HA95 is a protein of the chromatin and nuclear matrix regulating nuclear envelope dynamics

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Summary

We report a role for HA95, a nuclear protein with high homology to the nuclear A-kinase anchoring protein AKAP95, in the regulation of nuclear envelope-chromatin interactions. Biochemical and photobleaching data indicate that HA95 is tightly associated with chromatin and the nuclear matrix/lamina network in interphase, and bound to chromatin at mitosis. HA95 resides in a complex together with lamin B receptor (LBR), lamina-associated polypeptide (LAP)2 and emerin, integral proteins of the inner nuclear membrane. Cross-linking experiments, however, illustrate a tight association of HA95 with LBR and LAP2 only. Intra-nuclear blocking of HA95 with anti-HA95 antibodies abolishes nuclear breakdown in a mitotic HeLa cell extract. The antibodies inhibit nuclear membrane breakdown and chromatin condensation – the latter independently of nuclear membranes. However, lamina disassembly is not affected, as judged by immunological analyses of A/C- and B-type lamins. In contrast, immunoblocking of HA95 bound to condensed chromosomes does not impair chromatin decondensation, nuclear membrane reassembly or lamina reformation. Our results argue for a role in anchoring nuclear membranes and lamins to chromatin in interphase, and in releasing membranes from chromatin at mitosis. The data also suggest that HA95 is not involved in initial binding of membranes to chromatin upon nuclear reassembly. We propose that HA95 is a central platform at the chromatin/nuclear matrix interface implicated in regulating nuclear envelope-chromatin interactions during the cell cycle.

Key words: HA95, Chromatin, Nuclear envelope, Mitosis

Introduction

The nuclear envelope (NE) is a highly dynamic structure consisting of two concentric membranes underlaid by the nuclear lamina, a network of intermediate filaments called A/C- and B-type lamins (reviewed by Collas and Courvalin, 2000; Wilson, 2000). The outer nuclear membrane (ONM) is in direct continuity with the endoplasmic reticulum (ER) and shares biochemical and functional properties with the ER. The inner nuclear membrane (INM) harbors specific integral proteins that provide attachment sites for chromatin and the nuclear lamina. ONM and INM are connected by sharply bend membranes associated with nuclear pore complexes. The dynamic nature of the NE is best illustrated at mitosis when the NE breaks down and reassembles around daughter chromosomes. Disassembly of the NE at prophase correlates with phosphorylation of proteins of the chromatin, nuclear membranes and lamina, leading to solubilization of lamin A/C while B-type lamins remain mostly in a membrane-bound form (Gerace and Blobel, 1980). In late anaphase, ER-derived membranes associate with chromatin to reform nuclear membranes (Chaudhary and Courvalin, 1993; Ellenberg et al., 1997), while in telophase, lamins are dephosphorylated and repolymerize (Ottaviano and Gerace, 1985).

Proteins of the INM, lamina and chromosomes are extensively interconnected. The INM harbors specific integral proteins including lamin B receptor (LBR; Worman et al., 1990), lamina-associated polypeptides (LAP)1 and LAP2 (Foissner and Gerace, 1993), emerin (Nagano et al., 1996), nurim (Rolls et al., 1999) and MAN1 (Lin et al., 2000). LBR, LAP1, LAP2β and emerin bind to lamins A/C and/or B in vitro (reviewed by Wilson, 2000). LBR and LAP2β also bind chromatin via interactions with heterochromatin protein (HP)1 (Ye and Worman, 1996) and the small DNA-binding protein BAF (barrier to autointegration factor; Furukawa, 1999). An intranuclear LAP2 variant, LAP2α, also interacts with lamins A/C (Dechat et al., 1998) while H2-type histones bind lamins A/C and B in vitro (Goldberg et al., 1999). Thus, the chromatin provides multiple anchoring sites for the NE.

HA95, also called HAP95 or NAKAP95, is a novel nuclear protein recently cloned by us (Ørstavik et al., 2000) and others (Seki et al., 2000; Westberg et al., 2000). HA95 exhibits high homology to the human nuclear A-kinase (PKA) anchoring protein, AKAP95 (Eide et al., 1998). Like AKAP95, HA95 harbors two zinc fingers and a putative nuclear localization signal but does not contain the PKA-binding domain of AKAP95 (Ørstavik et al., 2000). HA95 and AKAP95 co-localize in interphase and at mitosis, yet the two proteins do
not interact. Nevertheless, HA95 is found in multiprotein complexes directly associated with one another or indirectly associated through other nuclear proteins. To support this view, HA95/HAP95 was recently shown to interact with RNA helicase A and enhance expression of CTE, an RNA element involved in nuclear export of retroviruses (Westberg et al., 2000).

We report here that HA95 is a chromatin- and nuclear matrix-associated protein implicated in the regulation of NE-chromatin interactions. HA95 directly interacts with the lamina and with a subset of integral proteins of the INM. Antibody-mediated blocking of HA95 inside purified nuclei inhibits chromatin condensation and disassembly of nuclear membranes, but not of the lamina, in mitotic cytosol. However, immunoblocking HA95 bound to mitotic chromosomes does not affect nuclear reformation. We propose that HA95 is a central element of the chromatin and the nuclear matrix implicated in regulating NE-chromatin interactions during the cell cycle.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Affinity-purified rabbit polyclonal antibodies directed against the NH2-terminal domain of human LBR, against a peptide of human lamin B (not distinguishing between lamin B isoforms), and against a peptide of human LAP2 (generous gifts from Drs J.-C. Courvalin and B. Buendia, Institut Jacques Monod, Paris, France) were described elsewhere (Buendia and Courvalin, 1997; Chaudhary and Courvalin, 1993; Collas et al., 1996). Anti-HA95 polyclonal antibodies (designated α-HA95) directed against amino acids 65-84 of human HA95 were produced and affinity-purified as described by Eurogentec. The anti-Myc antiseraum was from Invitrogen (cat. no. R950-25). Anti-emerin monoclonal antibodies (mAbs) were a generous gift from Dr G. E. Morris (N. E. Wales Institute, Wrexham, UK; Manilal et al., 1996).

**Cell culture and transfection**

The human fibroblast cell line 293T and the B cell line Bjab were cultured in RPMI1640 (Gibco BRL) as described previously (Ørstavik et al., 2000). HeLa cells were grown as monolayers in EEMEM (Gibco BRL) and synchronized in M phase with 1 μM nocodazole as reported earlier (Eide et al., 1998).

Bjab cells were stably transfected with an EcoRI/NoI fragment of full-length HA95 cDNA ligated to a c-myc epitope at the C' end (HA95-Myc) and cloned into pcDNA3 (Invitrogen) as described previously (Ørstavik et al., 2000). Fibroblasts (293T) were transiently transfected with an EcoRI/BamHI fragment encoding residues 1-347 of human HA95 and ligated into pEGFP-N1 (Clontech) as reported earlier (Ørstavik et al., 2000). Fibroblasts were also transiently transfected with a BglII-EcoRI fragment encoding amino acids 326-772 of the human transcription factor TCF11 (Luna et al., 1995) and ligated into pECFP-N1 (Clontech). This TCF11-CFP vector was a kind gift from Dr A. B. Kolstø (Biotechnology Center, University of Oslo; A. B. Kolstø, unpublished data). Cells were examined for HA95-GFP or TCF11-CFP expression after 24 hours.

**Mitotic and interphase cell extracts**

Cytosolic extracts were prepared from mitotic HeLa cells as described earlier (Collas et al., 1999). In short, mitotic cells were homogenized by sonication in 1 volume of lysis buffer and the lysate sedimented at 10,000 g for 15 minutes. The supernatant was cleared at 200,000 g for 3 hours in a Beckman SW55 rotor. The clear supernatant (mitotic cytosol) was aliquoted and frozen in liquid nitrogen and stored at −80°C. Interphase cytosol was prepared as above except that EDTA was omitted from the lysis buffer.

Mitotic membrane vesicles were recovered from the 200,000 g pellet following mitotic cytosol preparation, washed by resuspension and sedimentation at 100,000 g for 30 minutes in membrane wash buffer, frozen in liquid nitrogen and stored at −80°C (Collas et al., 2000).

**Isolation of nuclei, nuclear matrices and interphase chromatin**

Nuclei from Bjab cells used for fractionation or immunological procedures were purified as described elsewhere (Ørstavik et al., 2000). Briefly, cells were suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl2) and lysed by addition of 0.5% NP-40. Nuclei were sedimented, washed in buffer N (10 mM Hepes, pH 7.5, 2 mM MgCl2, 250 mM sucrose, 25 mM KCl, 1 mM DTT and protease inhibitors). HeLa nuclei were isolated by Dounce-homogenization as described previously (Collas et al., 1999). Bjab and HeLa nuclei were frozen at −80°C in buffer N containing 70% glycerol.

For biochemical experiments, high salt (2 M NaCl)-extracted nuclear matrices were prepared from purified Bjab nuclei after extractions of nuclei with 1 mg/ml DNase I and 0.5% Triton X-100 as reported previously (Reyes et al., 1997). Nuclear matrices were further extracted with 2 M NaCl for 30 minutes at 4°C before being solubilized in SDS sample buffer. Interphase chromatin was prepared from Bjab nuclei essentially as described (Collas et al., 1999). Briefly, approx. 106 nuclei were digested with 5 U micrococcal nuclelease and nuclei sedimented. The supernatant was collected and the pellet was resuspended in 2 mM EDTA and incubated for 15 minutes at 4°C. After sedimentation the supernatant was combined with the first supernatant to yield an interphase chromatin fraction. Proteins were precipitated with trichloroacetic acid and dissolved in SDS sample buffer. To solubilize mitotic chromatin, chromosomes were recovered from a mitotic cell lysate (Collas et al., 1999), extracted with 1% Triton X-100, sedimented and the detergent-insoluble fraction was digested with 5 U micrococcal nuclease. After sedimentation, proteins of the supernatant were precipitated and dissolved in SDS-sample buffer.

In situ extraction of nuclear matrices was carried out after affixing Bjab nuclei onto poly-L-lysine-coated glass coverslips. Nuclei were washed in PBS, extracted with 0.1% Triton X-100 in PBS for 5 minutes, incubated for 5 minutes with ice-cold cytoskeleton stabilization buffer (CSK; Collas et al., 1999) containing 0.1% Triton X-100 and washed twice with CSK buffer. DNA was digested for 30 minutes with 1 mg/ml DNase I made in CSK buffer. Digested nuclei were washed twice 5 minutes with PBS and fixed with 3% paraformaldehyde for immunofluorescence analysis.

**Cross-linking of anti-HA95 antibodies to nuclear localization signal peptides and introduction of antibodies-NLS conjugates into nuclei**

Synthetic nuclear localization signal (NLS) peptides of the SV40 large T antigen (CGGPKKKRKV-NH2) were conjugated to α-HA95 or pre-immune IgGs using the cross-linker MBS (Pierce Biochemicals). One hundred micrograms of immunoglobulins in 100 μl were added to 10 μl MBS (at 1 mg/ml in dimethylformamide) and the mixture stirred for 30 minutes. Antibodies derivatized with MBS were then washed with 100 μl PBS and fixed with 3% parafomaldehyde for immunofluorescence analysis.
A nuclear import assay for antibody-NLS conjugates typically consisted of 20 µl interphase cytosol, an ATP-generating system (2 mM ATP, 20 mM phosphocreatine, 50 µg/ml creatine kinase), 100 µM GTP, 2-x10^6 nuclei and 2 µl antibody-NLS import substrate (0.3 µg/µl final protein concentration). Reactions were incubated at 23°C for 1 hour. At the end of incubation, nuclei were recovered by sedimentation at 1,000 g through a 1 M sucrose cushion. Nuclei were either resuspended in buffer N for immunofluorescence or in mitotic cytosol for nuclear disassembly reactions. Nuclear import of antibodies was monitored by immunofluorescence using TRITC-conjugated anti-rabbit antibodies (data not shown).

**Nuclear disassembly and chromatin condensation**

A nuclear disassembly/chromatin condensation reaction typically consisted of 20 µl mitotic cytosol and 1 µl of nuclei (2-x10^6 nuclei, intact or loaded with antibodies). The reaction was started by addition of the ATP-generating system and allowed to proceed at 30°C for up to 2 hours (Collas et al., 1999). Condensation of chromatin into compact chromosomes was monitored by phase contrast microscopy or by staining DNA with 0.1 µg/ml Hoechst 33342 (Steen et al., 2000). Percent chromatin condensation was calculated as the ratio of condensed chromatin masses per number of nuclei examined. For biochemical analyses, the condensed chromatin was sedimented at 1,000 g through 1 M sucrose and recovered from the pellet.

**Nuclear reconstitution assay**

A chromatin substrate was prepared from interphase nuclei disassembled in mitotic cytosol as described above. Condensed, membrane-free chromatin masses were recovered by sedimentation through 1 M sucrose and resuspended in interphase cytosol to their initial concentration (approx. 2,000 chromatin units/µl extract). It was necessary to supplement the interphase cytosol with mitotic vesicles to provide membranes harboring proteins required for NE assembly (see Results; Collas et al., 1996; Pyrpasopoulou et al., 1996). An ATP generating system and 100 µM GTP were added to promote chromatin decondensation, nuclear membrane vesicles binding to chromatin and fusion of vesicles into nuclear membranes (Collas et al., 1996). In some instances, cytosol and chromatin masses were pre-incubated with antibodies for 30 minutes prior to adding the ATP-generating system. The reaction was incubated at 30°C for up to 2 hours and nuclear reassembly was monitored by phase contrast microscopy, DNA labeling with Hoechst 33342, membrane staining with 10 µg/ml of the lipophilic dye DiOC6 and immunofluorescence.

**Immunological procedures**

Immunoblotting analysis was performed as described earlier (Collas et al., 1999) using the following antibodies: anti-HA95 (1:250 dilution), anti-lamin B (1:1,000), anti-LBR (1:500), anti-LAP2 (1:500), anti-emerin (1:250). For immunofluorescence analysis (Collas et al., 1996) cells, nuclei or chromatin masses were sedimented onto coverslips, fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 (cells and nuclei only) and proteins blocked with 2% BSA in PBS/0.01% Tween-20. Primary and secondary antibodies were used at a 1:100 dilution and DNA was stained with 0.1 µg/ml Hoechst 33342. Observations were made and photographs taken as described earlier (Collas et al., 1999). For immunoprecipitations (Collas et al., 1999), whole mitotic or interphase cells were sonicated on ice in immunoprecipitation buffer (10 mM Hepes, pH 7.5, 10 mM KCl, 2 mM EDTA, 1% Triton X-100 and protease inhibitors) and the lysate centrifuged at 10,000 g for 15 minutes. The supernatant was pre-cleared with Protein A/G agarose. Immunoprecipitations were carried out with relevant antibodies (diluted 1:50) from the supernatant at room temperature for 2.5 hours, followed by incubation with Protein A/G-agarose for 1.5 hours and centrifugation at 4,000 g for 10 minutes. Immunocomplexes were washed three times and proteins eluted in SDS sample buffer.

**Immunoprecipitation of the HA95 complex after cross-linking**

Purified Bjab nuclei (approx. 2x10^5) suspended in buffer N were cross-linked with 1 mM of the cleavable cross-linker dithiobis-succinimidylpropionate (DSP) for 15 minutes at room temperature (Zha et al., 1998). The reaction was stopped with 50 mM Tris (pH 7.5) from a 1 M stock and the mixture incubated for 15 minutes at room temperature. Proteins were precipitated with 0.25 M (NH4)2SO4 and dissolved in 8 M urea. Urea was diluted to 2 M with immunoprecipitation buffer and HA95 was immunoprecipitated as described above.

**Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) experiments**

FRAP was carried out at room temperature with an Argon-Krypton laser (model 643LICA-A01, Omnicrome, Carlsbad, CA, USA) using a confocal scanning laser DM RXA Leica microscope fitted with a x63 water-immersion objective. Cells expressing HA95-GFP or TCF11-CFP (used as a control for fluorescence recovery) were illuminated at 488 nm and detection done at 495-585 nm. The indicated box in Fig. 2B was bleached by continuous scanning and recovery of fluorescence monitored by scanning the whole cell in 5-minute intervals. We noted that the diffusion rate of TCF11-CFP was so high that it was virtually impossible to detect photobleaching in nuclei of TCF11-CFP-expressing cells (data not shown). Thus, FLIP was carried out using TCF11-CFP-expressing fibroblasts by continuously photobleaching the area shown in Fig. 2C for five successive 50-second periods and monitoring fluorescence by scanning the entire cell between each bleaching sequence. In neither FRAP nor FLIP experiments did scanning laser intensity significantly photobleach the sample.

**RESULTS**

**HA95 is a non-diffusible nuclear protein**

The subcellular localization of HA95 was examined throughout the cycle in the human B cell line Bjab. Immunofluorescence analysis using affinity-purified anti-HA95 antibodies (α-HA95) indicated that HA95 was exclusively nuclear in interphase, co-localized with DNA and was excluded from nucleoli (Fig. 1A). During all mitotic stages, HA95 staining was restricted to chromosomes (Fig. 1A). Cell fractionation data confirmed these observations and showed that HA95 was nuclear in interphase and co-fractionated with micrococcal nuclease-soluble chromatin at mitosis (Fig. 1B). These results were verified by immunofluorescence analysis of 293T fibroblasts transiently expressing HA95-GFP, in which endogenous HA95 co-localized with HA95-GFP (Fig. 1C). Additionally, Myc-tagged HA95 stably expressed in Bjab cells also co-localized with endogenous HA95, as judged by double immunofluorescence using anti-Myc and anti-HA95 antibodies (Fig. 1D). Thus, HA95 is restricted to the nucleus in interphase and associates with chromatin at mitosis.

To address the extent of association of HA95 with nuclear structures, purified Bjab nuclei were extracted with incremental concentrations (0.05-1 M) of NaCl in the presence of 0.1% Triton X-100. Western blotting analysis of particulate and soluble fractions indicated that HA95 remained insoluble even at the highest salt concentration (Fig. 2A). Insolubility of HA95 after detergent and salt extraction was verified in a FRAP experiment to qualitatively examine the mobility of
HA95-GFP in living 293T fibroblasts. HA95-GFP was found to be highly immobile in interphase nuclei, as judged by lack of fluorescence recovery up to 15 minutes after photobleaching (Fig. 2B). In a control experiment, photobleaching fibroblast nuclei expressing residues 326-772 of the transcription factor TCF11 fused to CFP was followed by immediate recovery of fluorescence into the bleached area, such that the bleach was virtually impossible to monitor (data not shown). However, repeated 50-second bleaches elicited a loss of TCF11-CFP fluorescence in the entire nucleus over a 275-second period (Fig. 2C, right nucleus, arrow; the nucleus on the left was not bleached). This ascertained that a mobile nuclear protein was capable of diffusing under the present conditions. Taken together, the data of Fig. 2 argue that HA95 is a non-diffusible protein tightly anchored to nuclear structures.

HA95 is a protein of the chromatin and of the nuclear matrix scaffold

To address the subnuclear distribution of HA95, purified Bjab nuclei were fractionated into salt-extracted nuclear matrices (defined as a nuclear scaffold resistant to 1 mg/ml DNase I, 0.5% Triton X-100 and 2 M NaCl) and micrococcal nuclease-soluble chromatin. Western blots and densitometric analyses of each fraction revealed that approx. 30% of HA95 partitioned with the matrix while approx. 70% co-fractionated with chromatin (Fig. 3A).

These biochemical data were verified by in situ preparations of Bjab nuclei and nuclear matrices as described in Materials and Methods, followed by immunofluorescence analysis. As expected, intranuclear HA95 labeling was detected in intact nuclei, whereas antibodies against B-type lamins (referred to as ‘lamin B’ in the text), markers of the nuclear matrix/lamina network, decorated the NE (Fig. 3B, Nuclei). DNase digestion of nuclei however, removed most intranuclear HA95 labeling (Fig. 3B, Matrices), suggesting the association of a fraction of HA95 with nucleic acids. Nevertheless, strong perinuclear labeling of HA95 remained and co-localized with lamin B throughout the lamina/matrix network, as evidenced by varying the focal plane of examination (Fig. 3B, Matrices). Thus, although HA95 is primarily associated with DNase-sensitive chromatin, a significant fraction of HA95 also associates with the nuclear matrix/lamina network.

Association of HA95 with the nuclear lamina was verified biochemically after immunoprecipitation of HA95 or lamin B

**Fig. 1.** Subcellular distribution of HA95 during the cell cycle. (A) Distribution of HA95 was examined by immunofluorescence analysis of unsynchronized Bjab cells using an affinity-purified polyclonal anti-HA95 antibody. DNA (insets) was labeled with Hoechst 33342. (B) Interphase and mitotic Bjab cells were fractionated into nuclei or chromatin, cytosol and cytoplasmic membranes, and proteins of each fraction were immunoblotted using the anti-HA95 antibody. (C) Human 293T fibroblasts were transiently transfected with HA95-GFP. After 24 hours, cells were fixed, labeled with anti-HA95 antibodies and localizations of HA95-GFP and endogenous HA95 were examined by fluorescence microscopy. (D) Bjab cells stably expressing HA95 tagged with a Myc epitope (HA95-Myc) were analyzed by immunofluorescence using anti-Myc and anti-HA95 antibodies. DNA was stained with Hoechst 33342. Bars, 10 μm.
from Bjab cells. HA95 and lamin B co-precipitated from interphase cell lysates regardless of the precipitating antibody, whereas at mitosis the proteins were segregated (Fig. 3C). We noted however, that not all HA95 was co-precipitated with lamin B under conditions where all detectable lamin B could be immunoprecipitated, as judged on immunoblots of the non-precipitated material (Fig. 3C, Sup.). Interaction of HA95 with lamin B in interphase was verified by co-precipitation of the lamin from Bjab cells expressing Myc-tagged HA95, using anti-Myc antibodies (Fig. 3D). Lamin B also co-precipitated with HA95 after treating Bjab nuclei with the cleavable, membrane-permeable, cross-linker DSP (Zhao et al., 1998) and denaturation in 8 M urea (Fig. 3D, DSP). These results indicate a tight interaction between HA95 and the lamina that is cell cycle-regulated.

**HA95 interacts with integral proteins of the inner nuclear membrane**

The existence of HA95 at the NE-chromatin interface raises the possibility that HA95 interacts with the inner nuclear membrane (INM). To test this hypothesis, HA95 was immunoprecipitated from interphase cells and the precipitates were immunoblotted with antibodies against the INM markers LBR, LAP2β and emerin. These three markers co-precipitated with HA95, but not with pre-immune IgGs (Fig. 4A). Interaction of HA95 with INM proteins was confirmed by the co-precipitation of LBR, LAP2β and emerin with HA95-Myc from HA95-Myc-expressing cells, using anti-Myc antibodies (Fig. 4B). Moreover, anti-Myc antibodies co-precipitated HA95-Myc as well as endogenous HA95, suggesting that HA95 associates with itself (see also Ørstavik et al., 2000; Fig. 4B). Finally, to evaluate the strength of the interaction of HA95 with INM proteins, HA95 was immunoprecipitated from Bjab nuclei following reversible cross-linking with DSP under low efficiency cross-linking conditions and protein denaturation in 8 M urea (see Zhao et al., 1998). Neither LBR, LAP2β nor emerin co-precipitated with HA95 when proteins were denatured with urea without previous DSP cross-linking (data not shown). In contrast, both LBR and LAP2β co-precipitated with HA95 from DSP-treated nuclei (Fig. 4C). Interestingly however, emerin did not co-precipitate with HA95 under these conditions (Fig. 4C). These results argue that associations of HA95 with LBR and LAP2β are stronger than that with emerin.

**Functional HA95 is required for disassembly of nuclear membranes, but not of the lamina, in mitotic cytosol**

Disassembly of the NE at mitosis involves the disruption of INM-lamina-chromatin interactions. We determined whether HA95 played a role in NE disassembly by blocking HA95 function within purified HeLa nuclei using the affinity-purified
Fig. 3. HA95 is associated with chromatin and the nuclear matrix. (A) Purified Bjab nuclei were fractionated into chromatin and high salt-washed nuclear matrices and proteins were immunoblotted using anti-HA95 antibodies. Relative amounts (means ± s.d.) of HA95 in each fraction were determined by densitometric analysis of duplicate blots. (B) Bjab nuclei settled on coverslips were permeabilized with 0.1% Triton X-100 (Nuclei) or further extracted with DNase I to produce nuclear matrices (Matrices). Nuclei and matrix preparations were fixed and examined by immunofluorescence using anti-HA95 and anti-lamin B antibodies. DNA was labeled with Hoechst 33342. Photographs of matrices were taken in two different focal planes. Bar, 10 μm. (C) HA95 and lamin B (LB) were immunoprecipitated (IP) from interphase and mitotic cell lysates and immune precipitates immunoblotted with anti-HA95 and anti-lamin B antibodies. Sup., immunoblot of a reaction supernatant following complete immunodepletion of B-type lamins from interphase cell lysate. (D) Left panel, HA95-Myc was immunoprecipitated from Bjab cells expressing Myc-tagged HA95 using anti-Myc antibodies and precipitates were immunoblotted using anti-lamin B antibodies. Right panel, co-precipitation of HA95 and lamin B after cross-linking nuclear proteins with DSP and denaturing proteins with 8 M urea. HA95 was immunoprecipitated and precipitates immunoblotted with anti-lamin B antibodies. IgG, control immunoprecipitations using pre-immune rabbit IgGs.

Fig. 4. HA95 associates with integral proteins of the inner nuclear membrane. (A) HA95 was immunoprecipitated (IP) from interphase Bjab cell lysates and immune precipitates were immunoblotted with anti-LBR, anti-LAP2 and anti-emerin antibodies. Control immunoprecipitations were performed using pre-immune rabbit IgGs. (B) Co-precipitation of HA95 and INM proteins from Bjab cells expressing HA95-Myc. Interphase cell lysates were immunoprecipitated using anti-Myc antibodies and immune precipitates immunoblotted using anti-HA95, anti-LBR, anti-LAP2 and anti-emerin antibodies. (C) Immunoprecipitation of HA95 after cross-linking. Bjab nuclei were cross-linked with DSP, proteins were precipitated, denatured in 8 M urea and HA95 was immunoprecipitated. The precipitates were immunoblotted using anti-LBR, anti-LAP2 and anti-emerin antibodies.
anti-HA95 antibody, and monitoring nuclear breakdown in a cytosolic extract derived from mitotic HeLa cells. This mitotic cytosol was previously shown to support NE disassembly and chromosome condensation (Steen et al., 2000). Anti-HA95 antibodies (or pre-immune rabbit IgGs) were conjugated to NLS peptides and imported into purified HeLa nuclei using a cell-free nuclear import assay.

To address the effect of immunoblocking HA95 on nuclear disassembly, antibody-loaded interphase nuclei (Fig. 5A, Input) were exposed for 2 hours to mitotic cytosol and nuclear disassembly was assessed by phase contrast microscopy and membrane labeling with DiOC6. No Ab, control nuclei not exposed to any antibody; IgG, control nuclei loaded with pre-immune IgGs; α-HA95, nuclei loaded with α-HA95; α-HA95+pep., nuclei loaded with α-HA95 bound to the competitor HA95 peptide. Bar, 10 μm. (B) Proportions (± s.d.) of chromosome condensation in nuclei containing α-HA95 (●), preimmune IgGs (□), no antibodies (▲) or α-HA95 plus competitor peptide (▼) during incubation in mitotic cytosol.

Fig. 5. Intranuclear immunoblocking of HA95 inhibits nuclear breakdown in mitotic cytosol. (A) Anti-HA95 antibodies were introduced into purified HeLa nuclei as described in Materials and Methods (Input). After recovery by sedimentation through sucrose, nuclei were resuspended in mitotic cytosol and nuclear morphology was examined after 2 hours by phase contrast microscopy and membrane labeling with DiOC6. No Ab, control nuclei not exposed to any antibody; IgG, control nuclei loaded with pre-immune IgGs; α-HA95, nuclei loaded with α-HA95; α-HA95+pep., nuclei loaded with α-HA95 bound to the competitor HA95 peptide. Bar, 10 μm. (B) Proportions (± s.d.) of chromosome condensation in nuclei containing α-HA95 (●), preimmune IgGs (□), no antibodies (▲) or α-HA95 plus competitor peptide (▼) during incubation in mitotic cytosol.

To address the effect of immunoblocking HA95 on nuclear disassembly, antibody-loaded interphase nuclei (Fig. 5A, Input) were exposed for 2 hours to mitotic cytosol and nuclear disassembly was assessed by phase contrast microscopy and nuclear membrane labeling with the lipophilic dye, DiOC6. Control nuclei not exposed to immunoglobulins or loaded with pre-immune IgGs condensed into chromosomes while nuclear membranes disassembled (Fig. 5A,B, No Ab, IgG). In contrast, α-HA95 inhibited chromatin condensation and nuclear membrane solubilization (Fig. 5A, α-HA95) even after prolonged exposure to the cytosol (Fig. 5B). This inhibition was specific since it did not occur with α-HA95 introduced into nuclei with 160 μM of the competitor HA95 peptide used to generate the antibody (Fig. 5A,B α-HA95+pep.).

To ascertain the maintenance of the NE after immunoblocking HA95, markers of the INM and of the lamina were examined by immunofluorescence following nuclear disassembly in the presence or absence of α-HA95. Clearly, the INM persisted after blocking HA95 function as shown by LBR labeling (Fig. 6A, α-HA95). However both A/C- and B-type lamins were eliminated (Fig. 6A, α-HA95). Solubilization of B-type lamins, but not of LBR, was verified on western blots of nuclei and chromatin fractions (P) and reaction supernatants (S) at the end of a 2-hour incubation in mitotic cytosol (Fig. 6B). Thus, the absence of lamin B immunostaining resulted from solubilization of the lamin B isoforms rather than from antigen masking. Control nuclei not exposed to antibodies (not shown), or loaded with pre-immune IgGs or with α-HA95 bound to the competitor HA95 peptide underwent nuclear membrane breakdown, lamina disassembly and chromatin condensation (Fig. 6A, IgG, α-HA95+pep.). Additional immunoprecipitation experiments indicated that interactions of HA95 with lamin B, LBR and LAP2β were maintained in interphase nuclei loaded with α-HA95, indicating that the disruption of the interaction of HA95 with B-type lamins was not a mere consequence of introducing anti-HA95 antibodies into nuclei (data not shown). Rather, the results argue that intranuclear immunoblocking of HA95 prevents disassembly of the nuclear membranes, but not of the lamina, in mitotic cytosol.

HA95 function is required for chromatin condensation

Intranuclear blocking of HA95 prevents nuclear membrane breakdown and chromatin condensation. To determine whether the lack of chromosome condensation resulted from inhibition of membrane disassembly or from an effect of anti-HA95 antibodies on chromatin per se, we assessed the consequence of immunoblocking intranuclear HA95 after removal of nuclear membranes with detergent. HeLa nuclei were extracted with 0.1% Triton X-100 to solubilize nuclear membranes (verified by staining with DiOC6; data not shown), incubated with α-HA95 for 1 hour in nuclear buffer and subsequently exposed to mitotic cytosol for 2 hours. As anticipated, demembranated nuclei not exposed to immunoglobulins or exposed to pre-immune IgGs condensed normally, albeit at a lower frequency than intact nuclei (Fig. 7; compare with Fig.
However, pre-incubation of demembranated nuclei with α-HA95 abolished chromosome condensation (Fig. 7). As the nuclear lamina is solubilized in mitotic cytosol, this implies that inhibition of condensation after intranuclear blocking of HA95 (see Fig. 5) does not solely result from the persistence of nuclear membranes. Rather, immunoblocking of intranuclear HA95 also affects chromosome condensation.

Antibody blocking of HA95 does not inhibit nuclear reassembly in interphase cytosol

The role of HA95 in nuclear membrane disassembly in vitro, as judged from the antibody inhibition experiments, raises the possibility that HA95 might also be implicated in the reassembly of the NE (membranes or lamina, or both) at the end of mitosis. To test this hypothesis, we examined whether antibody-mediated blocking of HA95 associated with condensed chromosomes would affect nuclear reassembly in vitro. To this end, we developed a cell-free system that recapitulates nuclear reassembly at the end of mitosis. A condensed chromatin substrate was prepared by exposing purified HeLa nuclei to mitotic cytosol as described above. Nuclei breakdown took place and the condensed chromatin was purified by sedimentation through a sucrose cushion. The condensed chromatin was exposed to pre-immune IgGs, α-HA95 or no antibodies for 30 minutes. After sedimentation through 1 M sucrose to remove unbound antibodies, the chromatin was resuspended into an interphase cytosol containing an ATP-regenerating system, GTP and membrane vesicles to promote nuclear reformation. After 2 hours, chromatin decondensation and NE reformation were monitored. Phase contrast examination shows that nuclei reassembled whether the chromatin was exposed to preimmune IgGs or α-HA95 (Fig. 8). Moreover, all nuclei reformed membranes, as judged by DiOC6 labeling, and a nuclear lamina, as shown by immunofluorescence analysis of B-type lamins (Fig. 8). Based on these criteria, we concluded that antibody-mediated blocking of HA95 associated with condensed chromosomes does not impair nuclear reconstitution in vitro.

DISCUSSION

Using cell and nuclear fractionation techniques and in vitro nuclear disassembly and reconstitution assays, we report biochemical and functional characterizations of HA95, a ubiquitous nuclear protein recently cloned by us and others (Ørstavik et al., 2000; Seki et al., 2000; Westberg et al., 2000). HA95 is homologous to AKAP95, a PKA-binding protein of the nucleus, but lacks the RII-binding motif of AKAP95 and thus, does not bind PKA-type II. HA95 is primarily a chromatin-associated protein, although a significant proportion co-fractionates with the nuclear matrix/lamina network. This positions HA95 primarily at the chromatin-NE interface.

A previous study involving human-mouse heterokaryons suggested that HA95/HAP95 was able to shuttle in and out of...
the nucleus, although a steady state nuclear localization of HA95 was reported (Westberg et al., 2000). Our studies, however, do not support a shuttling property of HA95 for three reasons: (i) HA95 is not detected on western blots of cytosolic fractions even after overloading the gels (data not shown); (ii) HA95 remains insoluble after extraction of nuclei with detergent and high salt; (iii) FRAP experiments demonstrate that HA95-GFP does not roam in the nucleus. Thus, we concluded that HA95 is tightly anchored to insoluble nuclear structures throughout the cell cycle.

HA95 constitutes the third example to date of a chromatin protein interacting with integral proteins of the INM. First, heterochromatin protein HP1 was identified as a binding partner for LBR (Ye and Worman, 1996). Second, BAF, a small DNA-binding protein implicated in retroviral genome insertion into chromosomes (Cai et al., 1998; Lee and Craigie, 1998), was also found to bind LAP2β in a yeast two-hybrid screen (Furukawa, 1999) through a domain conserved in emerin and MAN1 (Lin et al., 2000). Third, cross-linking and immunoprecipitation experiments indicate that HA95 tightly interacts with LBR and LAP2β but not with emerin, although emerin lies in a multi-protein complex including HA95.

Selective interaction of HP1, BAF and HA95 with proteins of the INM and of the lamina may underline implications of these associations in intranuclear processes. Deletion analyses have shown that LAP2β plays a role in DNA synthesis and in expansion of in vitro reconstituted Xenopus nuclei (Gant et al., 1999). Furthermore, lamins have been proposed to be involved in nuclear functions such as DNA replication (Ellis et al., 1997; Spann et al., 1997) and organization of pre-mRNA splicing complexes (Jagatheesan et al., 1999). Interactions of HA95 with LAP2β and B-type lamins raise the possibility that HA95 acts as an effector of LAP2β and lamin functions. Other chromatin-associated proteins interacting with the INM or the lamina, such as LAP2α (Dechat et al., 1998) and BAF (Furukawa, 1999), may also participate in these effector functions (see Wilson, 2000). Additionally, a cross-talk between HA95- and BAF-associated complexes through HA95 and BAF may not be excluded. To support this view, preliminary data from our laboratory indicate that HA95 and BAF co-precipitate in interphase (S. Martins, K. Wilson and P. Collas, unpublished data), suggesting that the two proteins biochemically and perhaps functionally interact. It emerges from these observations that the multiplicity of protein complexes linking chromatin to the NE not only provides control mechanisms for mitotic NE disassembly and reformation, but also establishes functional links between the NE and the genome.

Attachments of heterochromatin to the NE are reversible and cell cycle-regulated. This dynamics is best illustrated at mitosis, when the NE breaks down in prophase and reassembles in anaphase/telophase (reviewed by Marshall and Wilson, 1997; Collas and Courvalin, 2000). In interphase, most heterochromatin is subjacent to the NE. However in late S phase, replicating heterochromatic DNA is released from the nuclear periphery, translocated towards the replication machinery and moves back to the nuclear periphery after replication (Li et al., 1998). Reversible heterochromatin anchoring to the NE may be regulated by LBR, LAP2β or lamins, which all bind chromatin. The role of HA95 in anchoring chromatin to the INM further suggests that HA95 provides an additional level of regulation of NE-heterochromatin interactions during interphase and at mitosis.

Immunoblocking of HA95 in interphase nuclei abolishes chromosome condensation and nuclear membrane disassembly in mitotic cytosol; however, lamina disassembly is not affected. Mitotic chromosome condensation requires multiple proteins, including the multiprotein condensin complex (Hirano et al., 1996).
1997) and the HA95-related PKA-binding protein AKAP95, acting as a targeting protein for the condensin complex (Collas et al., 1999; Steen et al., 2000). The putative role of HA95 in chromosome condensation may be independent of that of AKAP95, since anti-HA95 antibodies do not recognize AKAP95 (and vice-versa) and presumably maintain AKAP95 active. This suggests that the two proteins have distinct functions in chromosome condensation. This view is supported by our observation that anti-AKAP95 antibodies do not block NE disassembly in vitro (Collas et al., 1999), and by the lack of co-precipitation of HA95 and AKAP95 in interphase and mitosis (Ørstavik et al., 2000; our unpublished data).

Anti-HA95 antibodies interfere with interactions of HA95 with LBR or LAP2β such that disruption of these interactions in a mitotic environment is abolished. How anti-HA95 antibodies affect nuclear membrane disassembly remains speculative at present. A first possibility is that anti-HA95 antibodies mask mitotic phosphorylation sites of LBR and/or LAP2β that are implicated in NE disassembly (Courvalin et al., 1992; Foisner and Gerace, 1993). Whether HA95 is phosphorylated at mitosis is currently unknown. Alternatively, anti-HA95 antibodies may cross-link components of the HA95 complex, preventing their segregation. A third possibility is that, as anti-HA95 antibodies block chromatin condensation, LBR or LAP2β association with chromatin is regulated not only by phosphorylation but also by chromatin conformation, and that changes in both are required for NE breakdown. Thus, one can envisage that a role of lamin phosphorylation at mitosis is to alter lamin-lamin interactions, and that this is all that is required to eliminate the lamins from the NE. In contrast to nuclear membranes, disruption of HA95 from the lamina is not impaired by anti-HA95 antibodies as both A/C- and B-type lamins are solubilized in the mitotic cytosol.

Implications of anti-HA95 antibody-mediated inhibition of nuclear membrane – but not lamina – disassembly, are twofold. First, this inhibition strongly supports previous reports that membrane and lamina disassembly may occur independently and involve biochemically distinct pathways (Miake-Lye and Kirschner, 1985; Newport and Spann, 1987; Collas, 1998). Second, it argues for a separation of the INM and lamina originally proposed (Chaudhary and Courvalin, 1993).

It is intriguing that anti-HA95 antibody-mediated blocking of HA95 function on condensed chromosomes does not prevent any of the nuclear reassembly steps examined, namely, chromatin decondensation, nuclear membrane and lamina assembly. An implication is that the NH2-terminal domain of HA95 (in which the antigen resides) does not contribute to membrane anchoring to chromatin upon nuclear reformation. Alternatively, HA95 may not be involved at all in targeting or initial binding of nuclear membranes to chromatin. The role of LBR in this process (Collas et al., 1996; Pyrapopoulo et al., 1996) may be mediated by LBR interaction with HP1 (Ye and Worman, 1996) rather than with HA95. Instead, HA95 may conceivably anchor the INM to chromatin at a later stage of NE assembly, possibly by the time the nuclear lamina is reassembled.

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REFERENCES


Nuclear envelope anchoring to chromatin by HA95


