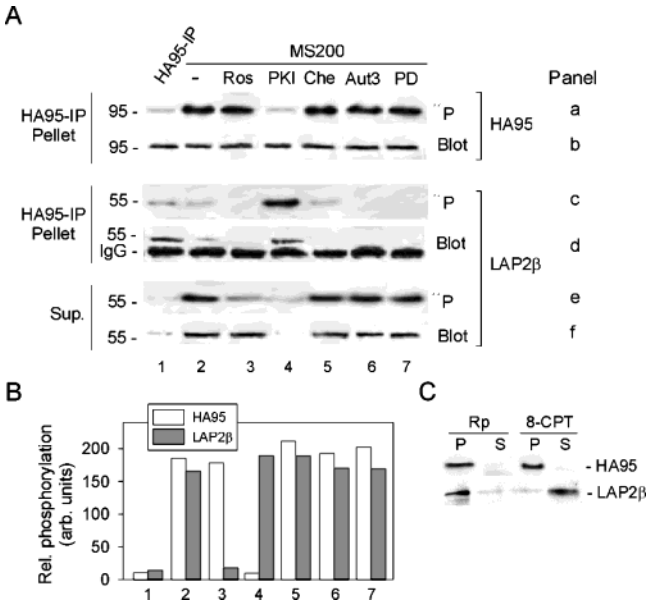

FIGURE 4: Inhibition of HA95 and LAP2β phosphorylation and LAP2β solubilization by protein kinase inhibitors in mitotic extract. (A) HA95-IPs were incubated in MS200 containing [γ-^{32}P]ATP and 1 mM roscovitine (Ros), 100 μM PKI, 10 μM chelerythrine (Che), 50 μM autocamtide 3 (Aut3), or 50 μM PD98059 (PD). HA95-IPs were sedimented, and HA95 and LAP2β were analyzed by autoradiography (^{32}P) and immunoblotting (Blot) of HA95-IP pellet and supernatant (Sup) fractions. (B) Densitometric analysis of autoradiograms in A showing relative phosphorylation level of HA95 (labeling intensities of each lane of panel Aa) and LAP2β (sum of labeling intensities within each lane of panels Ac and Ae). (C) Immunoblots of HA95 and LAP2β in pelleted (P) and supernatant (S) fractions after incubation of HA95-IPs in MS200 containing 100 μM Rp-8-Br-cAMPS (Rp) or 100 μM 8-CPT-cAMP (8-CPT) and sedimentation of HA95-IPs. Apparent M_r is shown in kDa.

hibitors, both LAP2β and HA95 were phosphorylated (Figure 4A, panels a and e, lane 2), and LAP2β was released from HA95-IP (Figure 4A, panels d and f, lane 2). Chelerythrine, autocamtide 3, or PD98059 did not prevent phosphorylation of HA95 or LAP2β and LAP2β solubilization (Figure 4A, lanes 5−7). Roscovitine did not inhibit HA95 phosphorylation (Figure 4A, panel a, lane 3) but greatly attenuated LAP2β phosphorylation (Figure 4A, panels c and e, lane 3). Nonetheless, LAP2β was dissociated from HA95-IP and found exclusively in the supernatant (Figure 4A, panels d and f, lane 3). Remarkably, inhibition of PKA with PKI nearly abolished HA95 phosphorylation (Figure 4A, panel a, lane 4) and LAP2β solubilization (Figure 4A, panel d, lane 4), although LAP2β phosphorylation was not inhibited (Figure 4A, panel c, lane 4). The AKAP-RII disruptor peptide, Ht31 (500 nM), had no inhibitory effect on HA95 and LAP2β phosphorylation and dissociation of LAP2β from HA95-IP in MS200 (data not shown), suggesting that RII anchoring to an AKAP is not required for LAP2β release from the HA95-IP in our system. Furthermore, dissociation of LAP2β from HA95-IP in MS200 was abolished by down-regulating cAMP signaling in the extract with 100 μM cAMP antagonist Rp-8-Br-cAMPS, whereas the cAMP agonist 8-CPT-cAMP had no effect (Figure 4C). Ht31, Rp-8-Br-cAMPS, and 8-CPT-cAMP had no effect on the release of LAP2β from HA95-IP in IS200 (data not shown). The relevance of CDK and PKA inhibition on HA95 and LAP2β phosphorylation in MS200 was substantiated by the detection of PKA and CDK1 (but not PKC) activities in MS200, measured as phosphorylation of recombinant PKA-RIIα subunit and histone H1, respectively (data not shown). We concluded that PKI and roscovitine abolish phosphorylation of HA95 and LAP2β, respectively, in MS200. PKI also prevents LAP2β dissociation from HA95 despite the phosphorylation of LAP2β. Inhibition of LAP2β (but not HA95) phosphorylation with roscovitine does not block LAP2β release from HA95. The data suggest a role of PKA in HA95 phosphorylation and dissociation of LAP2β from the HA95 complex in our system. However, PKA-RII anchoring to an AKAP seems to be dispensable.

PKA Phosphorylates HA95 and Solubilizes LAP2β in Vitro. To determine how PKA- and CDK-mediated phosphorylation in vitro affected the HA95 complex, HA95-IPs were incubated with the PKA catalytic subunit, CDK1, or purified rat $\alpha\beta\gamma$PKC and [γ-^{32}P]ATP. HA95-IPs were sedimented, and the pellet and soluble fractions were examined by autoradiography, immunoblotting, and densitometric analysis of autoradiograms and blots. HA95 was phosphorylated by PKA but not detectable by CDK1 or PKC (including human βII-PKC; data not shown), whereas LAP2β was phosphorylated by CDK1 and PKC but not by PKA (Figure 5A, ^{32}P and Figure 5B). Phosphorylation of HA95 by PKA elicited the release of LAP2β from HA95-IP into the supernatant, and this was inhibited with 100 μM PKI (Figure 5A, lanes 1−4 and Figure 5C). In contrast, CDK1 and PKC did not promote the dissociation of LAP2β from HA95-IP (Figure 5A, lanes 5−12 and Figure 5C). Thus, in vitro, PKA phosphorylates immunoprecipitated HA95, whereas HA95-associated LAP2β is a target for PKC and CDK1. The data also suggest that the interaction between HA95 and LAP2β is influenced in vitro by PKA-mediated phosphorylation of HA95 rather than by CDK1- or PKC-mediated phosphorylation of LAP2β.

DISCUSSION

We previously identified a direct interaction between NE and chromatin mediated by the INM protein LAP2β and the chromatin- and nuclear matrix-associated protein, HA95 (*19*). We now report conditions promoting the release of LAP2β from an anti-HA95 immune precipitate using an in vitro system supporting the dissociation of macromolecular assemblies. In an extract from mitotic cells, the interaction between LAP2β and immunoprecipitated HA95 is modulated by PKA-mediated phosphorylation of HA95 rather than by phosphorylation of LAP2β by PKC or roscovitine-inhibitable CDKs (CDK1/2) (ref *25*). The extract mimics mitotic conditions in that it supports (i) disassembly of exogenous nuclei, including NE breakdown and chromosome condensation (*22*), (ii) phosphorylation of LAP2β as judged by ^{32}P labeling and slower migration in SDS−PAGE (see also ref *4*), and (iii) phosphorylation of a set of HA95-associated proteins similar to those detected in ^{32}P-labeled mitotic cells.

The CDK1- and PKC-mediated in vitro phosphorylation of LAP2β is consistent with the identification of putative CDK1 phosphorylation sites (Thr256 and Thr320) (*6*) and of a phosphorylated PKC site (Ser179) in LAP2β purified from NEs and analyzed by mass spectrometry (*12*). Notably, Ser179 and Thr256/Thr320 lie within the NH_2- and COOH-

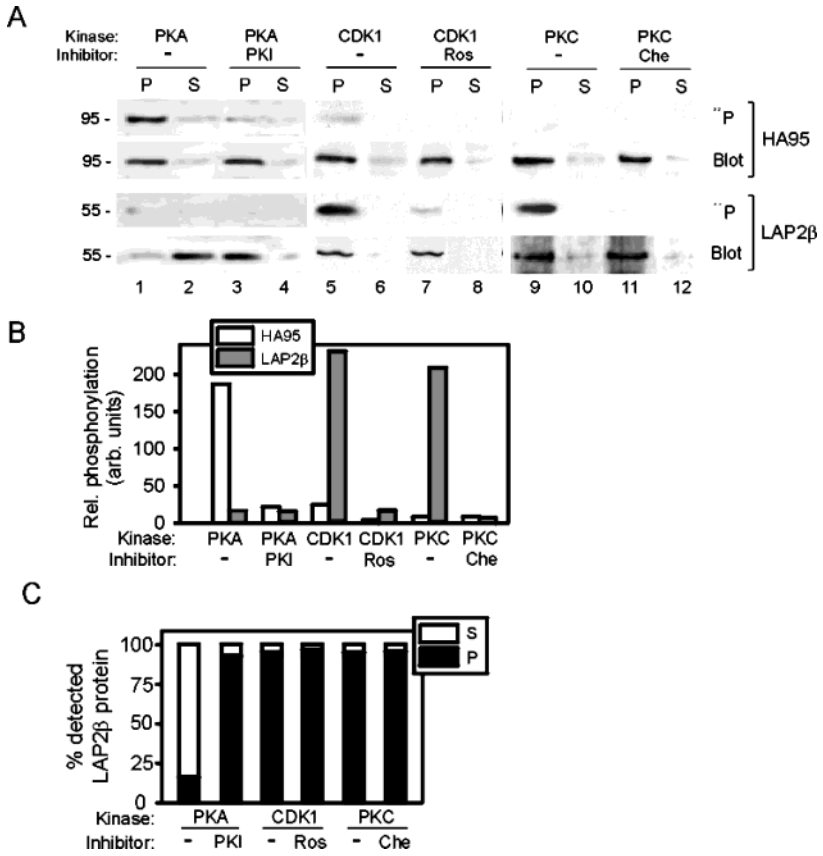

FIGURE 5: PKA phosphorylates HA95 and solubilizes LAP2β in vitro. (A) HA95-IPs were phosphorylated in vitro with PKA catalytic subunit, CDK1, or rat $\alpha\beta\gamma$PKC in the presence of [γ-^{32}P]ATP, and either 500 μM PKI, 1 mM roscovitine, or 10 μM chelerythrine, as indicated. HA95-IPs were sedimented, and HA95 and LAP2β were analyzed by autoradiography (^{32}P) and immunoblotting (Blot). (B) Densitometric analysis of autoradiograms illustrated in panel A, showing relative phosphorylation levels of HA95 and LAP2β (sum of labeling intensities of lanes 1+2, 3+4, 5+6, 7+8, 9+10, 11+12, each for HA95 and LAP2β). (C) Densitometric analysis of LAP2β immunoblots in pellet (P) and supernatant (S) fractions after treatment of HA95-IPs with protein kinases as in panel A. Bottom panel of Figure 5A was analyzed. Total labeling is given a reference value of 100%.

terminal HA95-binding regions of LAP2β, respectively; however, in vitro phosphorylation of these residues does not perturb the interaction of LAP2β with HA95 within HA95 immune complexes. In contrast, as Thr256 and Thr320 both lie within the B-type lamin binding domain of LAP2β (*18*), CDK1-mediated phosphorylation of these sites may be involved in disrupting interactions of the INM with the nuclear lamina (*4*). The phosphorylation of HA95 by PKA in vitro is consistent with a putative PKA phosphorylation at Ser522, downstream of the COOH-terminal zinc finger of HA95 (*13*). Furthermore, despite the detection of several putative PKC phosphorylation sites throughout the entire protein, rat $\alpha\beta\gamma$PKC (this paper) or purified human βII-PKC (data not shown) did not phosphorylate immunoprecipitated HA95 in our hands, ruling out these sites as PKC targets under the conditions tested. PKC, CDK1, and PKA had clearly distinct effects on the integrity of the HA95-IP in vitro. Whereas PKC and CDK1 phosphorylated LAP2β, but not HA95, only PKA was capable of triggering the phosphorylation of HA95 and the release of LAP2β from the HA95-IP. These results argue that at least in vitro, the association of LAP2β and HA95 is regulated by PKA-mediated phosphorylation of HA95 rather than CDK1- or PKC-mediated phosphorylation of LAP2β. The data provide a clear example of phosphorylation of a chromatin-associated ligand, rather than of a component of the NE, as the sole trigger for the release of an integral protein of the INM.

The discovery that PKA activity, rather than CDK or PKC activity, was sufficient to elicit the release of LAP2β from HA95-IPs was surprising in light of the previously reported involvement of cAMP signaling at mitosis entry and on nuclear disassembly. The PKA-mediated disruption of LAP2β from HA95 can be triggered in a mitotic extract and as such might reflect the disruption of nuclear membranes from chromatin that occurs at mitosis. However, mitosis entry has been shown to coincide with a down-regulation of cAMP levels and PKA activity (*26*, *27*), which is required for NE breakdown in cultured mitotic mammalian cells (*28*). Moreover, inhibition of PKA activity with PKI in a mitotic extract does not abolish the breakdown of exogenous nuclei (*22*). These observations would argue toward a minor involvement of PKA in the disruption of nuclear membrane−chromatin interactions at mitosis. Nonetheless, PKI may not necessarily fully inhibit all PKA activity in vitro, in particular within exogenous nuclei. Furthermore, the first trigger of nuclear breakdown has been shown to be a microtubule-mediated tearing of the NE, whereas the physical dissociation of the nuclear membranes from chromosomes only follows as membranes are pulled away from chromatin toward the minus ends of microtubules (*29*, *30*). As cAMP level and PKA activity gradually rise during mitosis (*27*), sufficient PKA activity might be present near chromosomes (*22*) to contribute to the phosphorylation of INM and/or chromatin-bound substrates and enable the release of nuclear mem-

branes. It is also possible that PKA activity at mitosis entry may be down-regulated only to a threshold level permissive for the phosphorylation of INM or chromatin-bound components. Clearly, an involvement of cAMP signaling via PKA in the dissociation of nuclear membranes from chromatin remains to be explored.

An alternative possibility is that cAMP signaling via PKA modulates INM−chromatin interactions during interphase. The interaction of LAP2β with HA95 is essential for the initiation phase of DNA replication (*19*). As cells progress into the S phase, however, a peptide-mediated disruption of the interaction is not inhibitory for DNA synthesis. It is conceivable that transient disruptions of INM−chromatin interactions may be implicated in or necessary for replication of, for example, heterochromatic DNA residing at the nuclear periphery. Alternatively, a dynamic interaction between INM and chromatin might be important for chromatin remodeling events involved in the control of gene expression (*31*, *32*) or for the dynamics of chromosome domains and proteins during interphase (*32*−*34*). An increase in PKA-type II activity reported in Chinese hamster ovary cells upon S phase entry (*35*) is compatible with an interphase phosphorylation of chromatin-associated proteins by PKA. Nevertheless, whether PKA is indeed involved in interphase disruptions of chromatin-associated components from integral INM proteins remains to be tested. Preliminary data illustrating the release of LAP2β from an HA95-IP in interphase cytosol supplemented with the cAMP agonist 8-CPT-cAMP supports this view (our unpublished data).

Where does PKA activity phosphorylating HA95 originate from? Local intracellular elevations of cAMP cause a release of the PKA catalytic (PKA-C) subunits from a regulatory subunit dimer (*36*). Free activated PKA-C subunits can translocate to the nucleus where they may activate cAMP-responsive genes (*37*). Thus, free intranuclear PKA-C subunits can transiently dock to HA95 to elicit phosphorylation. An alternative possibility is that HA95 itself acts as an anchoring protein for PKA-C. To support this view, HA95 has recently been shown to localize the PKA-Cα subunit at nuclear sites where it can modulate transcription from specific promoters in Epstein−Barr virus-transformed lymphoblastoid cells (*38*). Furthermore, we have also identified PKA-C in HA95-IPs from the lymphoid Bjab cell line and detected PKA activity in HA95-IPs from interphase HeLa cells (our unpublished data). Thus, it will be important to determine whether HA95 anchors the protein kinase activity eliciting its phosphorylation.

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