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Np95 Is a Histone-Binding Protein Endowed with Ubiquitin Ligase Activity

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Np95 is an important determinant in cell cycle progression. Its expression is tightly regulated and becomes detectable shortly before the entry of cells into S phase. Accordingly, Np95 is absolutely required for the G_1/S transition. Its continued expression throughout the S/G_2/M phases further suggests additional roles. Indeed, Np95 has been implicated in DNA damage response. Here, we show that Np95 is tightly bound to chromatin in vivo and that it binds to histones in vivo and in vitro. The binding to histones is direct and shows a remarkable preference for histone H3 and its N-terminal tail. A novel protein domain, the SRA-YDG domain, contained in Np95 is indispensable both for the interaction with histones and for chromatin binding in vivo. Np95 contains a RING finger. We show that this domain confers E3 ubiquitin ligase activity on Np95, which is specific for core histones, in vitro. Finally, Np95 shows specific E3 activity for histone H3 when the endogenous core octamer, coimmunoprecipitating with Np95, is used as a substrate. Histone ubiquitination is an important determinant in the regulation of chromatin structure and gene transcription. Thus, the demonstration that Np95 is a chromatin-associated ubiquitin ligase suggests possible molecular mechanisms for its action as a cell cycle regulator.

Ubiquitination is a frequent posttranslational modification with a vast impact on cell physiology. Ubiquitin (Ub) is a conserved 76-amino-acid polypeptide that is covalently attached to target proteins via an isopeptide bond between its carboxyl-terminal glycine and the ε-amino group of a lysine in substrate proteins (23). A complex enzymatic cascade leads to ubiquitination. Ub is first activated through the formation of a thiol ester bond with the Ub-activating enzyme (E1) and then transferred to a Ub-conjugating enzyme (E2). Finally, Ub is transferred to the substrate through the action of a Ub ligase (E3). Two major families of E3s exist, the HECT type and the RING type. In HECT-E3-mediated catalysis, Ub is transferred from E2 to HECT-E3 and then by E3 to the substrate. In RING-E3-mediated catalysis, E3 mediates the direct transfer of Ub from E2 to the substrate. The E3 ligases, therefore, are the substrate recognition components of the system and confer specificity on the process (23, 47).

The best-characterized type of ubiquitination is polyubiquitination, in which the substrate-bound Ub serves as an acceptor for further cycles of ubiquitination (23). By and large, polyubiquitin functions as a general device for targeting of the polyubiquitinated substrate to the proteasome, with ensuing proteolytic degradation (25, 48). An emerging body of evidence indicates, however, that when Ub is appended as a single moiety to a target protein (monoubiquitination), the posttranslational modification has a completely different biological impact and serves primarily to modulate protein function and/or interaction(s) (10, 24, 57).

Histones are among the major monoubiquitinated proteins in the cell, and histones H2A, H2B, and H3 have all been reported to be so modified in mammals (7, 18, 58), while ubiquitinated histone H1 has been detected in *Drosophila* (46). Despite the facts that histones were the first proteins to be discovered as monoubiquitinated (18) and that their modifications have profound effects on chromatin function (reviewed in references30 and 43), our understanding of the role of ubiquitinated histones is still preliminary. The major mechanistic understanding came from recent studies with yeast. In *Saccharomyces cerevisiae*, it was shown that the Rad6p/Bre1p complex (E2/E3 complex) mediates monoubiquitination of histone H2B (28, 52, 59). This event, in turn, causes methylation of lysines 4 and 79 on histone H3, a modification directly involved in gene silencing (4, 11, 55). These findings directly implicated histone ubiquitination in the control of transcription. In mammals, while histone ubiquitination has been correlated with transcriptional activation (8, 9, 27, 45), the molecular mechanisms are still obscure. In particular, limited knowledge is available as to the molecular machinery that executes and controls the process.

A recently identified murine protein, Np95 (17; known in humans as ICBP90 [26]), is a good candidate for this role. Np95 is a cell cycle-regulated protein that is expressed at the G_1/S boundary and in S phase and is absent in G_0 and G_1 (3,

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17, 41, 56). Functional studies have demonstrated that the function of Np95 is required for the G_1/S transition (3). In addition, overexpression of Np95, together with that of the cycE/cdk2 complex, can force reentry of terminally differentiated cells into the cell cycle (3). Finally, a possible role for Np95 in DNA damage response has been described (44). Np95 localizes exclusively to the nucleus (17), where it is found associated with PCNA in early and mid S phase (41, 56). The most striking feature of Np95 is the presence of a C3HC4-type carboxyl-terminal RING finger motif. RING finger proteins contain a characteristic C3HC4, or C3H2C3, amino acid sequence that binds two atoms of zinc with a unique ligation system (15, 16). A large body of evidence has demonstrated that the RING domain mediates Ub protein ligase activity (29, 32, 37). Thus, in summary, Np95 is an important regulator of the cell cycle that is probably associated with chromatin and possibly endowed with RING-type E3 activity. On the basis of these features, the present studies were undertaken to elucidate the molecular bases of the association of Np95 with chromatin and to test the possibility that Np95 is a mammalian Ub ligase displaying specificity for histones.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells and cells of a modified human embryonic kidney 293T cell line (Phoenix, a generous gift of G. Nolan) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Expression plasmids and mutagenesis. The full-length mouse Np95 cDNA (17) was subcloned into the pcDNA3.1/myc-His plasmid (Invitrogen) in frame and upstream of the myc epitope and of the polyhistidine (His_6) tag, yielding the pcDNA3-myc-His_6-Np95 construct. Np95 mutants were generated by recombinant PCR. The H730A mutant was generated by PCR-directed mutagenesis (QuikChange XL site-directed mutagenesis kit; Stratagene). All construct sequences were verified. The sequences of the oligonucleotides used are available on request.

Recombinant proteins and purification. For Np95 purification, pcDNA3-myc-His_6-Np95 wild-type and mutant constructs were transfected into Phoenix cells and the cells were harvested at 48 h posttransfection. Total cellular extracts were obtained by scraping the cells off the plate in buffer A (50 mM sodium phosphate buffer [pH 7.8], 0.5 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM antiproteolytic cocktail [Sigma]) supplemented with 0.1% Nonidet P-40, 1 mM β-mercaptoethanol, and 5 mM imidazole, followed by three freeze-thaw cycles and sonication. Lysates were then cleared by ultracentrifugation at 150,000 × *g* for 1 h at 4°C. The supernatant was incubated with Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) beads for 1 h at 4°C, followed by washing with 30 volumes of buffer A containing 5 mM imidazole, followed by 30 volumes of buffer A containing 30 mM imidazole. Proteins were eluted in buffer A containing 250 mM imidazole, 0.01% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM EDTA. The purity of the Np95 preparation was monitored by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. This procedure yielded preparations of >95% purity (see Fig. 2B).

In vitro translation of pcDNA3-myc-His_6-Np95 wild-type and mutant constructs was performed with the TNT coupled reticulocyte lysate system (Promega), in accordance with the manufacturer's instructions, in the presence of [^{35}S]adenosylmethionine (Amersham).

Glutathione *S*-transferase (GST) and GST-histone tail fusion proteins (21) were purified on glutathione Sepharose 4B beads (Pharmacia) in accordance with the manufacturer's instructions. Calf thymus histone proteins and pure histone H1 were from Roche; recombinant *Xenopus laevis* histones H2A, H2B, H3, and H4 were from Upstate Biotechnology. H1-depleted HeLa polynucleosomes were a generous gift of G. Schnitzler and R. Kingston. Nuclear factor YABC (NF-YABC) and NF-YBC recombinant protein complexes were obtained as described in reference 5.

Immunofluorescence and antibodies. NIH 3T3 fibroblasts were grown on glass coverslips in six-well dishes. Cells were washed twice with phosphate-buffered saline (PBS) and either fixed in 4% paraformaldehyde in PBS at room temperature (RT) for 10 min or permeabilized with 0.5% Triton X-100 in PBS for 10 min on ice before fixation. Fixed cells were then permeabilized with 1% Triton X-100 in PBS for 10 min, followed by incubation in blocking solution (2% bovine serum albumin in PBS) for 30 min. Staining with the primary antibody (1:1,000 dilution of polyclonal anti-Np95 serum [see below]) was done in blocking solution for 1 h, followed by two washes with PBS and incubation with fluorescein isothiocyanate-conjugated donkey anti-rabbit for 30 min at RT. DNA was detected by 4′,6-diamidino-2-phenylindole (DAPI) staining. Staining with secondary antibody alone yielded no signal.

A rabbit polyclonal anti-Np95 serum was raised against the purified full-length myc-His_6-tagged Np95 protein and affinity purified before use. The other antibodies used were goat anti-lamin B polyclonal antibody (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-MeCP2 antibody (ABCAM), rabbit polyclonal anti-myc epitope antibody (ABCAM), mouse anti-myc monoclonal antibody (our preparation), rabbit anti-histone H2A and anti-histone H2B polyclonal antibodies (Upstate Biotechnology), goat anti-histone H3 polyclonal antibody (N-20; Santa Cruz Biotechnology Inc.), mouse anti-NF-YB monoclonal antibody (5), anti-phosphohistone H3 antibody (ser 10, 6G3; Cell Signaling), and mouse anti-bromodeoxyuridine (BrdU) monoclonal antibody (Becton Dickinson).

Protein extraction, biochemical fractionation, and immunoprecipitation. For the experiment shown in Fig. 1A, nuclei from NIH 3T3 cells were prepared as previously described (51). Nuclei were then extracted with 200 μg of DNase I (Promega) per ml in buffer B [100 mM KCl, 300 mM sucrose, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 3 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, antiproteolytic cocktail (Sigma)] for 45 min at 33°C, followed by centrifugation at 1,500 × *g* for 10 min at 4°C. This early released fraction corresponds to the DNase I fraction. The pellet was extracted with 250 mM ammonium sulfate in buffer B and centrifuged at 4,000 × *g* for 5 min at 4°C, releasing the ammonium sulfate fraction. Finally, solubilization of the remaining pellet with 2 M NaCl in buffer B, followed by centrifugation at 8,000 × *g* for 30 min 4°C, released the high-salt fraction. Insoluble proteins represent the nuclear matrix.

Cell extracts (Fig. 2A; see also Fig. 3D and 6) were prepared by scraping the cells off the plate in buffer C (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 1 mM PMSF, antiproteolytic cocktail [Sigma]), followed by sonication at 4°C and centrifugation at 16,000 × *g* for 30 min at 4°C. For each immunoprecipitation, 100 μg of lysate was used with the indicated antibodies, followed by recovery with protein A–Sepharose CL-4B beads (Pharmacia). Beads were washed seven times with buffer C plus 50 μg of ethidium bromide per ml, and proteins were eluted by boiling in SDS sample buffer. For the experiment shown in Fig. 3D, cells were treated with 0.5% Triton X-100 in PBS for 10 min on ice, followed by three washes in PBS, before lysis.

Far-Western analysis. Far-Western analysis was carried out as described by Edmondson et al. (12, 13). The HeLa polynucleosomes (used in Fig. 2D) were digested with trypsin as previously described (20). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Protran; Schleicher & Schuell). Renaturation of the proteins and blocking of the membranes were performed in PBST-milk buffer (PBS, 0.1% Tween 20, 2% skim milk) for 2 h at RT. Membranes were incubated with purified myc-His_6-Np95 protein or mutant proteins (500 ng/3 ml) in PBST-milk buffer supplemented with 50 μg of ethidium bromide per ml for 2 h at RT, followed by detection with anti-myc (Upstate Biotechnology) and goat anti-rabbit secondary antibody in conjunction with the ECL system (Amersham) or by autoradiography (Fig. 3B). Staining with secondary antibody alone yielded no specific signal.

In vitro ubiquitination assays. Recombinant His_6-tagged Ub (His-Ub), engineered to contain a protein kinase recognition site (LRRASV), and His_6-tagged UbcH5B were purified by Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) affinity chromatography. His-Ub was labeled with [γ-^{32}P]ATP as previously described (50).

Unless stated otherwise, standard ubiquitination reaction mixtures (30 μl) contained 100 ng of purified Np95 protein, 100 nM mammalian Ub-activating enzyme (E1; AFFINITI Research Products Ltd.), 300 ng of His-tagged UbcH5B, and 3 μg of [^{32}P]His-Ub in 25 mM Tris-HCl (pH 7.6)–5 mM $MgCl_2$–100 mM NaCl–1 mM dithiothreitol–2 mM ATP. The mixture was incubated at 30°C for 1 h, and the reaction was stopped by adding 1× final SDS sample buffer. The reaction mixture was resolved by SDS-PAGE, and the ^{32}P-labeled products were visualized by autoradiography. One microgram of protein substrate was added to the reaction mixture when appropriate, as indicated in the figure legends.

For ubiquitination assays with myc-Np95 immunoprecipitates from Phoenix cells (see Fig. 6), whole cell lysates from transfected cells and anti-myc immunoprecipitates were prepared as described above. myc immunoprecipitates substituted for purified Np95 in standard ubiquitination reactions.

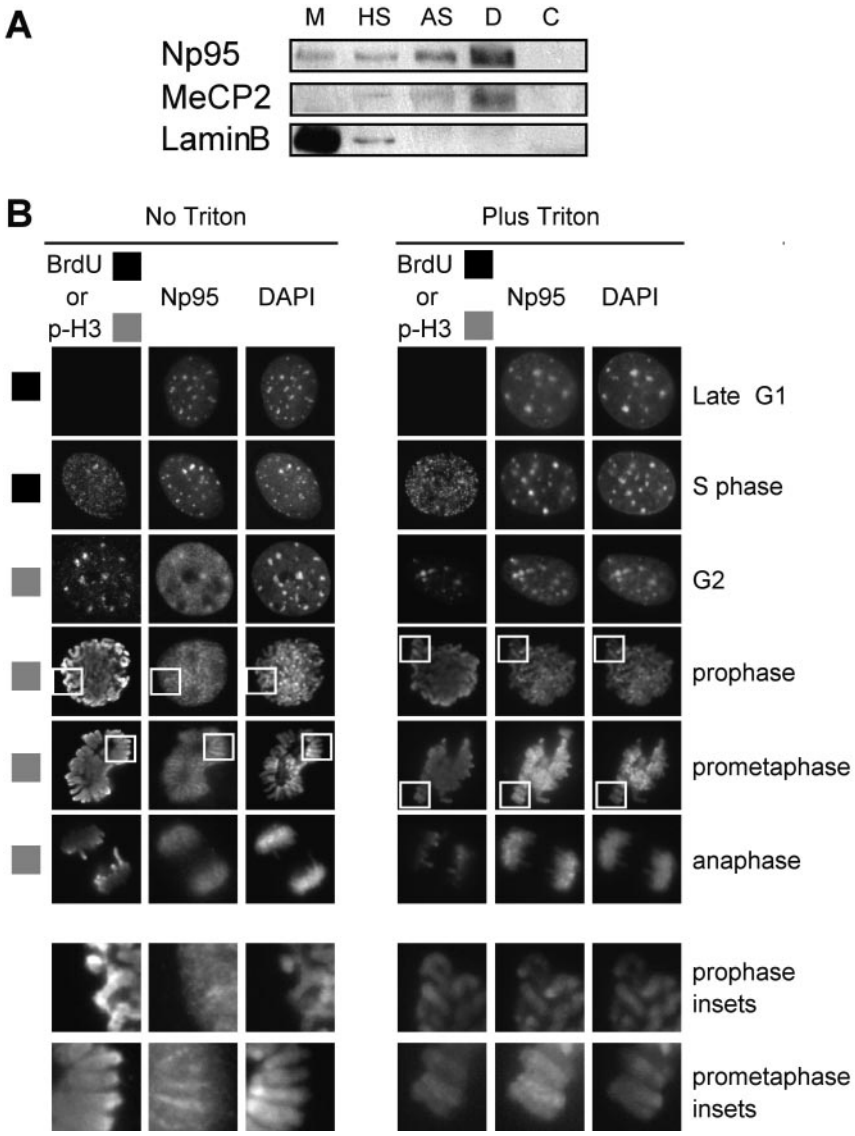

FIG. 1. Np95 is a chromatin-associated protein. (A) Biochemical fractionation of NIH 3T3 cell extracts. Fractions, prepared as described in Materials and Methods, are as follows: C, cytoplasmic; D, DNase I; AS, ammonium sulfate; HS, high salt (2 M NaCl); M, nuclear matrix. Fractions were immunoblotted with the antibodies indicated on the left. Anti-MeCP2 and anti-lamin B antibodies were used as controls to verify the purity of the fractions. (B) Triton-resistant association of Np95 with chromatin. NIH 3T3 cells were grown on coverslips and serum starved for 48 h. Ten percent FBS was then added to stimulate reentry into the cell cycle. After 16 h, 10 μM BrdU was added and the mixture was incubated for 10 min to identify cells in S phase. Cells were then harvested 16 and 23 h after FBS addition. Before fixation, half of the coverslips were treated with 0.5% Triton X-100 (Plus Triton) to remove soluble proteins or mock treated (No Triton). Cells were then stained with anti-Np95 antibody, together with either anti-BrdU (for cells harvested at 16 h [marked with black squares]) or anti-anti-pH3 (specific for histone H3 phosphorylated at serine 10) antibody to reveal cells in G_2/M (for cells harvested at 23 h [marked with grey squares]). Counterstaining was done with DAPI. Representative pictures of the indicated phases of the cell cycle are shown. The insets (last two rows, corresponding to magnifications of the areas indicated in the prophase and prometaphase panels) show details of the distribution of Np95 on the mitotic chromosomes.

RESULTS

Np95 is a chromatin-associated protein. To gain insight into the function of Np95, we initially studied its subcellular localization. Np95 displays almost exclusively nuclear localization (56); accordingly, it was undetectable in the cytosolic fraction of NIH 3T3 cells and only present in nuclear fractions (Fig. 1A). Further subnuclear fractionation revealed that the bulk of Np95 was released from intact nuclei by DNase I treatment, while residual amounts were also present in the nuclear matrix, high-salt, and ammonium sulfate fractions (Fig. 1A).

We next evaluated the staining pattern of Np95 during the cell cycle. Serum-starved NIH 3T3 cells were induced to reenter the cycle by serum stimulation and harvested at 16 and 23 h, corresponding to the S and G_2/M phases of the cell cycle. The exact position of the analyzed cells in the cycle was assessed by combined analysis of DAPI staining, BrdU incorporation (which indicates DNA replication), and phosphorylation of

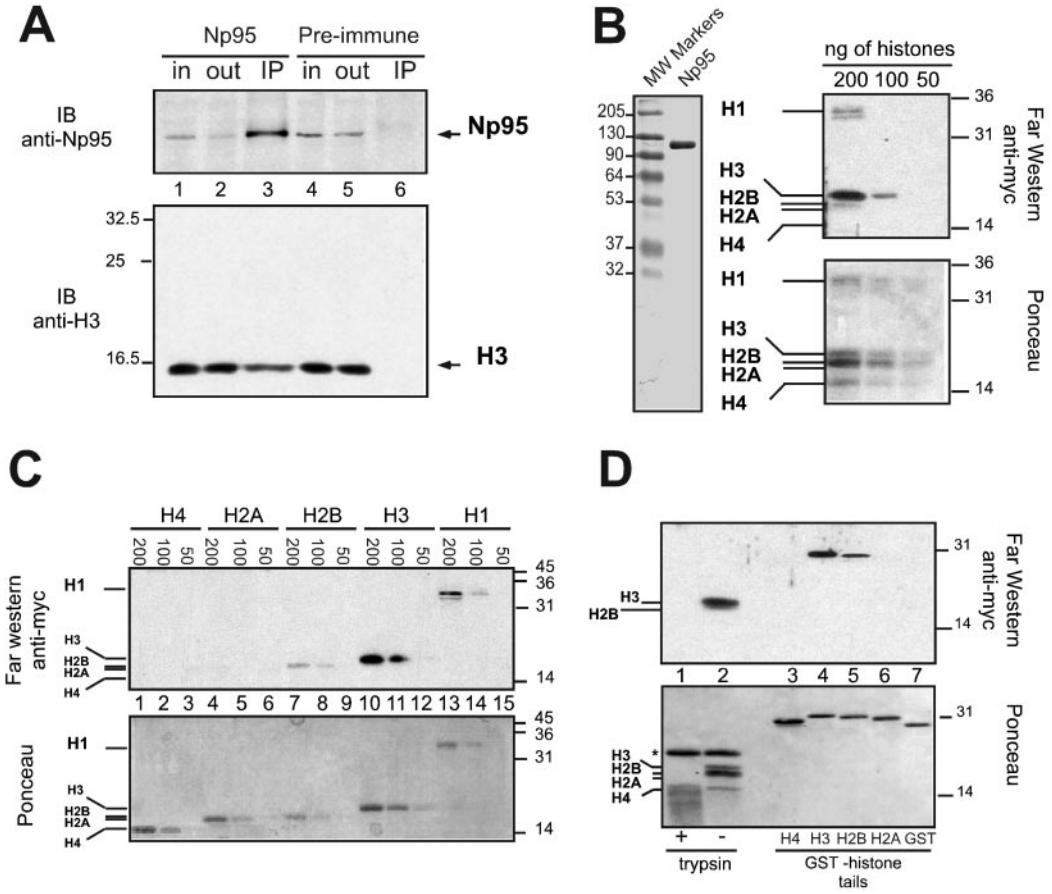

FIG. 2. Np95 interacts with histones in vivo and in vitro. (A) Endogenous Np95 coimmunoprecipitates with histone H3. Total extracts from asynchronously growing NIH 3T3 cells were immunoprecipitated with either anti-Np95 or preimmune serum (indicated at the top). The immunoprecipitates (IP) were washed under stringent conditions and immunoblotted (IB) with anti-Np95 (top) or anti-H3 (bottom) serum. A fraction (10%) of the starting material (in, input) and of the supernatant of the immunoprecipitates (out, output) is also shown. (B) Np95 interacts with H3, H2B, and H1. (Left) Coomassie staining of the purified myc-His_6-Np95 (Np95) protein used in all subsequent far-Western assays and enzymatic assays (details are given in Materials and Methods). (Right) The indicated amounts (shown at the top) of a mixture of commercially available core histones and H1 were detected in a far-Western assay with purified Np95, followed by immunostaining with anti-myc. Ponceau staining of the blot is also shown (bottom). (C) Np95 interacts with individual histones. Recombinantly produced histones, in various amounts (indicated at the top), were detected in a far-Western assay with pure Np95 as in panel B. Ponceau staining of the blot is also shown (bottom). (D) Np95 interacts with histone tails. Four micrograms of polynucleosomes treated with trypsin for 30 min (lane 1) or mock treated (lane 2) or 1 μg of GST fusion proteins of each of the core histone tails (lanes 3, 4, 5, and 6, also indicated at the bottom), or of GST (lane 7) was detected in a far-Western assay with pure Np95 as in panel B. Ponceau staining of the blot is also shown (bottom). The asterisk indicates a band corresponding to the trypsin inhibitor used to terminate the reaction in the first two lanes. In all panels, where appropriate, the positions of histones are indicated and molecular mass (MW) markers are shown (sizes are in kilodaltons).

histone H3 (which marks G_2/M cells [22]). As shown in Fig. 1B, Np95 was tightly associated with chromatin in all phases in which its expression was detectable, as indicated by its resistance to Triton treatment (Fig. 1B, Plus Triton). During mitosis, the staining of Np95 showed two patterns: a Triton-sensitive one interspersed among chromosomes that was lost upon detergent treatment (Fig. 1B, insets) and a Triton-resistant one tightly associated with chromosomes (Fig. 1B, insets). Together, these results indicate that Np95 is strongly bound to chromatin at all times of its expression.

Np95 interacts with histones in vitro and in vivo. The above results prompted us to test the association of Np95 with histones. Initially, we tested whether endogenous Np95 coimmunoprecipitates with histones. As show in Fig. 2A, histone H3 was readily recoverable in anti-Np95 immunoprecipitates (lane 3, IB anti-H3), indicating association in vivo between Np95 and the histone complex. To assess whether this interaction is direct and to establish which histone contacts Np95, we performed far-Western blotting assays. In order to do this, myc-His_6-tagged Np95 was expressed in eukaryotic cells and purified to near homogeneity (Fig. 2B, left, and Materials and Methods). This preparation of purified Np95 was used as a probe in the far-Western experiments and in the enzymatic assays described below.

Initially, we fractionated an equimolar mixture of histones, purified from calf thymus, by SDS-PAGE. Purified Np95 was able to interact specifically and directly with histones H3, H1, and H2B, while no binding to H2A or H4 was detectable (Fig. 2B, right). A hierarchy of strengths of interaction (H3 $\gg$ H1 $>$ H2B) was also evident (Fig. 2B). The interaction was further

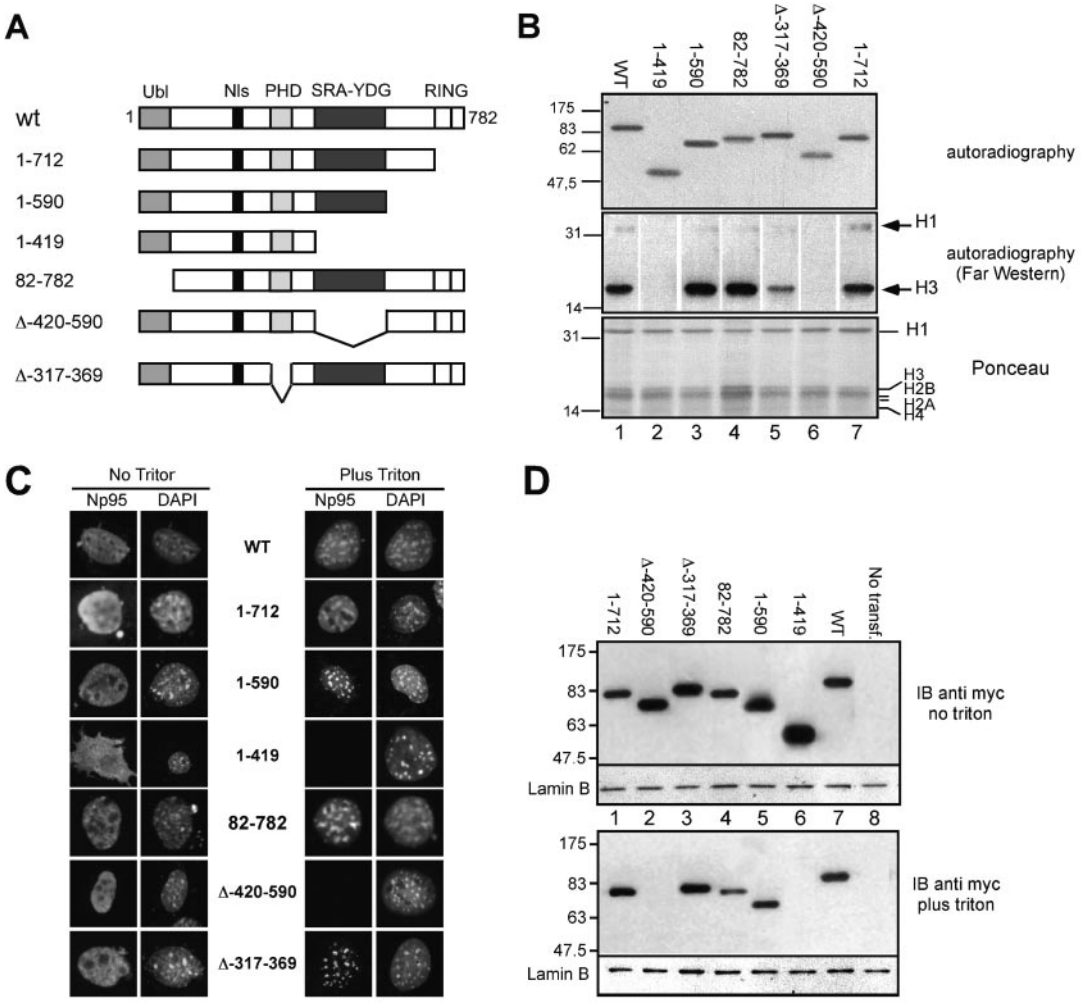

FIG. 3. The SRA-YDG domain is essential for the interaction of Np95 with histones in vitro and with chromatin in vivo. (A) Schematic representation of wild-type (wt) and mutant Np95 proteins. Nls, putative nuclear localization signal; PHD, PHD domain; SRA-YDG, SRA-YDG domain; RING, RING finger domain. All constructs were myc-His_6 tagged. (B) Interaction of wild-type (WT) and various mutant proteins with histones in vitro. (Top) The Np95 constructs indicated in panel A were in vitro translated in the presence of [^{35}S]methionine. The autoradiogram shows the labeled reaction products. (Middle and bottom) One microgram of a mixture of commercially available core histones and H1 was detected in a far-Western assay with ^{35}S-labeled mutant Np95 proteins (as at the top), followed by autoradiography. Ponceau staining of the blot is also shown (bottom). The middle part represents an assembly of individual strips. (C) The SRA-YDG domain is necessary for interaction of Np95 with chromatin in vivo. NIH 3T3 cells were grown on coverslips and transfected with 2 μg of each of the myc-His_6-tagged Np95 constructs indicated in panel A. Before fixation, cells were either treated with 0.5% Triton X-100 (Plus Triton) or mock treated (No Triton). The cells were then stained with anti-myc (Np95) and counterstained with DAPI. Representative pictures are shown. (D) NIH 3T3 cells were transfected (transf.) with the indicated constructs as in panel C. Cells were then either treated with 0.5% Triton X-100 or mock treated in situ (see Materials and Methods). Lysates of Triton-extracted whole cells were then obtained and analyzed in an immunoblot (IB) assay with anti-myc. An antibody against lamin B was used as a control. In all panels, where appropriate, the positions of histones are indicated and molecular mass markers are shown (sizes are in kilodaltons).

confirmed by far-Western experiments in which individual recombinantly produced histones were used (Fig. 2C). Also in this case, Np95 displayed a preference for binding to histone H3.

Core histones are schematically composed of a so-called histone fold that is folded up into the nucleosome core and of an NH_2 tail that provides a grip for complexes that regulate chromatin structure and function (19, 54). To map the region of core histones responsible for binding to Np95, we prepared tailless polynucleosomes by limited digestion with trypsin. In far-Western experiments, these partially trypsinized histones could not bind to purified Np95 (Fig. 2D, lane 1) while non-trypsinized control polynucleosomes efficiently did so (Fig. 2D, lane 2). In addition, we detected direct binding of purified Np95 to GST fusion proteins harboring the NH_2 tails of H3 and H2B (Fig. 2D, lanes 4 and 5) but not to the tails of H2A and H4 (Fig. 2D, lanes 3 and 6). Thus, Np95 binds directly and specifically to histones; within the histone core, the tail of histone H3 likely represents the highest-affinity binding site.

The SRA-YDG domain is essential for binding to histones in vitro and for Triton-resistant binding to chromatin in vivo. Np95 contains several domains: from the N to the C terminus,

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CHP/Dr                MPRHFGAVPGVVPGMAFVNRQELRDAGVHLPTQAGISGSA--------SEGADSIVLS
SUV2/AraTh            DKHIVGPVTGVEVGDIFFYRMELCVLGLHGQTQAGIDCLTAE-RSATGEPIATSIVVS
SUV3/AraTh            MKKRVGTVPGIEVGDIFFSRIEMCLVGLHMQTMAGIDYIISK-AGSDEESLATSIVSS
SUV5/AraTh            GTQIIGTVPGVEVGDEFQYRMELNLLGIHRPSQSGIDYMKD----DGGELVATSIVSS
SUV9/AraTh            DKRIVGSIPGVQVGDIFFFRFELCVMGLHGHPQSGIDFLTGS-LSSNGEPIATSVIVS
Similar G9a1/AraT     GVHILGEVPGVEVGDEFQYRMELNILGIHKPSQAGIDYMK-----YGKAKVATSIVAS
Similar G9a2/AraTh    ----------------------MCLVGLHRNTAGGIDSLLAK-ESGVDGPAATSVVTS
C3HC4-zf/AraTh        AEHDPVRNQGVLVGESWENRVECRQWGVHLPHVSCIAGQE--------DYGAQSVVIS
Hyp. Prot/AraTh       ----------------------MGLVGLHSGTIDMEFIGVEDHGDEEGKQIAVSVISS
Np95/Mm               PANHFGPIPGVPVGTMWRFRVQVSESGVHRPHVAGIHGRS--------NDGAYSLVLA
Consensus (60%)       s.+bhGslPGVbVG-bFb.RbElsllGlHbs*btGIDhbp......s.c.lATSlVsS

CHP/Dr                GGYEDDRDEGDVILYTGEGGRDP-----LTGHQVKPQQLVRGNLALAISHRDGL----
SUV2/AraTh            GGYEDDEDTGDVLVYTGHGGQDH------QHKQCDNQRLVGGNLGMERSMHYGI----
SUV3/AraTh            GRYEGEAQDPESLIYSGQGGNAD------KNRQASDQKLERGNLALENSLRKGN----
SUV5/AraTh            GGYNDVLDNSDVLIYTGQGGNVG---KKKNNEPPKDQQLVTGNLALKNSINKKN----
SUV9/AraTh            GGYEDDDDQGDVIMYTGQGGQDR------LGRQAEHQRLEGGNLAMERSMYYGI----
Similar G9a1/AraT     GGYDDHLDNSDVLTYTGQGGNVM(4)KGEELKEPEDQKLITGNLALATSIEKQT----
Similar G9a2/AraTh    GKYDNETEDLETLIYSGHGGK------------PCDQVLQRGNRALEASVRRRN----
C3HC4-zf/AraTh        GGYKDDEDHGEWFLYTGRSRG--------RHFANEDQEFEDLNEALRVSCEMGY----
Hyp. Prot/AraTh       GKNADKTEDPDSLIFTGFGGTDM------YHGQPCNQKLERLNIPLEAAFRKKS----
Np95/Mm               GGYEDDVDNGNYFTYTGSGGRDLS-GNKRTAGQSSDQKLTNNNRALALNCHSPI(14)
Consensus (60%)       GGY-D-pDstDsLlYTGpGGps.......phcQscDQ+LpsGNLALcsSb+csh....

CHP/Dr                PLRVTRGHR--HSSQFSPQSGYQYAGLYRVDDHWREVGRSGFLIWRFRLTRLENQ
SUV2/AraTh            EVRVIRGIK---YENSISSKVYVYDGLYKIVDWWFAVGKSGFGVFKFRLVRIEGQ
SUV3/AraTh            GVRVVRGEE---DAASKTGKIYIYDGLYSISESWVEKGKSGCNTFKYKLVRQPGQ
SUV5/AraTh            PVRVIRGIKNTTLQSSVVAKNYVYDGLYLVEEYWEETGSHGKLVFKFKLRRIPGQ
SUV9/AraTh            EVRVIRGLK---YENEVSSRVYVYDGLFRIVDSWFDVGKSGFGVFKYRLERIEGQ
Similar G9a1/AraTh    PVRVIRGKHKSTHDKSKGG-NYVYDGLYLVEKYWQQVGSHGMNVFKFQLRRIPGQ
Similar G9a2/AraTh    EVRVIRGEL------YNNEKVYIYDGLYLVSDCWQVTGKSGFKEYRFKLLRKPGQ
C3HC4-zf/AraTh        PVRVVRSYKDR-YSAYAPKEGVRYDGVYRIEKCWRKARFP---VCRYLFVRCDNE
Hyp. Prot./AraTh      IVRVVRCMK---DEKRTNGNIYIYIYDGTYMITNRWEEEGQNGFIVFKFKLVREPDQ
Np95/Mm               PVRVVRNMKGGKHSKYAPAEGNRYDGIYKVVKYWPERGKSGFLVWRYLLRRDDTE
Consensus (60%)       sVRVlRGbK...bpppsstcsYlYDGLYpls-pWp.sG+SGFhVFKF+LhRbsGQ
VRV(I/V)RG motif        ******
YDG motif                                  ***
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FIG. 4. Alignment of SRA-YDG domains. Shown are alignments of various SRA-YDG domains and the consensus sequence of the domain (obtained at a plurality of 60%) based on the domain architecture, named the SET and RING finger-associated domain, present in the SMART database (accession no. SM00466). The domain is also described in detail in reference 2 with its 13 conserved G's (underlined in the alignment) and the VRV(I/V)RG and YDG motifs (indicated by asterisks in the alignment). Abbreviations: Dr, *Deinococcus radiodurans*; AraTh, *Arabidopsis thaliana*; Mm, *Mus musculus*. The sequences shown are as follows: CHP (conserved hypothetical protein), accession no. Q9RU61; SUV2, accession no. AAK28967; SUV3, accession no. Q9C5P4 and Q9SSL7; SUV5, accession no. O82175; SUV9, accession no. AAK28974; Similar G9a1, accession no. AAD26896; Similar G9a2, accession no. AAD15582; C3HC4-zf, accession no. AAC28190; Hyp. Prot (hypothetical protein), accession no. AAC95167; Np95, accession no. Q9Z1H6.

a Ub-like domain (Ubl), a putative nuclear localization signal, a PHD domain, an SRA-YDG domain (a novel domain whose function is uncharacterized) (2, 53), and a RING finger domain (Fig. 3A). We mapped the region of Np95 that interacts with histones. Various deletion mutant forms (Fig. 3A) of Np95 were produced as in vitro-translated radiolabeled proteins and used to detect a pool of histones in far-Western experiments. Only those constructs retaining the SRA-YDG domain displayed detectable binding, which was preferential for histone H3 (Fig. 3B, lanes 1, 3, 4, 5, and 7).

The above result strongly suggests that Np95 is directed to chromatin in vivo through a specific interaction between its SRA-YDG domain and the tail region of histone H3. If this is true, then the presence of the SRA-YDG domain should be indispensable for the association of Np95 with chromatin in vivo. A panel of myc-tagged mutant Np95 proteins (as in Fig. 3A) was transfected into NIH 3T3 cells. Before fixation, the cells were either treated with 0.5% Triton or mock treated. Immunofluorescence staining, in the absence of Triton treatment, shows that progressive deletions from the carboxyl terminus of the protein do not affect the nuclear staining until the SRA-YDG domain is removed (Fig. 3C). Internal deletion of the SRA-YDG domain, however, did not determine the loss of the nuclear staining, indicating that additional nuclear localization signals are present in the carboxyl terminus of the protein (Fig. 3C). In Triton-treated cells, instead, whenever the SRA-YDG domain was removed, the corresponding mutant protein was no longer detectable in the cell (Fig. 3C, 1-419 and Δ-420-590). Western blot analysis of the same samples confirmed this finding (Fig. 3D, lanes 2 and 6). Altogether, these results indicate that the presence of an intact SRA-YDG domain is required for the interaction of Np95 with chromatin in vivo. An alignment of the SRA-YDG domain is shown in Fig. 4.

Np95 is an E3 Ub protein ligase. In our search for a biological function for chromatin-bound Np95, our attention was attracted by its RING finger domain. Several RING finger domain-containing proteins appear to function as E3 Ub ligases (16, 32, 37). We thus used recombinant Np95 in ubiquitination reaction mixtures. A pure preparation of myc-His_6-

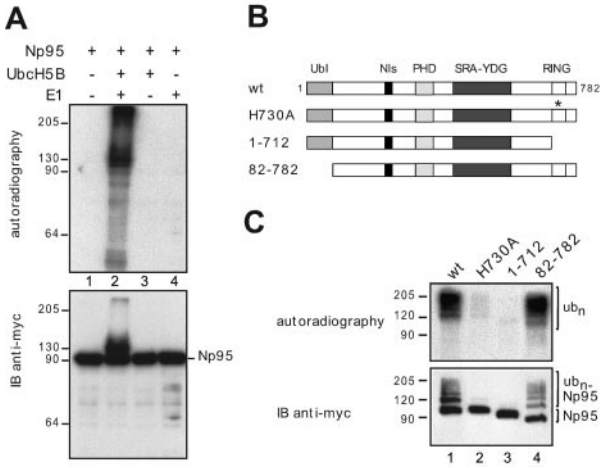

FIG. 5. Np95 has E2-dependent Ub ligase activity in vitro. (A) In vitro ubiquitination assay. Purified Np95 (as in Fig. 2B; see also Materials and Methods) was incubated with ATP, radioactively labeled Ub, and the indicated combinations (+/−) of mammalian E1 and recombinant E2 (UbcH5B) enzymes and assayed for Ub ligase activity. High-molecular-weight Ub conjugates were detected by autoradiography (top). Np95 was detected by immunoblotting (IB) with anti-myc (bottom). (B) Schematic representation of wild-type (wt) and mutant Np95 proteins. All constructs were myc-His_6 tagged. Domains are as in Fig. 3A. (C) The RING finger domain of Np95 is required for Ub ligation. Wild-type and mutant Np95 proteins were incubated in an in vitro ubiquitination reaction mixture containing ^{32}P-labeled Ub, E1, and UbcH5B enzymes, followed by autoradiography (top). Ub conjugates (ub_n) are indicated. Np95 proteins and their Ub conjugates (ub_n-Np95) were detected by immunoblotting with anti-myc (bottom). In all panels, where appropriate, the positions of Np95 and its Ub conjugates (ub_n-Np95) are indicated and molecular mass markers are shown (sizes are in kilodaltons).

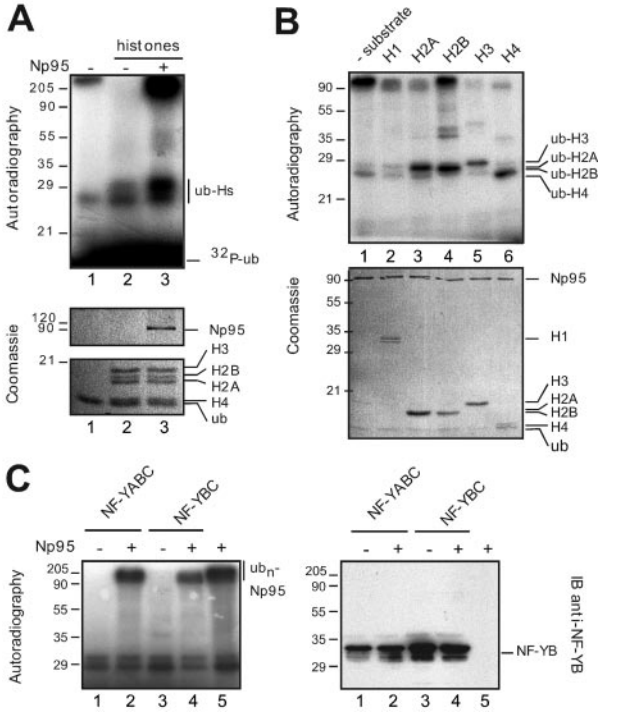

FIG. 6. Np95 is an E3 ligase for core histones in vitro. (A) Np95 ubiquitinates core histones in vitro. Ubiquitination reaction mixtures containing 4 μg of histones purified from calf thymus, ^{32}P-labeled Ub (^{32}P-ub), E1, and UbcH5B were prepared with (+) or without (−) purified Np95. Ubiquitinated histones (ub-Hs) were detected by autoradiography (top). (B) Purified Np95 was incubated with the indicated substrate (1 μg of H1 purified from calf thymus or recombinant H2A, H2B, H3, or H4) or no substrate (− substrate) in a ubiquitination assay, followed by autoradiography (top). In both panels A and B, Coomassie staining of the top parts shows Np95 and the substrates present in the reaction mixtures (bottom parts). (C) NF-Y is not a substrate for Np95. NF-YABC or NF-YBC complexes were incubated in ubiquitination reaction mixtures in the presence (+) or absence (−) of Np95 as indicated. High-molecular-weight products of Np95 self-ubiquitination (ub_n-Np95) were detected by autoradiography (top). The NF-YB subunit was detected by immunoblotting (IB) with anti-NF-YB (bottom). Note the lack of a gel-retarded product in the anti-NF-YB blot, which would be indicative of Ub conjugates. In all panels, where appropriate, the positions of individual proteins or of their Ub conjugates (ub_n-) are indicated and molecular mass markers are shown (sizes are in kilodaltons).

tagged Np95 (the same as that shown in Fig. 2B) was incubated with radioactive Ub in the presence of purified mammalian E1 and recombinant E2 (UbcH5B) enzymes. High-molecular-weight Ub conjugates were readily detectable under these conditions (Fig. 5A, top, lane 2) but not when either E1 or E2 was omitted (Fig. 5A, top, lanes 1, 3, and 4). Western blotting analysis with anti-myc antibodies (Fig. 5A, bottom) detected a ladder of ubiquitinated proteins, indicating that the Ub-containing bands represent covalent conjugates between Np95 and Ub (Fig. 5A, bottom, lane 2). Thus, Np95 is an E3 Ub ligase that can, under the conditions of the in vitro assay used, mediate its own ubiquitination, consistent with what has been reported for several RING finger E3 enzymes (32, 38).

Np95 harbors two conserved domains functionally related to the ubiquitination pathway, an amino-terminal Ubl domain and a carboxyl-terminal C3HC4 RING finger motif (17). We investigated whether the Ubl and RING domains are required for Ub ligation by Np95. To this end, constructs encoding myc-His_6-Np95 mutant forms were generated (Fig. 5B) and the corresponding proteins were purified from eukaryotic cells (data not shown). In the same ubiquitination assay described above, a mutant Np95 protein lacking the Ubl domain efficiently supported in vitro self-ubiquitination indistinguishably from wild-type Np95 (Fig. 5C, lane 4). In contrast, mutants harboring a deletion of the RING finger domain or a single amino acid substitution in one of the predicted zinc coordination sites (H730A) showed no detectable E3 activity (Fig. 5C, lanes 3 and 2, respectively). Thus, Np95 is a RING-type E3 Ub ligase.

Np95 specifically ubiquitinates histones in vitro. Histones are known to be modified by monoubiquitin in vivo (7, 18, 30, 58). The specific interaction of Np95 with histones, coupled to its Ub ligase activity, prompted us to test whether histones are substrates for Np95-mediated ubiquitination in vitro. A strong ubiquitination signal in the proximity of 29 kDa was visualized when histones purified from calf thymus were incubated with recombinant Np95 in ubiquitination assays (Fig. 6A, lane 3). We then added individual histones to ubiquitination reaction mixtures and found that all four nucleosomal core histones were efficiently ubiquitinated in the presence of Np95 (Fig. 6B, lanes 3, 4, 5, and 6, top). On the contrary, linker histone H1,

which is known to constitute a substrate for ubiquitination (46), was not ubiquitinated by the Np95 E3 activity (Fig. 6B, lane 2, top).

As an additional specificity control, we used the trimeric transcriptional activator NF-Y. NF-Y could be a potential substrate for Np95, since two of its three protein subunits, namely, NF-YB and NF-YC, harbor a histone fold motif and belong to the H2A-H2B subfamily (39). However, no NF-Y-ubiquitinated products (Fig. 6C, left, lanes 2 and 4) were detected in reaction mixtures in which Np95 efficiently promoted its own ubiquitination (Fig. 6C, left, lanes 2, 4, and 5). Western blot analysis with antibodies against NF-YB (Fig. 6C, right side) confirmed that this subunit was not ubiquitinated by the Np95 ligase activity. These data support substrate specificity for Np95 E3 activity in vitro, indicating a strong preference for core histones.

Np95 promotes ex vivo ubiquitination of interacting histone H3. The above results indicate that all core histones, when individually tested, are good substrates for Np95 in vitro. However, the situation in vivo may be different, as Np95 interacts preferentially with histone H3 and histones themselves may be selectively available to Np95 in the nucleosomal structure. To gain insight into this issue, we established an ex vivo ubiquitination assay in which Np95 was immunoprecipitated from total cellular lysates and the native histones coimmunoprecipitating with it were used as substrates in the ubiquitination reaction.

Myc-tagged wild-type Np95 ($Np95^{wt}$) or the catalytically inactive H730A mutant ($Np95^{H730A}$) was immunoprecipitated, and the specific immunoprecipitates were incubated in the presence of Ub, E1, and UbcH5B and then analyzed in a Western blot assay with antibodies specific for various histones. Under these conditions, ubiquitinated H3, but not H2A or H2B, was readily detectable despite efficient coimmunoprecipitation of all three core histones (Fig. 7, left side). In addition, the H730A mutant, while capable of coimmunoprecipitating with all three histones, could not exert E3 ligase activity on H3 (Fig. 7, left side), strongly arguing that histone ubiquitination was dependent on Np95 E3 activity and did not derive from an Np95-associated protein.

As a further control, we added to parallel aliquots of $Np95^{wt}$ or $Np95^{H730A}$ immunoprecipitates a mixture of purified histones in a ubiquitination reaction. Under these conditions, which resemble those of the assays with pure Np95 shown in Fig. 5A, core histones H2A, H2B, and H3 were all efficiently ubiquitinated by $Np95^{wt}$ but not by $Np95^{H730A}$ (Fig. 7, right side). Thus, Np95 could ubiquitinate H2A, H2B, and H3 with similar efficiencies when a mixture of purified histones was used as a substrate. In contrast, in a setting more closely mimicking the physiological conditions, when only Np95-associated endogenous histones were present, histone H3 was the preferred substrate. The nucleosomal conformation of the histone octamer is most likely not altered by our mild salt conditions for cell extract preparation and immunoprecipitation. In addition, the possibility that Np95-mediated ubiquitination occurs on free H3 is unlikely, since more than 0.9 M NaCl is needed to extract H3 from chromatin (36). Thus, the combined analysis of our data indicates that endogenous nucleosomal histone H3 constitutes a preferred substrate for Np95-dependent ubiquitination.

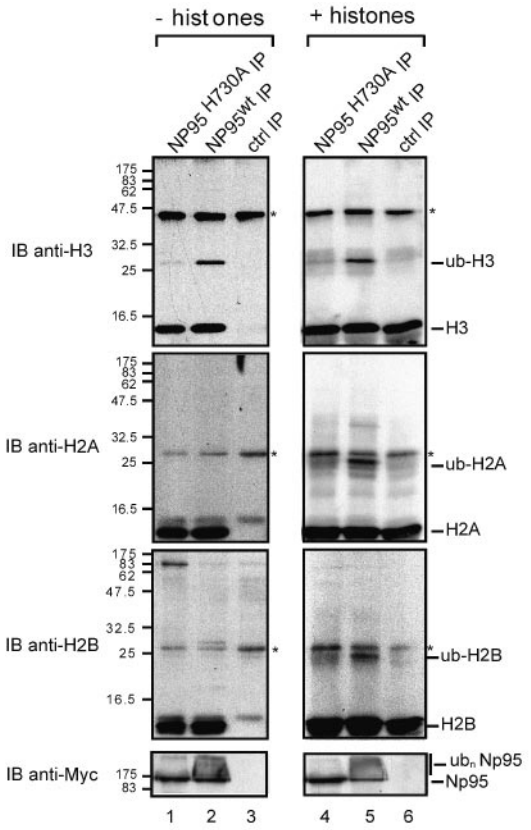

FIG. 7. Np95 promotes ubiquitination of interacting histone H3 ex vivo. Phoenix cells were transfected with myc-tagged $Np95^{wt}$, $Np95^{H730A}$, or the vector alone (control [ctrl]), and equivalent amounts of cell lysates were immunoprecipitated with anti-myc (see Materials and Methods). Immunoprecipitates (IP) were assayed for ubiquitination activity in the presence of Ub, E1, and UbcH5B in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of histones purified from calf thymus. Detection in an immunoblot (IB) assay was done with the antibodies indicated on the left. In all panels, where appropriate, the positions of individual proteins or of their Ub conjugates are indicated and molecular mass markers are shown (sizes are in kilodaltons). Asterisks in all panels indicate nonspecific bands.

DISCUSSION

In this report, we provide evidence that Np95 is a new chromatin binding protein that interacts with histones in vivo and in vitro and functions as an E3 Ub ligase for histones in vitro with a strong preference for H3.

Np95 binds in vitro to histones with an H3 $\gg$ H1 $>$ H2B hierarchy. Core histones are bound by their NH_2 tails, which are the sites of various posttranslational modifications that regulate access to the underlying DNA and control the dynamic transitions between transcriptionally active and inactive chromatin states (31). The most relevant feature of the Np95-histone interaction is that it is lost when the SRA-YDG domain is removed from Np95, indicating that this new domain is essential for the interaction. Experiments are currently ongoing to establish if the SRA-YDG domain is also sufficient for binding to histones or if additional domains are required. SRA-YDG (also known as SIN) is a recently described ~170-amino-acid-long domain whose function has not been charac-

terized (2, 53). In mammals, it is present only in Np95/ICBP90 and in Np97, the product of a related gene (42). Surprisingly, no proteins displaying this domain are predicted in the genomes of *S. cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*. However, in fish, plants, and bacteria, several proteins displaying the domain are found (2, 53). While this may have interesting implications for evolutionary studies, some attractive hypotheses can be envisioned on the basis of the structures of SRA-YDG-harboring proteins. In particular, the domain is found frequently in association with any of the following three domains: (i) SET, of the family of the histone methyltransferase SUV39H1, in plants; (ii) RING fingers, most of which possess Ub ligase activity, in plants and vertebrates; and (iii) HNH, a nonspecific nuclease motif, in bacteria. Thus, and in light of our present data, one could speculate that the SRA-YDG domain is necessary to direct to the chromatin a number of heterogeneous proteins with associated enzymatic activities. In this respect, but possibly with different specificity, the SRA-YDG domain might function similarly to chromo and bromo domains, which bind to modified histones and are frequently associated with SET or RING domains in proteins that are central to the epigenetic regulation of chromatin, gene expression, and DNA replication (for reviews, see references 6, 14, and 34).

It has been reported that ICBP90, the human homologue of Np95, is a CCAAT binding protein (26). Our preliminary results suggest that Np95 also interacts with DNA but does not seem to have the same specificity for the CCAAT box as ICBP90 does (R. Papait et al., unpublished data).

Our results also show that Np95 is a RING-type Ub ligase. In vitro, Np95, is able to ubiquitinate all core histones and shows, under certain conditions, a marked preference for histone H3. Of course, core histones have been known for a long time to represent good general substrates in vitro for a variety of ubiquitinating enzymes (49). Thus, our results do not prove that Np95 is a core histone-specific (or H3-specific) E3 ligase in vivo. However, a circumstantial case can be made on the basis of the facts that (i) Np95-bound endogenous histone H3 constitutes a preferential substrate for Np95-dependent ubiquitination under conditions in which the entire core octamer is efficiently coimmunoprecipitated; (ii) despite the interaction with H1, Np95 in unable to ubiquitinate this linker histone, which is known to represent a ubiquitination substrate both in vitro and in vivo (46); (iii) a further degree of specificity is shown by the lack of in vitro activity toward proteins showing a histone fold, such as NF-YB and NF-YC; (iv) Np95 strongly associates with chromatin in vivo; and (v) Np95 directly binds to histones in vivo and in vitro, with a strong preference for histone H3.

Vis-à-vis the long-standing knowledge of histone ubiquitination in mammals, our understanding of the molecular machinery involved is very limited. HHR6A and HHR6B are the two mammalian homologues of yeast Rad6p (33); however, it is not clear whether they are involved in histone ubiquitination in vivo (1). In addition, two homologues of Bre1p are predicted to be encoded by the human genome and represent good candidates for histone-specific Ub ligases (28). Finally, the complex BRCA1/BARD1 has been recently shown to promote histone ubiquitination in vitro (38). Np95 now adds to this growing list of candidates and suggests that multiple histone ubiquitination pathways may exist.

In mammals, ubiquitination of H3 in vivo has only been described in elongating spermatids. It was suggested that this histone modification might be associated with relaxation of the nucleosome structure, to increase the accessibility of the transcription machinery, or with chromatin restructuring (7). Np95 is widely expressed (17); thus, if it were to work as an H3 Ub ligase, one would predict a much broader distribution of ubiquitinated histone H3. We note, however, that Np95 is expressed, at a steady state, in only a fraction of cycling cells, since it is absent in G_1, the longest phase of the cell cycle, and it is totally absent in noncycling cells (3, 56). In addition, the action of Np95 might be exerted in a very restricted temporal window, i.e., at the G_1/S border (3), and/or in particular situations, such as following DNA damage (44). Thus, and on the basis of our data, a systematic reevaluation of the state of ubiquitination of histone H3 in somatic cells under these conditions appears warranted.

Np95 plays a key role in the regulation of cell proliferation, being essential for entry into S phase (3). The histones of actively transcribed chromatin are monoubiquitinated (8, 9, 27, 35, 45), while this modification is absent in metaphase chromosomes (40). This argues that ubiquitination may aid in the relaxation of compact chromatin and nucleosomal structures to increase accessibility to the transcription machinery (9, 30, 35). We speculate that Np95-mediated histone ubiquitination may play a role in chromatin reorganization in order to facilitate the transcription of specific genes during the G_1/S transition. The demonstration of Np95 as a chromatin-associated Ub ligase therefore suggests possible molecular mechanisms for its action as a cell cycle regulator.

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