Vps75, A New Yeast Member of the NAP Histone Chaperone Family

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Homologues of nucleosome assembly protein 1 (NAP1) are found throughout eukaryotes. Here we identify and characterize a new NAP family histone chaperone from budding yeast, named Vps75. Purified Vps75 preferentially binds histone H3/H4 tetramers and is capable of assembling nucleosomes in vitro. In vivo, Vps75 is associated with the chromatin of both active and inactive genes and telomeres. Others have previously reported that Vps75 forms a complex with Rtt109, required for acetylation of histone H3 lysine 56 (H3 Lys-56). Cells lacking RTT109 are sensitive to hydroxyurea, pointing to a role in replication. We show that VPS75 is not required for H3 Lys-56 acetylation and that vps75Δ cells are insensitive to hydroxyurea, suggesting that although Rtt109 and Vps75 associate and are likely to be functionally connected, they also have separate roles.

In eukaryotic cells, DNA is present in a highly compacted form called chromatin. The repeating unit of chromatin is the nucleosome, formed from two histone H2A-H2B dimers and one histone H3-H4 tetramer around which 147 bp of DNA is wrapped (1). Despite the tight packaging of its structure, chromatin is highly dynamic, a characteristic that is vital in regulating nuclear processes, such as transcription and replication, which require access to DNA. Processes that positively influence chromatin fluidity include post-translational modifications of histones, incorporation of histone variants, and histone exchange by dedicated histone chaperones (for review, see Ref. 2).

Histone chaperones are proteins that regulate the interaction of histones with other proteins and DNA and also act to prevent the highly basic histones from forming inappropriate complexes. In addition to playing an important role in histone exchange during nuclear processes, histone chaperones function in nucleocytoplasmic shuttling of histones, histone storage, nucleosome assembly, and as a link between chromatin remodeling factors and histones (for reviews, see Refs. 3 and 4). Given the diversity of these roles, it is not surprising that many different histone chaperones have been identified, including nucleoplasmin, ASF1, HIR proteins, Spt6, FACT, ACF, CAF1, and NAP1.

Members of the NAP family of proteins, found from yeast to mammals, possess particularly diverse roles in histone metabolism (4). These proteins are characterized by a conserved central domain (the NAP domain), a non-conserved N-terminal region of variable length, and in general, a highly acidic C-terminal domain. The NAP domain is required and sufficient for histone binding, although the acidic domain may enhance this activity (5). The best characterized member of the NAP family is NAP1, which has been found in yeast, plants, flies, and mammals. Other NAPs include SET (also known as TAF-Iβ), CINAP, TSPY, and a steadily expanding group of NAP1-like proteins (NAP1L1–NAP1L5) (4).

In this study, we describe the isolation and characterization of a novel NAP family member from yeast. This protein, Vps75, was previously identified in genomic screens for vacuolar protein sorting genes and for factors that affect telomere length (6, 7). Our data indicate that Vps75 is a bona fide histone chaperone with a preference for H3-H4 tetramers and that it may have a general role in chromatin assembly and rearrangement since it is found in both transcribed and non-transcribed regions in vivo.

EXPERIMENTAL PROCEDURES

Yeast Manipulation—The following strains (genotype) were used for genetic experiments: W303A (MATa leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-1); vps75Δ (as W303A, but vps75Δ::LEU2); BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0); and rtt109Δ and asf1Δ (as BY4741 but rtt109Δ::KANMX or asf1Δ::KANMX). The strain expressing C-terminal His6-TEV2-Myc9 (HTM)-tagged Vps75 had a wild-type phenotype. Details of affinity tagging are available on request.

Expression and Purification of Recombinant Proteins—To purify Vps75 from yeast, HTM-tagged Vps75 cells were harvested, resuspended in an equal volume (w/v) of lysis buffer (40 mM Hepes (pH 7.5), 250 mM potassium acetate, 20% glycerol, 5 mM dithiothreitol, 0.1% Nonidet P-40, protease inhibitors), and lysed using a freezer mill. Lysate was clarified at 12,000 × g for 10 min and again in an ultracentrifuge using a Beckman Ti45 rotor (40,000 rpm, 1 h, 4 °C). The supernatant was incubated with 9E10 affinity resin for 90 min at 4 °C, and the resin was washed with 40 bed volumes lysis buffer and 10 volumes elution buffer (40 mM Tris-Cl (pH 7.5), 250 mM potassium acetate, 10% glycerol). Vps75-His was eluted twice by incubating with 1 volume elution buffer containing 40 μg of TEV protease for 2 h at 12 °C and further purified by MonoQ chromatography. GST-Vps75 was expressed in Escherichia coli BL21 cells from pGEX-6P-1 and purified on glutathione-Sepharose using standard techniques.

Histone expression vectors were a kind gift from Brad Cairns. Individual yeast histones were purified from E. coli, and histone

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2The abbreviations used are: NAP, nucleosome assembly protein; yNAP1, yeast NAP1; HTM, His6-TEV2-Myc9; TEV, tobacco etch virus; GST, glutathione S-transferase; ChIP, chromatin immunoprecipitation; NES, nuclear export signal; AD, accessory domain.
complexes (H2A-H2B dimers, H3-H4 tetramers, and octamers) were prepared as described (8). HeLa core histones used in nucleosome assembly assays were prepared as described (9).

Mass Spectrometry—Protein samples were analyzed by matrix-assisted laser desorption/ionization-time of flight using an Applied Biosystems 4700 proteomics analyzer.

Binding Reactions and GST Pull-down Assays—To assess Vps75 binding to core histones, 9 pmol of Vps75-His purified from yeast was incubated with 20 μl of core histones immobilized on 4% agarose (Sigma H3889) or empty 4% agarose for 2 h at 4 °C in 500 μl of buffer A (20 mM Tris-HCl (pH 7.5), 10% glycerol, 50 μg/ml bovine serum albumin, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM diithiothreitol) containing 200 mM NaCl. Resin was washed in buffer A, and bound protein was subjected to SDS-PAGE and Western blotting using an antibody to the His tag (Sigma).

GST pull-down assays were done by incubating GST-Vps75 (or GST) and yeast histone complexes with 20 μl of glutathione-Sepharose (Amersham Biosciences) in 250 μl of buffer A containing 100 mM NaCl for 3 h at 10 °C. After extensive washing, bound proteins were subjected to SDS-PAGE and visualized by silver staining.

Nucleosome Assembly Assays—Vps75 nucleosome assembly activity was measured essentially as described (5). 60 fmol of pRS316 plasmid was relaxed by incubation with 3 units of calf thymus topoisomerase I (Invitrogen) in assay buffer (10 mM Tris-HCl (pH 8.0), 150 mM potassium acetate, 1 mM EDTA, 0.1 mg/ml bovine serum albumin) for 30 min at 37 °C. HeLa core histones (0.5–2 pmol) and Vps75 (from yeast; 4.5–18 pmol) were preincubated in assay buffer for 30 min at 37 °C. Samples were then mixed with 60 fmol of the relaxed pRS316 and further incubated at 37 °C for 45 min. The reactions were analyzed by agarose gel electrophoresis as described (7).

Histone Association and Chromatin Immunoprecipitation (ChIP) Assays—Qualitative histone association assays were carried out as described (10). Chromatin was immunoprecipitated with an antibody specific for the C terminus of histone H3 (a kind gift of Alain Verreault), and Vps75 was detected using a monoclonal antibody (9E10) directed against the Myc tag. ChIP assays were carried out essentially as described (11) but with minor modifications; DNA co-precipitated with 9E10 was analyzed by quantitative real-time PCR using a Bio-Rad MyIQ iCycler in standard conditions and ABsolute™ QPCR SYBR Green reagents (ABgene). Primer sequences are available on request. Immunoprecipitation efficiency was determined by dividing the amount of precipitated DNA by the amount in the input sample.

Growth Sensitivity Assays—The effect of hydroxyurea on growth was tested as described (12).

RESULTS

Sequence Analysis of Vps75—Although several NAP family members have been described in human and mouse cells, only one, NAP1, has been identified in yeast. However, homology searches revealed that yeast contains at least one other likely NAP protein, Vps75. A BLAST query of the Vps75 protein sequence indicated that a region encompassing amino acids 12–229 shares homology with the NAP domain. The structure of the NAP domain of yeast NAP1 (yNAP1) at 3.0 Å resolution was recently solved (13). It comprises two distinct domains (I and II), each of which can be further delineated into two subdomains (Fig. 1A). Sequence alignment of Vps75 with various other NAP family members revealed that it is more closely related to the Drosophila and human SET/TAF-I and human TSPY proteins than yNAP1 (data not shown). This is supported by analysis of its domain architecture (Fig. 1A). Like SET and TSPY, Vps75 completely lacks subdomain B, termed the accessory domain (AD; shown in yellow in yNAP1). The AD has been implicated in regulating access to a functional nuclear export signal (NES; shown as a blue/white stripe) embedded in A (shown in blue) (13). Vps75, SET, and TSPY also lack a recognizable NES (data not shown), suggesting an evolutionary link between the conservation of the NES and the AD and supporting the idea that this domain plays a role in regulating access to the NES. These observations raise the possibility that unlike yNAP1 (14), Vps75 does not function in nucleocytoplasmic shuttling of histones but possesses a primarily nuclear role. This theory is consistent with the observations that Vps75 is found in the nucleus (15) and that it lacks the phosphorylation sites implicated in translocation of Drosophila NAP1 between the cytoplasm and the nucleus (16).

In contrast to the apparent differences observed between NAP domain I of Vps75 and yNAP1, domain II appears to be structurally conserved, apart from a small acidic region found near the start of subdomain D in yNAP1 that is lacking in Vps75. However, Vps75 contains a highly acidic region near its C terminus (Fig. 1A), a characteristic of most NAP family proteins, and has a theoretical pI of 4.47, which is also similar to other members of the family.

Purification of Vps75—To experimentally address the possibility that Vps75 is a NAP family histone chaperone, we created a yeast strain expressing Vps75 tagged at the C terminus with HTM from the endogenous VPS75 promoter. Crude extract was passed over a 9E10 (anti-Myc) affinity column, and Vps75—

FIGURE 1. Sequence analysis and purification of Vps75. A, domain structure of yNAP1 and Vps75 showing the conserved NAP domain (I + II), comprising subdomains A (blue), B (yellow), C (green), and D (red). The C-terminal (C-term) acidic domains are also indicated. NLS, nuclear localization sequence. B, Vps75 was purified from yeast whole cell extracts by affinity purification (lane 1) and subsequent MonoQ chromatography (lane 2). Proteins identified by mass spectrometry are indicated on the right.
Vps75 Preferentially Binds H3-H4 Tetramers in Vitro—The defining feature of a histone chaperone is its ability to bind histones. To determine whether Vps75 possesses such an activity, we incubated it with resin-immobilized core histones. Vps75-His bound efficiently to this matrix but not to the control matrix lacking histones (Fig. 2A).

A number of histone chaperones exhibit preferential binding to either H3-H4 tetramers (H3-H4)₂ or H2A-H2B dimers (H2A-H2B) (3). To determine the binding preference of Vps75, the interaction of GST-Vps75 with H3-H4 tetramers (H3-H4)₂ or H2A-H2B dimers was analyzed using a GST pull-down assay (Fig. 2B). GST-Vps75 isolated from E. coli bound efficiently to (H3-H4)₂ tetramers (lanes 4–6) but exhibited only weak binding to (H2A-H2B) complexes (lanes 1–3) under the conditions used in this experiment. This was clearly evident when the different nucleosome subcomplexes were mixed prior to incubation with Vps75 (lanes 7–9).

Nucleosome Assembly Activity of Vps75—A number of NAP family members are capable of assembling chromatin on naked DNA in vitro (4). The plasmid supercoiling assay, which measures the assembly activity on the basis of the introduction of negative supercoils into a relaxed DNA template (18), was used to test whether Vps75 also possesses such activity. Vps75-His introduced negative supercoils on plasmid DNA, an effect that was dose-dependent (Fig. 2C, lanes 3–5) and that required histones (lanes 7–9). This is indicative of nucleosome assembly and suggests that Vps75 may function in chromatin dynamics in vivo.

Association of Vps75 with Chromatin in Vivo—To determine whether Vps75 associates with histones and/or chromatin in vivo, we first utilized a histone association assay (10). Protein-DNA complexes in yeast cells producing HTM-tagged Vps75 were cross-linked by formaldehyde treatment, and the resulting chromatin was sheared by extensive sonication to yield pieces averaging 500 bp in length. Immunoprecipitation with a histone H3-specific antibody was carried out, and following reversal of the cross-links, co-precipitated Vps75 was detected by immunoblotting. A proportion of cellular Vps75 was indeed found to be associated with histone H3 (Fig. 3A). In theory, this association could be mediated by DNA or via a direct interaction between Vps75 and histone H3. Because of the resistance of cross-linked chromatin to nuclease treatment (10), we were unable to distinguish between these possibilities. Furthermore, this assay was unable to reveal whether Vps75 is associated with histones in chromatin or with free histones in the cytoplasm or nucleoplasm. To try and gain a more complete understanding of the putative interaction between Vps75 and chromatin, we carried out ChIP experiments. Vps75 was detectable at the pro-
**DISCUSSION**

In this report, the identification and characterization of a new NAP family member from yeast, Vps75, is described. Using a GST pull-down assay, we demonstrated that Vps75 has a preference for (H3-H4)$_2$ tetramers over (H2A-H2B) dimers. Chromatin assembly occurs by a two-step process in which a tetramer is deposited first followed by two dimers, and it is generally thought that different histone chaperones catalyze each step (4). Our experiments suggest that Vps75 belongs to the H3/H4 family of histone chaperones. This contrasts with many in vivo and in vitro studies that have generally described NAP family members as chaperones for H2A and H2B (for review, see Ref. 4), although a recent study reported that γNAP1 preferentially binds (H3-H4)$_2$ tetramers in vitro (19). We are currently conducting further experiments to elucidate the histone preference of Vps75 in vivo.

Using ChIP, we found Vps75 at all genomic regions tested. Our data suggest that the presence of Vps75 is not strictly correlated with transcription since we detected it at a repressed gene (GAL10) and at high levels at the telomere of chromosome VI. It is important to point out, however, that a role in transcription cannot be ruled out on the basis of these experiments. However, the fact that Vps75 is detected at telomeres is particularly interesting in light of the finding that vps75Δ mutants display shortened telomeres (6). Thus, our data might suggest a role for Vps75 in assembly or maintenance of telomere chromatin. It is clear, however, that more work needs to be done to define a role for Vps75 in specific chromatin-related processes.

We and others have found that Vps75 co-purifies with Rtt109, a protein recently implicated in H3 Lys-56 acetylation, and thus processes such as DNA repair, replication, and transcription (12, 20, 21). Two lines of evidence from our work indicate that Vps75 is not required for deposition of this histone mark. First, a vps75Δ strain did not exhibit the dramatic reduction in bulk 43 Lys-56 acetylation observed in rtt109Δ and asf1Δ. Second, the vps75Δ strain was also not sensitive to hydroxyurea, which is toxic to strains with defects in H3 Lys-56 acetylation. Preliminary ChIP experiments with vps75Δ cells have also failed to uncover significant differences in the level of histone H3 Lys-56 acetylation in chromatin (data not shown).
Although we cannot rule out the possibility that Vps75 is important for H3 Lys-56 acetylation at a certain subset of genes or genomic locations, it is equally plausible that the Vps75/Rtt109 complex possesses a distinct function. One possibility is that Rtt109 modulates the interaction of Vps75 with histones, thereby serving to regulate the chromatin assembly/disassembly activity of Vps75. Rtt109 is a positively charged protein (theoretical pI 9.41) with some highly basic regions, which could conceivably compete with histones for binding to the acidic Vps75 protein. This is presently under investigation.

In conclusion, we describe here the identification of Vps75 as a new member of the NAP domain family. This information should prove useful in unraveling the complexities of histone metabolism and important associated processes, such as DNA replication and transcription.

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