The nuclear-envelope protein and transcriptional repressor LAP2β interacts with HDAC3 at the nuclear periphery, and induces histone H4 deacetylation

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Summary

Nuclear-envelope proteins have been implicated in diverse and fundamental cell functions, among them transcriptional regulation. Gene expression at the territory of the nuclear periphery is known to be repressed by epigenetic modifications such as histone deacetylation and methylation. However, the mechanism by which nuclear-envelope proteins are involved in such modifications is still obscure. We have previously shown that LAP2β, an integral nuclear-envelope protein that contains the chromatin-binding LEM domain, was able to repress the transcriptional activity of the E2F5-DP3 heterodimer. Here, we show that LAP2β's repressive activity is more general, encompassing various E2F members as well as other transcription factors such as p53 and NF-κB. We further show that LAP2β interacts at the nuclear envelope with HDAC3, a class-I histone deacetylase, and that TSA (an HDAC inhibitor) abrogates LAP2β's repressive activity. Finally, we show that LAP2β is capable of inducing histone-H4 deacetylation. Our data provide evidence for the existence of a previously unknown repressive complex, composed of an integral nuclear membrane protein and a histone modifier, at the nuclear periphery.

Key words: HDAC3, LAP2β, Histone modification, Nuclear envelope, Transcriptional repression, Chromatin architecture

Introduction

The nuclear lamina of eukaryotic cells is a protein mesh that lines the nucleoplasmic side of the inner nuclear membrane. It is known to be composed of lamins and a growing number of lamin and nuclear-envelope-binding proteins (for review, see Mattout-Drubezki and Gruenbaum, 2003). The lamina is known to be involved in diverse essential cellular processes such as maintenance of nuclear morphology, chromosome organization, cell-cycle control, differentiation and DNA replication and transcription (for review, see Gruenbaum et al., 2005). Its involvement in transcription can be viewed in two ways.

(1) Mechanical. Because lamins provide docking sites for chromatin at the nuclear periphery and act as a scaffold for DNA-related processes such as replication and transcription, perturbations of various lamina proteins cause abnormal chromatin attachment and disruption of transcription and replication (Favreau et al., 2003; Ognibene et al., 1999; Spann et al., 2002; Spann et al., 1997).

(2) Regulatory. Two general observations indicate that the nuclear lamina has a, mainly repressive, role in transcriptional regulation. First, there is a correlation between peripherally located chromatin or transcription factors and repression of gene expression. This is primarily demonstrated by the fact that heterochromatin, which is transcriptionally inactive, is preferentially located in the vicinity of the nuclear envelope (NE). Additionally, several experiments in various model systems have shown that the translocation of chromatin regions to the nuclear periphery results in silencing of the genes in these regions. In *Drosophila*, insertion of the *gypsy* insulator into a DNA sequence caused translocation of that sequence to the nuclear periphery, correlated with changes in gene expression (Gerasimova et al., 2000). In mammalian cells, the Ikaros transcriptional regulator, which activates lymphocyte-specific gene expression, was found to associate with transcriptionally inactive genes at centromeric loci (Brown, 1997). Immunoglobulin loci in inactivated pro-T cells preferentially co-localized with lamin B to the nuclear periphery, whereas they were centrally configured and active in pro-B cells (Kosak et al., 2002). Similarly, dissociation of the transcriptional repressor Oct-1 from lamin B and the nuclear periphery was correlated with reduced inhibitory activity (Imai et al., 1997).

Second, various components of the nuclear lamina interact directly with specific transcription regulators or chromatin-modifying proteins (for review, see Mattout-Drubezki and Gruenbaum, 2003), including the lamina-associated polypeptide 2 (LAP2) family of proteins (Nili et al., 2001). The six known mammalian LAP2 proteins are alternatively spliced products of the same gene (Berger et al., 1996). They share a constant N-terminus that harbors a ~40-residue-long LAP2

Emerin MAN1 (LEM) motif. This domain is essential for binding to barrier-to-autointegration factor (BAF), a DNA-binding protein that has roles in chromatin organization (for review, see Segura-Totten and Wilson, 2004). LAP2α, the largest isoform is nucleoplasmic and binds both lamin A/C and the repressive hypophosphorylated form of the retinoblastoma protein (pRb). Reduced levels of LAP2α or its aberrant localization caused mislocalization of pRb, suggesting that LAP2α and lamin A/C are anchoring sites for this protein (Markiewicz et al., 2002). LAP2β is the most ubiquitous LAP2 isoform. It is a type-II integral membrane protein that binds lamin B and chromatin via a specific region at its C-terminus. LAP2β shares homology with emerin, another LEM-domain protein that is responsible for the X-linked cases of Emery-Dreifuss muscular dystrophy (EDMD) (Bione et al., 1994). We have previously shown (Nili et al., 2001) that LAP2β interacts with germ-cell-less (GCL), a conserved BTB/POZ transcriptional repressor that is essential for proper germ-line formation (Jongens et al., 1994; Kimura et al., 2003). Mammalian GCL binds the E2F-DP3 heterodimer and represses its activity (de La Luna et al., 1999). We have shown that LAP2β also represses the activity of E2F-DP3, either co-operatively with GCL or, intriguingly, in an independent fashion, without GCL expression (Nili et al., 2001). The mechanism of this transcriptional repression is not clear.

Higher-order chromatin structure, mainly occurring via histone modifications, plays a crucial role in regulating gene expression. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two groups of enzymes with opposing activities and are the main enzymes responsible for histone modification by modulating their acetylation status. Furthermore, they have also been shown to affect transcription by interacting directly with various transcription factors (Grewal and Moazed, 2003; Somech et al., 2004). Mammalian HDACs are classified into three groups based on their similarity to their yeast orthologs. Class-I HDACs include HDACs 1, 2, 3 and 8, and are closely related to the yeast transcriptional regulator RPD3 (Emiliani et al., 1998). Classes II and III are homologous to the yeast Hda1 and Sir2 HDACs, respectively. Human HDAC3 is ubiquitously expressed (Emiliani et al., 1998). Its activity depends on its association with multisubunit repressor complexes containing the silencing mediator for retinoid and thyroid hormone receptors (SMRT) and with nuclear-receptor co-repressors (N-CORs) (Li et al., 2000). These complexes are unique compared with those formed by either HDAC1 or HDAC2. HDAC3 also differs from HDACs 1 and 2 in being essential for cell viability and localized to both the nucleus and cytoplasm of cells (Yang et al., 2002). HDAC3 has been linked to the transcriptional regulation of various factors, including GATA-2, YY1, TFII-I and RelA, a subunit of NF-κB, which has been reported to be exported from the nucleus in an acetylation-dependent manner (Chen et al., 2001; Ozawa et al., 2001; Wen et al., 2003; Yao et al., 2001). It has also been found to associate with class-II HDACs, which depend on its association for their activity (Fischle et al., 2002). Association of HDAC3 with components of the NE has not been previously described.

In this study, we report a repressive effect by LAP2β on various transcription factors, including p53, NF-κB and members of the E2F-DP family, indicating that it is a more general phenomenon than first suspected. We provide a clue to the mechanism of this repression by showing that LAP2β specifically interacts and co-localizes with HDAC3 but not HDAC1, and that its repressive effect is alleviated by the HDAC inhibitor trichostatin A (TSA). Finally, we show that overexpression of LAP2β induces deacetylation of histone H4 both in vitro and in vivo. Our results provide evidence for the existence of a novel repressive complex at the nuclear envelope. These findings broaden our view of the nuclear envelope as a transcriptionally repressive environment and might have implications for the understanding of the mechanisms underlying various pathologies in which lamina proteins are defective.

Materials and Methods

Tissue culture

Human osteosarcoma (U2OS) and human lung carcinoma (H1299) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Paisley, UK) and RPMI 1640 medium (Sigma-Aldrich), respectively, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 mg ml^{-1} streptomycin and 100 units ml^{-1} penicillin (Gibco BRL) at 37°C in a humidified incubator with 5% CO_2.

Transfections

The following plasmids were used: pcDNA3-HA-E2F5 (Lindeman, 1997); pCMV-HA-E2F-4 (Krek et al., 1994); pcDNA1-HA-E2F1 (Ginsberg et al., 1994); pCMV-HA-DP2 (Wu et al., 1993); pcDNAI-HA-DP-1 and E2F-Luciferase (Ginsberg et al., 1994); pCMV-Rb (Qin, 1992), pcDNA3HA-DP3, pcDNA-HA-LAP2β and pcDNA-HA-LAP2ζ (Nili et al., 2001); pGL2-KB-Luciferase, pCMV-p65 (Rel-A) and pCMV-Ikb (DiDonato et al., 1995); and pCMV-p53wt, pGL2-hMDM2, pGL2-p53-Luciferase, p300, FLAG-HDAC3 and MYC-HDAC1 (Taplick et al., 2001).

Transient transfections were performed using either the calcium-phosphate method, as described previously (Nili et al., 2001), or the Lipofectamine method (Lipofectamine™ 2000, Invitrogen). In brief, cells were plated 24 hours before transfection at 2×10^5 cells per well in a six-well plate (transcription assays) or 10^6 cells per 10 cm plate (protein expression and deacetylation assays). DNA amounts were kept constant by adding vector DNA (pCDNA3) when required.

Luciferase assay

Cells were harvested 24 hours after glycerol shock, washed twice with cold PBS and lysed with reporter lysis buffer (Promega, Madison, WI, USA). Expression of luciferase (Promega) and β-galactosidase were assayed immediately. Luciferase values were normalized with the aid of β-galactosidase values to correct for variations in transfection efficiencies. All luciferase and β-galactosidase activities were measured in triplicate plates for each point in several repeated experiments. For the endogenous expression of NF-κB, cells were treated with tumor necrosis factor α (TNFα) (200 units, Sigma-Aldrich, USA) 12 hours before harvesting.

For HDAC3 inhibition, 0.1 mM TSA (Sigma-Aldrich) was added to the relevant wells 12 hours before harvesting.

Yeast two-hybrid screening

The two-hybrid screening was performed using, as the bait, amino acids 219-328 of LAP2β (LAP2β-specific region) fused to the DNA-binding domain of GAL4 in PAS2 as previously described (Nili et al., 2001). Database searches and sequence comparisons were done using the BLAST program provided by the National Center for Biotechnology Information (Altschul, 1990).

In-vitro protein interaction

PGEX-4T1-GST, GST-HDAC3 and GST-HDAC1 were previously described (Taplick et al., 2001). Bacterial P-lys BL21 cells expressing glutathione-*S*-transferase (GST) or GST fused to HDAC3 (GST-HDAC3) or HDAC1 (GST-HDAC1) were grown overnight at 37°C and diluted 1:100. The cells were then grown to an optical density of 0.6 at 30°C (GST-HDAC3 and GST-HDAC1) or 37°C (GST). For GST expression, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added for an additional 3 hours. Cells were harvested and mildly sonicated (twice for 10 seconds each), and the recombinant proteins were extracted from the bacteria at 4°C using 1% Triton X-100 and 50 mM EDTA in the presence of protease-inhibitor cocktail (complete; Roche, Mannheim, Germany). The final volume of cell lysate that contained the recombinant proteins was 1/25th of the starting culture. The expressed proteins were detected by western-blot analysis using specific anti-GST antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). [^{35}S]-Labeled LAP2β and LAP2ζ were synthesized in vitro using the TNT-T7 quick system (Promega) in the presence of [^{35}S]-labeled methionine. For interaction with the in-vitro-translated products, GST and GST fusion proteins were diluted in 1 ml PBS and incubated by shaking for 2 hours at room temperature with 50 μl reduced glutathione-Sepharose beads (Amersham Pharmacia, NJ). Protein-bound beads were washed three times with 1 ml PBS, diluted in 0.5 ml PBS and incubated with 10 μl in-vitro-translated products for 1 hour at room temperature. The beads were then washed three times with 1 ml PBS and pelleted. Bound proteins were eluted by boiling the beads in 50 μl 2× SDS sample buffer, separated on SDS-PAGE and detected by autoradiography ([^{35}S]-labeled proteins) and western blot analysis (GST and GST fusion proteins).

Immunoprecipitation and western blotting

U2OS cells, plated at 10^6 cells in a 10 cm plate were co-transfected with LAP2β and HDAC3 as described above. Cells were washed with cold PBS and lysed by rapid freezing and thawing of cells immersed in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 10 μl dithiothreitol in the presence of protease-inhibitor cocktail (complete; Roche, Mannheim, Germany). Antibody-conjugated beads were prepared using 50 μl 50% anti-mouse IgG agarose beads (Sigma-Aldrich) and 5 μl monoclonal antibody against LAP2β (clone 6G11, generous gift from G. Goldstein, NJ, USA). Lysates were precleared by incubation with 50 μl 50% anti-rabbit or anti-mouse beads (Sigma-Aldrich) and then immunoprecipitated by incubation with the antibody-conjugated beads overnight at 4°C. Beads were washed three times with lysis buffer and PBS and proteins were eluted by boiling the beads in 50 μl 2× SDS sample buffer.

Proteins were separated by 12% gradient SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell) and detected using the western blot chemiluminescence Reagent plus (Pharmacia Biotech). The following primary antibodies were used: anti-LAP2β monoclonal 1:10,000; anti-HDAC3 1:5000 (Sigma-Aldrich); and anti-GST 1:5000. Secondary antibodies used were peroxidase-conjugated goat anti-mouse and peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories) at a dilution of 1:10,000.

Immunofluorescence

U2OS cells were grown on coverslips and transiently transfected with LAP2β and HDAC3 or HDAC1. Cells were fixed with ice-cold methanol (5 minutes) and ice-cold acetone (5 minutes), washed with TBS (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and blocked using 5% skimmed milk in TBS containing 0.1% Tween 20 (TBS-T) for 30 minutes. Incubations with primary and secondary antibodies were done in blocking solution for 1 hour each. Between and after the incubation with the antibodies, cells were washed using TBS-T. The coverslips were mounted in immunofluore (ICN, Costa Mesa, CA) and cells photographed with a confocal microscope.

The primary antibodies were used as follows: anti-LAP2β was used in a 1:100 dilution and anti-HDAC3 and anti-HDAC1 was used in a 1:500 dilution. Cy2- and Cy3-conjugated goat anti-mouse and donkey anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as secondary antibodies in a 1:100 and 1:500 dilution, respectively.

Mitogenic stimulation of peripheral-blood mononuclear cells

Blood samples were obtained from a consenting healthy donor. Peripheral-blood mononuclear cells (PBMCs) were separated on Ficoll-Hypaque gradient (IsoPrep, Robbins Scientific, Sunnyvale, CA, USA) and washed twice with PBS. For mitogenic stimulation, cells were grown in RPMI 1640 supplemented with 15% FCS and 12.5 mg ml^{-1} phytohemagglutinin (PHA) (Gibco BRL). Cells were harvested 72 hours after stimulation and whole-cell lysates were analysed by western blot and coimmunoprecipitation of endogenous LAP2β and HDAC3, as described above.

Histone H4 deacetylation assays

For the detection of deacetylation using western-blot approach, U2OS cells were transfected with LAP2β (0.1 μg or 1.0 μg) or HDAC1 (1 μg) using the Lipofectamine method and treated 24 hours later with 5 mM sodium butyrate. 12 hours later, cells were harvested and prepared for western-blot analyses as described above. For the detection of deacetylation by immunofluorescence, U2OS cells were grown on coverslips and transiently transfected with LAP2β or HDAC1. Cells were prepared as described above for this analysis. The acetylation status of histone H4 in both methods was detected using antibodies against acetylated histone-H4 (Upstate Biotechnology) in a dilution of 1:500 (western blot) or 1:100 (immunofluorescence). For the detection of deacetylation of histone H4 using the histone-deacetylase assay kit (Upstate Biotechnology), U2OS cells were transfected with 1 μg LAP2β, 1 μg HDAC3, 1 μg LAP2β and HDAC3 or 1 μg p300 and pCDNA3 control plasmid. 12-16 hours later, cells were harvested and prepared for the analysis. 100 μg purified histone-H4 peptide was radioactively labeled with [^{3}H]-acetyl CoA. We determined that the proportion of incorporation of [^{3}H]-acetate into histone-H4 peptide was more than 50%, as recommended in the kit protocol. Cell lysates were thoroughly mixed for 4 hours at 37°C with or without 50 mM sodium butyrate and 25,000 counts per minute (cpm) [^{3}H]-acetylated histone-H4 peptide and the release of [^{3}H]-acetate was then measured.

Results

LAP2β represses various transcription factors in a dose-dependent manner

We have previously shown that LAP2β reduces the transcription activity of the E2F5-DP3 complex (Nili et al., 2001). In order to determine whether this activity is specific to the E2F5-DP3 pathway, we analysed its effect on other E2F-DP transcription factors. LAP2β was transfected into U2OS cells together with various E2F-DP members (E2F1-DP3α, E2F4-DP1, E2F4-DP2 and E2F5-DP2). Its effect on the activity of a luciferase-encoding reporter gene driven by a minimal promoter containing three E2F binding sites was measured. As seen in Fig. 1A, LAP2β consistently reduced the activity of all E2F-DP heterodimers to 33-66% of their maximal activities.

The effect of LAP2β on other transcription factors, not confined to the E2F family, was assessed. Similar assays were

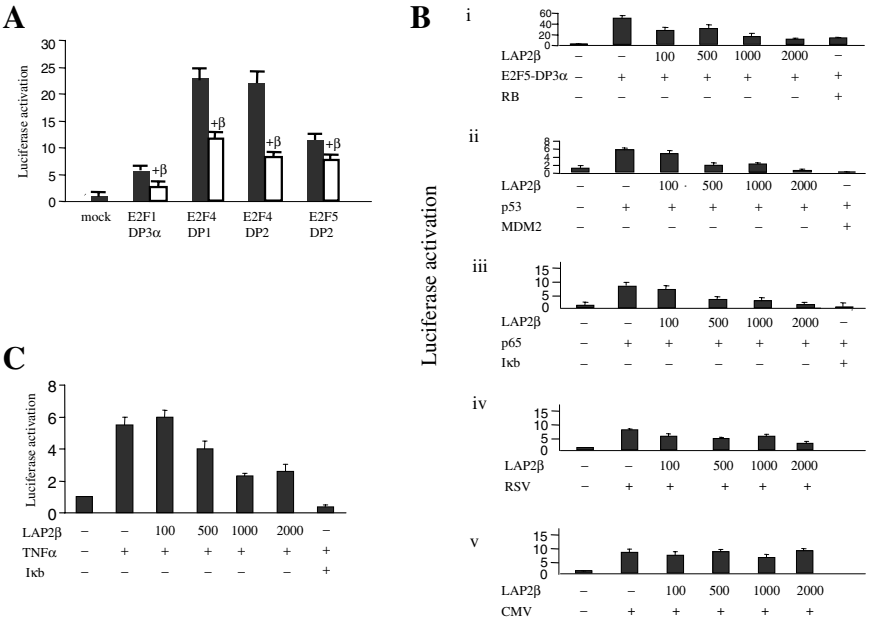

Fig. 1. LAP2β represses transcriptional activity in a dose-dependent manner. (A) LAP2β represses the activity of E2F transcription factors. The E2F reporter (0.5 μg) and pCMV-β-gal (0.5 μg) were co-transfected with expression vectors for E2F1 (0.15 μg), E2F4 (0.15 μg), E2F5 (0.15 μg), DP1 (0.15 μg), DP2 (0.15 μg), DP3α (0.3 μg), and LAP2β (1 μg) into U2OS cells as indicated. (B) LAP2β represses transcriptional activity of E2F5-DP3, p53, NF-κB and the constitutive RSV promoter in a dose-dependent manner, but not that of the constitutive CMV promoter. The specific reporters (0.1 μg) and pCMV-β-gal (0.5 μg), together with expression vectors for E2F5 (0.15 μg) and DP3α (0.3 μg, i), p53 (0.1 μg, ii), p65 (0.5 μg, iii), constitutive RSV promoter (0.1 μg, iv), constitutive CMV promoter (0.1 μg, v), and increasing doses of LAP2β (0.1-2.0 μg) were transfected into U2OS cells as indicated. For each factor, its known endogenous inhibitor was assayed [Rb (1.0 μg), MDM2 (0.5 μg) and IκB (1 μg)]. (C) LAP2β represses the activity of p65 upon TNFα stimulation. U2OS cells were transfected with increasing doses of LAP2β (0.1-2.0 μg) and IκB (1.0 μg). 12 hours before harvesting, the cells were stimulated with TNFα (200 units). For all reporter assays, the values shown represent the averages ± S.D. of duplicate (A) or triplicate (B,C) readings after the luciferase values were normalized to the β-galactosidase values and compared with the mock control.

performed using p53, NF-κB, the constitutive RSV and CMV promoters, and E2F5-DP3α as a positive control. U2OS cells were transfected with the luciferase-encoding reporter gene under the control of the RSV (Fig. 1Biv) or CMV (Fig. 1Bv) promoters, or co-transfected with E2F5-DP3α (Fig. 1Bi), p53 (Fig. 1Bii) or p65 (a subunit of the NF-κB transcription factor; Fig. 1Biii), together with plasmids encoding the luciferase-encoding reporter gene under the control of a promoter activated by the relevant transcription factor. The activity of each transcription factor was assayed on its own, with increasing concentrations of LAP2β or with its known endogenous inhibitor serving as a positive control (Rb for E2F5-DP3, MDM2 for p53 and IκB for p65). As seen in Fig. 1B, LAP2β repressed the activity of all examined transcription factors, including endogenous factors that activate the constitutive RSV promoter. However, it did not affect endogenous transcription factors that activate the constitutive CMV promoter (Fig. 1Bv), indicating that this repressive activity does not reflect a toxic effect of LAP2β. The effect of LAP2β was dose-dependent, reaching maximal reduction upon transfection with 2 μg LAP2β-encoding cDNA. Using this amount of cDNA, activation was reduced to 10-33% of the maximal activity of the various factors.

We next tested the transcription-repressive effect of LAP2β in a more physiological system in which expression of endogenous NF-κB was induced by TNFα. As depicted in Fig. 1C, LAP2β repressed the transcriptional activity of NF-κB. Again, this effect was dose dependent, reaching a ~60% reduction when cells were transfected with the maximal amount of LAP2β-encoding cDNA.

LAP2β and HDAC3 interact and co-localize to the NE

In order to elucidate the mechanism by which LAP2β represses transcription, we analysed positive clones obtained in a yeast two-hybrid screens for LAP2β-interacting proteins (Nili et al., 2001). This screen was performed using the LAP2β-specific region (amino acids 219-328) fused to the GAL4 DNA-binding domain as the bait protein (Fig. 2A). This region in LAP2β partially overlaps the lamin-binding and NE-targeting domains of LAP2β. Two of the LAP2β-interacting clones (0.6 kb and 1.2 kb long, designated clones 4.4 and 4.2, respectively) included partial cDNAs of two isoforms of murine HDAC3 (Fig. 2B). Interestingly, the two positive clones encoded only 37 overlapping amino acid residues near the N-terminus (amino acids 33-70 in HDAC3, Fig. 2B, striped bars) followed by different numbers of isoform-specific residues. We conclude that these 37 residues are part of the LAP2β-binding domain in HDAC3.

To prove that full-length LAP2β and HDAC3 interact directly with each other, full-length HDAC3 was expressed as a GST fusion protein in the PGEX bacterial expression system. GST pull-down experiments were then performed using in-vitro-synthesized [^{35}S]-labeled full-length LAP2β (Fig. 2C). As a control, we also tested [^{35}S]-labeled LAP2ζ (Fig. 2D), an alternatively spliced LAP2 isoform that lacks the LAP2β-specific region (Fig. 2A) and is therefore not expected to bind to HDAC3. [^{35}S]-Labeled LAP2β specifically bound GST-HDAC3 (Fig. 2C, lane 2) but did not bind GST alone (Fig. 2C, lane 1) or GST-HDAC1 (Fig. 2C, lane 3). As expected, [^{35}S]-labeled LAP2ζ did not bind either GST-HDAC3 or GST alone (Fig. 2D).

To demonstrate the in-vivo binding of LAP2β and HDAC3 in mammalian cells, cDNAs encoding both proteins were co-transfected into U20S cells followed by immunoprecipitation using anti-LAP2β antibodies. As seen in Fig. 2E, anti-LAP2β antibodies immunoprecipitated LAP2β- and HDAC3-containing complexes. A control experiment using mouse

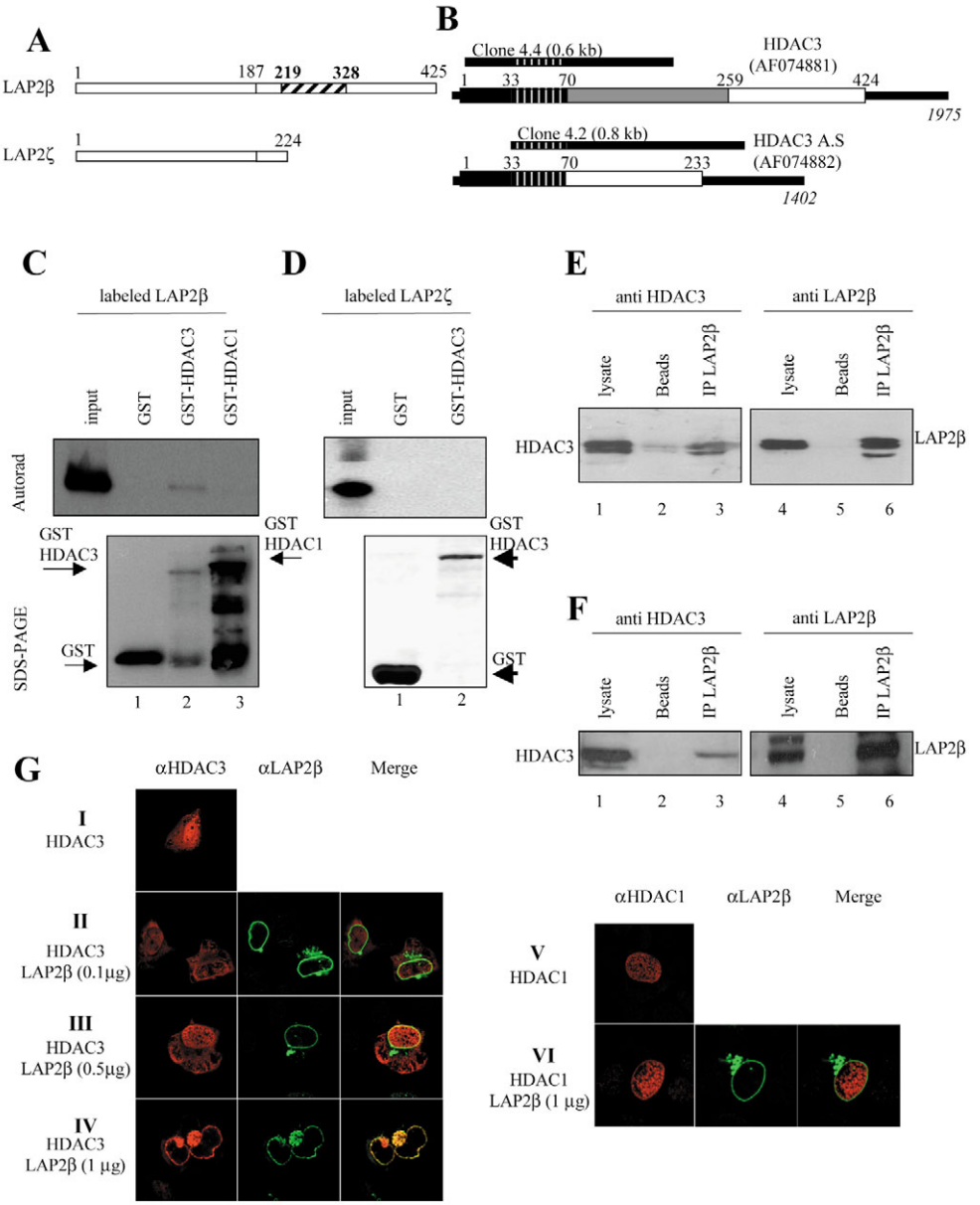

Fig. 2. LAP2β binds HDAC3 and both proteins are co-localized to the nuclear membrane. (A) Diagrams representing the LAP2β and LAP2ζ protein structures. Numbers represent the corresponding amino acids. The LAP2β-specific region (amino acids 219-328) that served as bait in the two-hybrid screen is delineated in bold stripes. (B) Schematic representation of the two-hybrid positive clones: 4.4 (0.6 kb) and 4.2 (0.8 kb), and the corresponding full-length cDNAs of HDAC3 and alternatively spliced HDAC3 (GenBank database accession numbers AF074881 and AF074882, respectively). The numbers above the two isoforms represent the corresponding amino acids and the numbers in italics below represent the corresponding nucleotides. The gray-striped area (amino acids 33-70 in both HDAC3 isoforms) represents the sequence common to both positive clones. (C) HDAC3 and LAP2β bind in vitro. HDAC3 and HDAC1 (as a control) were expressed as GST fusion proteins in bacteria. LAP2β was translated in vitro in the presence of [^{35}S]-labeled methionine (10% of input, lane 1). Mixtures of [^{35}S]-labeled LAP2β with GST, GST-HDAC3 or GST-HDAC1 (lanes 1, 2 and 3, respectively) were incubated with glutathione-Sepharose beads. The bound proteins were eluted, separated by SDS-PAGE and identified by autoradiography (top) and Coomassie-Blue staining (bottom). (D) LAP2ζ does not bind HDAC3. HDAC3 was expressed as a GST fusion protein in bacteria. LAP2ζ was translated in vitro in the presence of [^{35}S]-labeled methionine (lane 1). Mixtures of [^{35}S]-labeled LAP2ζ with GST or GST-HDAC3 (lanes 1 and 2, respectively) were incubated with glutathione-Sepharose beads. The proteins were eluted, separated by SDS-PAGE and identified by autoradiography (top) and Coomassie-Blue staining (bottom). (E) Overexpressed HDAC3 and LAP2β bind in vivo. U2OS cells were co-transfected with HDAC3 (1 μg) and LAP2β (1 μg). Cell extracts were immunoprecipitated with anti-LAP2β antibodies (lanes 3 and 6) followed by immunoblotting with anti-HDAC3 (lanes 1-3) or anti-LAP2β (lanes 4-6) antibodies. Beads only were used as a control for non-specific binding (lanes 2 and 5). A whole-cell lysate was used as a control marker for HDAC3 and LAP2β (lanes 1 and 4, respectively). (F) Endogenous binding of LAP2β and HDAC3 upon PHA stimulation of PBMCs. 2×10^6 PBMCs were stimulated with 25 μg PHA. After 72 hours, cellular extracts were immunoprecipitated with anti-LAP2β antibodies (lanes 3 and 6), followed by immunoblotting with anti-HDAC3 (lanes 1-3) or anti-LAP2β (lanes 4-6) antibodies. Beads only were used as a control for non-specific binding (lanes 2 and 5). A whole-cell lysate was used as a control marker for HDAC3 and LAP2β (lanes 1 and 4, respectively). (G) Co-localization of transfected HDAC3 and LAP2β. U2OS cells were transfected with either HDAC3 alone (1.0 μg, i), HDAC3 and increasing doses of LAP2β (0.1 μg, ii; 0.5 μg, iii; 1.0 μg, iv), HDAC1 alone (1.0 μg, v) or HDAC1 and LAP2β (1.0 μg, vi). The cells were fixed and immunostained with anti-HDAC3, anti-HDAC1 and anti-LAP2β antibodies, as indicated, and analysed by confocal microscopy.

antiserum beads alone revealed a weak band suggestive of nonspecific binding (Fig. 2E, lane 2). We further examined the interaction of the two endogenous proteins in PHA-stimulated PBMCs. These cells were chosen as they express high levels of both HDAC3 (Dangond et al., 1998) and LAP2β (data not shown) upon PHA stimulation. Similar to the immunoprecipitation results upon LAP2β and HDAC3 transfections (Fig. 2E), anti-LAP2β antibodies immunoprecipitated endogenous LAP2β and HDAC3 in PBMCs (Fig. 2F). It is worth realizing that only a small proportion of HDAC3 immunoprecipitated with LAP2β (Fig. 2F, compare lanes 1 and 3), probably indicating

a low affinity of binding between the two proteins under these conditions.

Transfection of HDAC3 into U2OS cells revealed its localization to both cytoplasm and nucleus, similar to findings in previous studies (Yang et al., 2002) (Fig. 2Gi). Co-transfection of HDAC3 and increasing amounts of LAP2β (100 ng to 1000 ng) resulted in a dose-dependent shift of HDAC3 to the nuclear membrane (Fig. 2Gii-iv). The LAP2β-dependent recruitment of HDAC3 correlates with the dose-dependent transcriptional repressive effect of LAP2β (Fig. 1B). By contrast, the nuclear localization of transfected HDAC1 was not affected by co-transfection with LAP2β (Fig. 2Gv,vi). These results support the specificity of the interaction between LAP2β and HDAC3.

TSA reduces transcriptional repression by LAP2β

The above-described findings led us to hypothesize that repression of transcription by LAP2β is mediated at least in part by HDAC3. Therefore, it was expected that TSA, which inhibits HDAC activity through binding to the enzyme catalytic site, would alleviate the repressive effect of LAP2β. U2OS cells were transfected with various transcription factors (E2F5-DP3, p53 or p65) or RSV promoter as described above, with or without LAP2β, and then treated with TSA. Transcriptional activity was measured in both treated and untreated cells (Fig. 3). The addition of TSA enhanced the activity of E2F5-DP3α, p65 and the RSV promoter but did not enhance the activity of p53 (Fig. 3, lane 3 in all graphs). When LAP2β-transfected cells were treated with TSA (Fig. 3, lane 5 in all graphs), the repressive activity of LAP2β was partially abrogated in all cases (Fig. 3, compare lanes 4 and 5 in all graphs). These results support the hypothesis that deacetylation is involved in regulation of transcription by LAP2β.

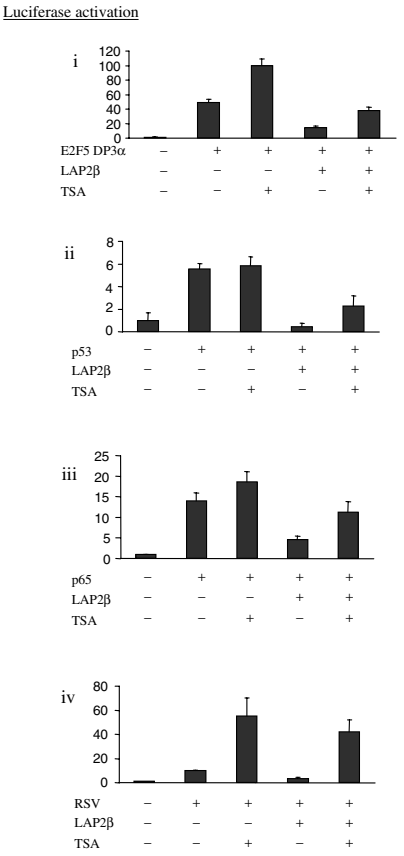

Fig. 3. TSA reduces the transcriptional repression induced by LAP2β. U2OS cells were transfected with pCMV-β-gal (0.5 μg) and expression vectors for E2F5 (0.15 μg)-DP3α (0.3 μg, i), p53 (0.1 μg, ii), p65 (0.5 μg, iii) together with their specific reporters, or constitutive RSV promoter (0.1 μg, iv) with or without LAP2β (1.0 μg), as indicated. Cells were treated with TSA (0.1 mM) as indicated 12 hours before harvesting. The values shown represent the average ± S.D. of triplicate readings after the luciferase values were normalized to the β-galactosidase values and compared with the mock control.

LAP2β induces deacetylation of histone H4

We further investigated whether LAP2β is capable of inducing deacetylation. We first examined the acetylation status of p53 and NF-κB, whose activities are repressed by LAP2β (Fig. 1) and are known to be regulated by their acetylation state (Bode and Dong, 2004; Chen and Greene, 2004). No change in acetylation of either p53 or NF-κB was detected upon LAP2β overexpression (data not shown). We then checked the effect of LAP2β on histone deacetylation. As a putative target, we chose histone H4, which was previously reported to reside in the vicinity of the NE and to form complexes with histone H3, HP1 and LBR, another inner NE protein (Polioudaki et al., 2001). The ability of LAP2β to induce deacetylation was demonstrated both in vivo and in vitro (Fig. 4). For in vivo studies, U2OS cells transfected with LAP2β and treated with 5 mM sodium butyrate were immunostained using antibodies against LAP2β and acetylated histone H4. LAP2β-transfected cells (Fig. 4A, green) demonstrated significantly reduced staining for acetyl histone H4 (Fig. 4A, top), while the untransfected cells retained their H4 acetylation (Fig. 4A, red). Cells transfected with LAP2β showed similar staining to that observed in cells transfected with HDAC1, which served as a positive control for induction of deacetylation (Fig. 4A, bottom). Similar results were obtained by western blot analysis using the same antibodies (Fig. 4B) and in other cell lines such as H1299 (data not shown). For the in-vitro studies, a biochemical deacetylation assay was performed. Nuclear extracts were prepared from U2OS cells transfected with LAP2β, LAP2β and HDAC3, HDAC3, or p300. These extracts were then incubated with a purified histone-H4 peptide labeled with [^{3}H]-acetyl-CoA, which served as a substrate for deacetylation activity. Release of [^{3}H]-acetate was measured, with elevated counts indicating high deacetylation activity. Fig. 4C shows that the deacetylation level of H4 in LAP2β-transfected cells was three times higher then that measured in untransfected cells, and about half the effect of HDAC3 that served as a positive control. The addition of HDAC3 to LAP2β more than tripled the deacetylation level achieved by LAP2β alone. H4 deacetylation induced by the p300 negative control was lower than that in untransfected cells. These results suggest that histone H4, and possibly other histones, is a substrate for the activity of a putative LAP2β-HDAC3 transcriptional repression complex at the NE.

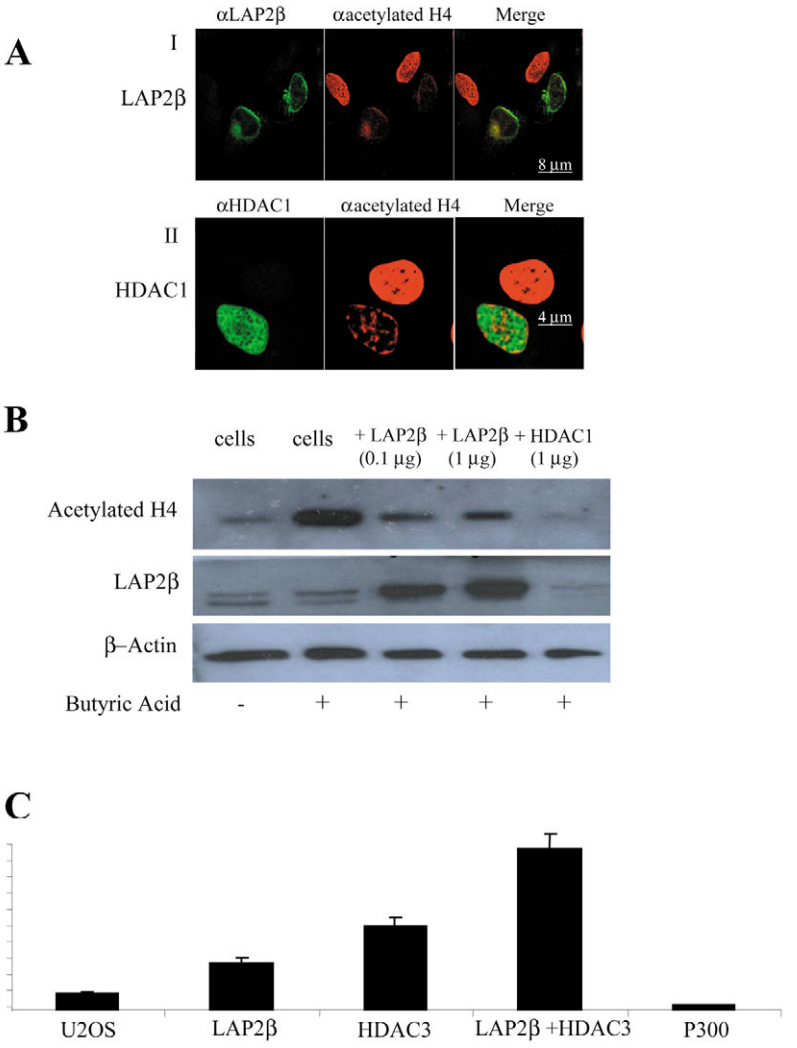

Fig. 4. LAP2β induces deacetylation of histone H4 in vitro and in vivo. (A) U2OS cells were transfected with either 1 μg LAP2β (i) or HDAC1 (ii). Cells were fixed and immunostained with antibodies against acetylated histone H4, LAP2β or HDAC1, as indicated, and analysed by confocal microscopy. (B) U2OS cells were transfected with LAP2β (0.1 or 1.0 μg) or HDAC1 (1.0 μg), followed by treatment with 5 mM sodium butyrate. Cellular extracts were detected by western blot analysis, using antibodies against acetylated histone H4, LAP2β and β-actin, as indicated. (C) Sodium-butyrate-treated U2OS cells were transfected with 1 μg LAP2β, HDAC3, LAP2β and HDAC3, or p300, as indicated. Cellular extracts were prepared and protein amount was detected in each sample. Equal amounts of lysates were incubated with [^{3}H]-acetylated histone H4 peptide. Released [^{3}H]-acetate was measured in cpm using a β-radiation counter. The experiment was repeated three times.

Discussion

We have previously shown that LAP2β is associated with transcriptional repression of the E2F5-DP3α heterodimer (Nili et al., 2001). Here, we demonstrate that LAP2β is capable of repressing the transcriptional activity of other E2F-DP pairs, as well as that of the transcription factors p53 and NF-κB and the RSV constitutive promoter. These results indicate that the activity of LAP2β is not restricted to a specific pathway but rather that it can function as a more general regulator of transcription. The fact that LAP2β repressed some, but not all, factors assayed, as well as the repressive effect seen on the endogenous activity of NF-κB upon TNFα stimulation, leads us to believe that these results reflect a physiological phenomenon and not toxicity caused by overexpression.

Various studies have established the correlation between the positioning of genes and transcriptional regulators close to the nuclear periphery and repression of transcription (for a review, see Spector, 2003). The peripheral localization of heterochromatin is believed to be a result of its interactions with NE proteins and lamins. However, the specific proteins mediating its anchorage and silencing have not yet been defined (Cohen et al., 2001). Our findings of the interaction between HDAC3 and LAP2β might provide one of the links between NE localization and functional repression.

Four lines of evidence confirm that HDAC3 and LAP2β are genuine partners and that HDAC3 is an 'effector' of LAP2β-mediated transcriptional repression. First, two different clones of HDAC3 were found to interact with LAP2β in the two-hybrid system. Second, both proteins co-immunoprecipitated from U2OS cells and specifically bound in a GST pull-down assay, providing evidence for their existence in the same complex and their direct interaction both in vivo and vitro. Third, overexpressed LAP2β drove co-overexpressed HDAC3 to the nuclear membrane in a dose-dependent manner. However, overexpression of LAP2β did not change the localization of HDAC1, indicating that LAP2β interacts selectively with HDAC3. Finally, endogenous LAP2β and HDAC3 bind each other in actively proliferating PBMCs after mitogenic stimulation (Fig. 2E) but not in resting lymphocytes (data not shown). Only a relatively small amount of endogenous HDAC3 immunoprecipitated with endogenous LAP2β in PBMCs, which might indicate that, under certain physiological conditions, only a small proportion of HDAC3 is situated at the NE, or that its localization is transient. Thus, the localization of HDAC3 at the NE could be exemplified by regular immunofluorescence techniques only after overexpression of LAP2β, which probably shifted intranuclear HDAC3 to the nuclear periphery.

Our results are in accordance with a growing number of studies linking histones and histone modifiers to the nuclear envelope. The chromatin modifier HP1 was shown to interact with both LBR, an inner nuclear membrane (INM) protein that binds lamin B1, and histones H3 and H4, to form a tetramer at the NE (Polioudaki et al., 2001). Notably, binding of HP1 and LBR to core histones and the peripheral localization of HP1 could only occur when histones were deacetylated and was strongly inhibited under acetylating conditions. Because we

show that LAP2β-mediated the deacetylation of histone H4, histones that are bound by HP1 and LBR might serve as substrates for the LAP2β-HDAC3 complex. Furthermore, although it has mostly been described as an intranuclear protein, microinjection studies showed HP1 to reside the at the nuclear periphery in a transient fashion, before dispersing in the nucleus (Kourmouli et al., 2000). This finding might shed light on our difficulties demonstrating the localization of endogenous HDAC3 at the NE.

The interactions between LBR and peripheral chromatin were further analysed in a recent study in which LBR was shown to associate with nucleosomes that cofractionated with the NE. Histones H3 and H4 isolated from the LBR-associated chromatin showed a specific acetylation/methylation 'modification signature' reminiscent of heterochromatin (Makatsori et al., 2004). Finally, in a study using a subtractive proteomic approach to identify new NE proteins, histones and histone modifiers such as HP1 and HDACs as well as transcription factors were identified in the isolated nuclear fractions (Schirmer et al., 2003). Thus, a pattern arises in which various NE proteins sequester chromatin remodelers and histone deacetylases in a defined nuclear subcompartment, and might act in concert to facilitate the establishment and propagation of repressive chromatin and hence transcriptional repression.

Finding the LAP2β-HDAC3 repressive complex at the NE might shed light on the mechanisms underlying the various nuclear envelopathies (also called laminopathies), which are an expanding group of pathological states that have been described in recent years (for review, see Somech et al., 2005). The clinical manifestations and affected tissues in these disorders are variable, although most of them are types of dystrophy (Burke and Stewart, 2002). The genes involved in most of these disorders encode emerin (an INM protein responsible for the X-linked cases of EDMD, the prototype of the nuclear envelopathies), MAN1 or lamin A/C, various mutations in which cause either the autosomal dominant form of Emery-Dreifuss muscular dystrophy (EDMD) or other laminopathies, amongst them Hutchison-Gliford progeroid syndrome (HGPS) (for review, see Mounkes et al., 2003). There are two models that try to explain how nuclear envelopathies arise. The structural model implicates the defective envelope proteins in stress-induced damage, leading to loss of cell viability. The gene-expression model suggests that, because NE proteins and lamins are involved in transcriptional regulation, mutations in these proteins will promote disease by compromising various gene regulatory pathways in different tissues. This model is mainly supported by studies that implicate lamin A in the proper localization and function of pRb (Markiewicz et al., 2002). As well as being involved in inhibition of proliferation, pRb was also shown to be important for skeletal-muscle and adipose-tissue differentiation, two tissues that are frequently affected in nuclear envelopathies (Johnson et al., 2004). In order to mediate at least some of its effects, pRb was shown to recruit various histone-modifying agents to its target promoters, including HDAC1, HDAC2 and HDAC3. Interestingly, a pRb-HDAC3 complex was shown to be important for the regulation of adipocyte differentiation by peroxisome proliferator activated receptor γ (PPARγ) (Fajas et al., 2002). Further support for the gene expression model comes from the observation that cells from EDMD patients and lamin-A-knockout mice showed an altered organization of heterochromatin at the nuclear periphery (Sabatelli et al., 2001). In about half of subjects with a clinical diagnosis of EDMD, no mutations were found in the genes encoding either emerin or lamin A (Hutchison and Worman, 2004). This, taken together with the high homology between emerin and LAP2β, and the emerging role of pRb in these pathologies make it imperative to examine the activity of the LAPβ-HDAC3 complex in these disorders. It would also be logical to check whether emerin, lamin A and possibly other LEM-domain or INM proteins have similar effects on transcription to LAP2β, and whether they interact with histone-modifying proteins. If so, this might provide the mechanism that we seek and help us to plan targeted therapies to treat nuclear envelopathies.

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