Preferential binding of the histone (H3-H4)$_2$ tetramer by NAP1 is mediated by the amino terminal histone tails

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Running title: yNAP1 binds histone folds and H3/H4 tails.
SUMMARY

The yeast nucleosome assembly protein 1 (yNAP1) participates in many diverse activities, such as the assembly of newly synthesized DNA into chromatin, and the rearrangement of nucleosomes during transcriptional activation. yNAP1 does not require ATP hydrolysis to perform these functions, and is a valuable tool for *in vitro* chromatin assembly. Using recombinant histone complexes, we show that yNAP1 has a preference for binding the \((H3-H4)_2\) tetramer over the \((H2A-H2B)\) dimer. We find that the loss of the histone tails abrogates this preference for H3 and H4, and demonstrate a direct interaction between yNAP1 and the N-terminal tails of H3 and H4. yNAP1 binds to one histone-fold domain, thus specifying the stoichiometry of the complexes formed with the histone dimer and tetramer. Finally, we provide evidence that the acidic carboxyl terminal region of yNAP1, while dispensable for nucleosome assembly *in vitro*, contributes to binding via structure-independent electrostatic interactions. Our results are consistent with recent mechanistic investigations of NAP1, and expand our understanding of the histone-chaperone family of assembly factors.
INTRODUCTION

The organization of DNA with an equal mass of proteins to form highly organized chromatin is a hallmark of all eukaryotes. It is at this level that the majority of the processes involving the DNA substrate (such as replication, transcription, repair, and recombination) are enacted. The fundamental repeating unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped in 1.65 supercoiled turns around a core of eight histone proteins, the histone octamer (1). The histone octamer is composed of two copies each of the four histone proteins; H2A, H2B, H3, and H4. At physiological salt concentrations, histones exist either as (H2A-H2B) dimers or (H3-H4)$_2$ tetramers (2). The latter can be described as a dimer of dimers. Histones dimerize via the structurally conserved histone-fold motif that is common to all four histones to form the histone fold dimer (3,4). A single tetramer binds the central 60 bp of DNA within a nucleosome, while two (H2A-H2B) dimers each organize 30 bp of DNA on either side of the tetramer, with the dimers making contacts with opposing faces of the central tetramer.

DNA binding is mainly afforded by the structured regions of the histones (histone fold and extensions (5)). The N-terminal basic tails of each histone protein are largely unstructured in solution as determined by NMR spectroscopy (6), and are too disordered to be observed in electron density maps from crystals of the histone octamer and the nucleosome (1,7-9). The tails do not appear to contribute to the stability of the nucleosome (10-13), though they appear to be important for the folding of chromatin fibers in vitro (14,15). The regulation of their involvement in transcription via
combinatorial post-translational modification of selected amino acid residues is currently a topic of intense research (16-18).

The transport of histones into the nucleus and the ordered deposition of histone sub-complexes onto DNA are carried out by highly regulated assembly proteins (reviewed in (19-21). In vivo, these events often involve the synchronous activity of ATP-driven and ATP-independent assembly factors to deposit histones onto the DNA, and to organize regularly spaced nucleosomal arrays (20,22,23). The (H3-H4)$_2$ tetramer acquires a unique set of acetyl groups on lysines in the histone tails followed by binding and deposition by the chromatin assembly factor CAF-1 (21). The replication-coupled assembly factor (RCAF) also catalyzes this activity (24).

Deposition of the (H2A-H2B) dimer is facilitated by an ATP-independent nucleosome assembly protein (NAP1; reviewed in (20,23,25)). NAP1, which can be classified as a ‘histone chaperone’ in the true sense of the word, was initially purified from Hela cells (26), and has since been isolated from a diverse set of organisms, such as soybeans, yeast, Paramecium, Drosophila and Xenopus (27-30). NAP1 has been isolated from a drosophila extract ((30) in complex with H2A and H2B, and from human cells in complex with H2A (31), and has thus been considered an (H2A-H2B) histone chaperone. However, in vitro, Drosophila NAP1 (dNAP1), human NAP-1 (AP-1) and yeast NAP1 (yNAP1) also bind the (H3-H4)$_2$ tetramer (32,33).

NAP1 is involved not only in the assembly of nucleosomes onto DNA (34,35), but also in the remodeling of nucleosomes during transcriptional activation (36,37), and in the recruitment of coactivators and the activation of transcription by facilitating the interaction between co-activators and chromatin (38,39). NAP1 has been shown to be
phosphorylated by cellular kinases in a cell-cycle dependent manner (40), and to be associated with components of the cell-cycle regulatory machinery (41,42). Thus, NAP1 likely plays a role in the onset and regulation of mitotic events.

An unusually high content of acidic amino acids is characteristic of histone chaperones (25). yNAP1 has four highly acidic regions distributed throughout its entire length. The most prominent acidic domain is located near the C-terminus (aa 366-403), where 28 out of 38 amino acids are either aspartic or glutamic acid residues. Surprisingly, this region, as well as a portion of the N-terminus, is dispensable for chromatin assembly (43). The basic histone N-terminal tails are considered to be likely targets for NAP1 binding, possibly through an interaction with one or more of the acidic domains within the NAP1 protein. Consistent with this idea, a previous study reported that yNAP1 interacts exclusively with the N-terminal tails of the histone octamer, and that the tails are required for yNAP1-mediated chromatin assembly (33). However, this same study showed that yNAP1 fails to bind mono- or poly-nucleosomes, despite the fact that the histone tails are likely to be accessible for such interactions in this structural context (4). More recently, it was shown that in vitro chromatin assembly using the structurally and functionally homologous Drosophila NAP1(30) occurs in the absence of the histone tails (44,45). Thus, the exact role of the N-terminal histone tails in chromatin assembly by NAP1 remains to be elucidated.

Here we investigate the preference and relative affinity of yNAP1 for recombinant histones, and dissect the contribution of the histone fold and histone tails toward this interaction. In contrast to previous findings (32,33), we show that yNAP1 has a preference for the (H3-H4)_2 tetramer over the (H2A-H2B) dimer. This preference is
dependent on the N-terminal tails of H3 and/or H4. We find that yNAP1 binds directly to the tails of both H3 and H4, but we do not observe yNAP1 binding to the N-terminal tails of H2A or H2B, despite similarities in residue content. In the absence of the N-terminal tails, yNAP1 binds the two core histone sub-complexes with nearly equal affinity. The stoichiometry of yNAP1 – histone sub-complex interactions is a consequence of each yNAP1 molecule binding a single histone molecule. Finally, we extend previous observations (43) and examine a series of yNAP1 truncations by circular dicroism (CD), topological assembly assays, and direct binding assays. We conclude that the chromatin assembly activity of NAP1 resides solely in the central core of the protein, which also is responsible for histone-fold-specific binding. The acidic C-terminus of yNAP1, while dispensable for chromatin assembly, contributes to binding via structure-independent, electrostatic interactions.
EXPERIMENTAL PROCEDURES

Expression and purification of recombinant proteins. Full-length yNAP1 was expressed and purified from the pTN2 expression vector (kind gift from Dr. Kikuchi) as described (43), with the following changes. The dialyzed fraction from the 35-65% ammonium sulfate cut was centrifuged, and the supernatant applied to a 10 ml Q-Sepharose FF anion-exchange column (Amersham Biosciences). The column was then washed and eluted with a linear NaCl gradient in buffer A (20 mM Tris pH 7.6 [4°C], 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMSF). Fractions were assayed for yNAP1 by SDS-PAGE, and peak fractions were pooled. The peak was diluted with buffer A to a final NaCl concentration of 150 mM, and applied to a 1 ml Mono-Q anion-exchange column (Amersham Biosciences). yNAP1 was eluted with 500 mM NaCl. Peak fractions were dialyzed into buffer A with 150 mM NaCl, and stored in aliquots at –80°C. All amino acid coordinates are reported using the sequence specified by accession number J05759.

GST-yNAP1 deletion mutants were generated by PCR amplification from the pTN2 plasmid, with PCR primers encoding BamH I and Sma I restriction sites. His6-tagged proteins were generated by PCR amplification of the pTN2 plasmid with primers encoding Nde1 and BamH1 restriction sites. Restriction enzyme digested and purified PCR products were ligated into similarly digested pGEX4T2 (Amersham Biosciences) for GST-fusions, or pET15b (Novagen) for His6-fusions. These constructs were transformed into DH5α cells, and screened by restriction digestion and by sequencing to confirm the identity of the clones. GST-fusions were purified by standard methods, dialyzed into buffer A (100 mM NaCl) and stored in aliquots at –80°C. His6 fusions were
expressed and purified over Ni\(^{2+}\)-NTA resin and further purified by Mono-Q chromatography. Peak fractions were dialyzed against buffer A (150 mM NaCl) and stored in aliquots at \(\sim80^\circ\) C. Insoluble His\(_6\)-74-293 and His\(_6\)-74-353 were extracted from inclusion bodies by solubilization of lysed, centrifuged cell pellets in 8M urea. The solution was centrifuged, and the supernatant applied to Ni\(^{2+}\)-NTA as described above. Full-length yNAP1, when subject to a similar denaturation/renaturation, recovers full chromatin assembly activity (43), as does His\(_6\)-74-365.

GST-fusion proteins for the \textit{S. cerevisiae} N-terminal tails (46) were expressed and purified by standard methods (see above). These expression plasmids encoded H2A (aa 1-35), H2B (aa 1-35), H3 (aa 1-46), and H4 (aa 1-34). Recombinant yeast and \textit{Xenopus laevis} histones (full length and truncated versions) were purified and refolded to (H2A-H2B) dimer and (H3-H4)\(_2\) tetramer as described (47-49). Native yeast and \textit{Drosophila} core histones were purified as described (50,51). The amino acid coordinates of the N-terminally deleted histone cores have been previously described (52). Histones were stored in 1M NaCl, 50% glycerol at \(-20^\circ\)C. Native \textit{Drosophila} histone H1 was purified as previously described (53).

**GST pull-down assays.** Coomassie stained GST pull-down assays were performed by incubating 100 pmoles of GST or GST-fusion proteins with 20 µl glutathione agarose (Sigma) in 200 µl TPD-25 (20 mM Hepes pH 7.9, 0.5 mM EDTA, 10% glycerol, 0.05% NP-40, 5 µM Zinc sulfate, 2.5 mM MgCl\(_2\), 25 mM KCl, 1 mM DTT) for 2-3 hours at 4°C. After extensive washing, secondary (target) proteins were added (the amounts are indicated in the figure legends), and incubated for 16 hours at 4°C. Bound proteins were washed extensively with TPD-250 (250 mM KCl) and briefly with
1X PBS prior to loading on SDS-PAGE. Proteins were visualized by staining with Coomassie Brilliant Blue. For GST pull-down assays using detection by western blot (anti-His<sub>6</sub> [H-15], Santa Cruz Biotechnology), 10 pmoles of GST or GST-fusion was bound to 10 µl of glutathione agarose, and 20-50 pmoles of target protein was added prior to 16 hour incubation in TPD (25 mM KCl). Following washing as described above, proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose and detected by ECL (Amersham Biosciences).

**Gel Filtration Chromatography.** Fractionation was performed on a FPLC Purifier® using a Superdex S-200 prep-grade 13/30 column (Amersham Biosciences). Samples were prepared and run in buffer A (150 mM NaCl) at 0.5 ml/min. Fractions were precipitated by TCA prior to SDS-PAGE. Elution volumes were measured by peak integration using Unicorn software (Amersham Biosciences), and Kav's were calculated by \( \frac{V_r - V_o}{V_c - V_o} \), where \( V_r \) is retention volume, \( V_o \) is void volume for the column (8.16 ml) and \( V_c \) is the column volume (24 ml). Kav was plotted against the molecular weights (log) for standards (Gel Filtration Calibration Kit; Amersham Biosciences) run in buffer A (150 mM NaCl). Best-fit exponential for the plot was determined in Cricket Graph III and the MWapp was determined by substitution of Kav into the equation for the line.

**Electrophoretic mobility shift assays.** EMSA's were performed by incubating yNAP1 at 10 µM in a 20 µl reaction with the indicated concentrations of histones at 4°C for 16 hours to establish equilibrium. All proteins were previously dialyzed into EMSA buffer (20 mM Tris pH 7.6 [4°C], 100 mM NaCl, 1 mM EDTA, 1 mM DTT). One-half of the binding reactions were transferred to a chilled tube containing 5 µl 20% sucrose, and the samples were loaded onto a 5% acrylamide, 0.2X TBE gel (8 x 10 cm, 1.5 mM
thick), electrophoresed for 60 min at 150 V (negative → positive), and stained with Coomassie brilliant blue. Molar ratios of histones to yNAP1 (where bound yNAP1=100%) were determined following digitization of the Coomassie stained gels and quantitation using ImageQuant (Molecular Dynamics). The stoichiometry of yNAP1/histone primary complex was determined by extrapolation of a linear fit of the plot of unbound yNAP1 (abscissa) versus molar ratio of histone to yNAP1. The intersection on the ordinate (histone: yNAP1 ratio) represents the molar ratio at which all yNAP1 is in complex. Though all data points are shown, only the lower, most linear portion of the data was utilized in the fit. Digitization noise led to the poor fit of data points as the percent of free yNAP1 approached zero.

**Proteolytic Cleavage.** Trypsin digestion of yNAP1 was performed at an enzyme concentration of 1µg/mg yNAP1 protein (1:1000 w/w), at a yNAP1 concentration of 1mg/ml, and at 22°C for the indicated time. The digestion products were resolved by 18% SDS-PAGE and Coomassie staining. For N-terminal sequencing, proteins were transferred to PVDF membrane (Bio-Rad) prior to sequencing (Applied Biosystems, Procise 491 protein sequencer, Perkin Elmer Cetus). Maldi-TOF mass spectrometry was performed on a Voyager DE Pro (Perseptive Biosystems).

**Chromatin assembly.** *In vitro* assembly reactions were performed at yNAP1 to core histone mass ratios of 4:1, as described (36), and assembled DNA was separated on 1.2% agarose gels and stained with SYBR-Gold (Molecular Probes). The use of mass ratios caused a progressive increase in the moles of yNAP1 utilized as construct molecular weight decreased (76 pmoles yNAP1, 88 pmoles 74-417, 102 pmoles 74-
365, 108 pmoles 74-353, 136 pmoles 74-293). Thus, the relative assembly activity of smaller constructs is over-estimated in this assay.

**Circular dichroism:** CD spectra were collected on a Jasco 720 spectrometer at 5°C. 20 spectra were obtained and averaged for each polypeptide in 20 mM NaH₂PO₄ pH 7.0, 50 mM NaCl. The molar ellipticity [\( \Theta \)] was obtained by normalization of the measured ellipticity (in mdeg) \( \Theta \), using \( [\Theta] = \Theta *100 / (n*l*c) \), where \( n \) is the number of residues, \( c \) is the total concentration in mM and \( l \) is the cell pathlength in cm. Percent helix is estimated as described previously (54).
RESULTS

**yNAP1 preferentially binds the (H3/H4)\textsubscript{2} tetramer over the (H2A/H2B) dimer.**

Recombinant histones of both yeast and *Xenopus* origin were refolded to (H2A-H2B) dimers, (H3-H4)\textsubscript{2} tetramers, and octamers [consisting of two (H2A-H2B) dimers and one (H3-H4)\textsubscript{2} tetramer]. The formation of correct histone complexes was verified by gel filtration (49), and the purified histone complexes were assayed for yNAP1 binding using a GST pull-down assay (Fig. 1A). While yNAP1 showed robust and nearly equivalent binding to (H2A-H2B) dimers and (H3-H4)\textsubscript{2} tetramers in separate reactions (Fig. 1A, lanes 3 and 6, respectively), a clear preference for the tetramer was observed when dimers and tetramers were allowed to compete directly in the form of ‘octamers’ (Fig 1A, lane 8). Note that the histone octamer dissociates into two (H2A-H2B) dimers and one (H3-H4)\textsubscript{2} tetramer under physiological and assay conditions (2). Since identical results were obtained with recombinant histone complexes from *Xenopus laevis* (Fig. 1A) and yeast (data not shown), we utilized *Xenopus laevis* recombinant histones in subsequent experiments.

To investigate whether assembly-specific posttranslational modifications affect the relative affinity of yNAP-1 for different histone pairs, we directly compared yNAP1 binding to recombinant and native *Drosophila* core histone octamers in the GST pull-down assay (Fig. 1B). A clear preference for the (H3-H4)\textsubscript{2} tetramer over the (H2A-H2B) dimer was observed for both preparations, although it is not quite as pronounced as that observed for *Xenopus laevis* histones. To rule out replication-dependent *Drosophila* core histone modifications different from those found in yeast, we performed an identical
experiment with native and recombinant *S. cerevisiae* octamers, with identical results (data not shown).

We used analytical gel filtration chromatography (GFC) to compare the types of complexes formed between yNAP1 and the two types of histone pairs. Consistent with previous observations for *Drosophila* NAP1 (30), yNAP1 (49.15 kDa) eluted with an anomalously high apparent molecular weight (MWapp) of 220 kDa (Fig. 1C). Subsequent hydrodynamic measurements have identified this species as yNAP1 dimers, and a fraction of larger oligomers (S.J. McBryant and O.B. Peersen, manuscript in preparation). Only a modest change in the elution position was observed when yNAP1 and an equimolar amount of histone dimer were pre-incubated. The observed MWapp of 251 kDa is consistent with a single yNAP1 dimer in complex with a single (H2A-H2B) dimer (MWapp 28.6 kDa by gel filtration, data not shown). This ratio is also apparent from visual analysis of gel filtration peak fractions by SDS PAGE (Fig. 1D). In contrast, the yNAP1 – (H3-H4)$_2$ tetramer complex eluted near the void volume for this column (>1 MDa). SDS-PAGE evaluation of peak fractions indicated a mass ratio that is inconsistent with a large excess of (H3-H4)$_2$ (Fig. 1D). The observed elution volume for the yNAP1/tetramer complex thus suggests a substantial change in either the shape or aggregation state of yNAP1. Gel filtration of yNAP1 with the recombinant histone octamer also resulted in a significant change in the elution profile when compared to yNAP1 alone, again eluting near the void volume (data not shown). When fractions were evaluated by SDS-PAGE, preferential binding of (H3-H4)$_2$ to yNAP1 was again observed (Fig. 1D, inset).
yNAP1 forms distinct complexes with (H2A-H2B) and (H3-H4)2.

We next determined the stoichiometry of the yNAP1 histone complexes. An electrophoretic mobility shift assay (EMSA) was developed to directly visualize and quantitate yNAP1/histone complexes (Fig. 2). Increasing concentrations of histone pairs, in the presence of a constant amount of yNAP1, resulted in distinct bands. As the ratio of (H3-H4)2 tetramer to yNAP1 monomer approached ~0.25:1.0 (Fig. 2A, lane 10, Fig. 2C, lane 4) all of yNAP1 was incorporated into the primary complex. In contrast, a (H2A-H2B) dimer to yNAP1 ratio of ~0.5:1 was required for complete supershift of yNAP1 (Fig. 2B, lane 6, Fig 2D, lane 3). Note that unbound histones are positively charged and will not enter the gel.

At increased histone ratios, higher-ordered complexes between yNAP1 and the (H2A-H2B) dimer (or the (H3-H4)2 tetramer) are formed. These appeared as histones were titrated above the 0.25:1 ratio of tetramer to yNAP1, and 0.5:1 dimer to yNAP1 (denoted by #: Fig. 2B, lanes 8-10, Fig. 2C, lanes 4-6). The slower migrating yNAP1 band observed in figure 2D (lane 1, asterisked) is routinely observed when using a concentrated solution of yNAP1 (>150 µM), and persists in the presence of reducing agent. It possibly represents a higher association state also observed by other methods (S.J. McBryant and Peersen, O.B., manuscript in preparation). Although we were able to eliminate this in subsequent experiments by dilution to concentrations less than 20 µM, it is noteworthy that this oligomer is competent for histone binding as a slight decrease in mobility is seen in the presence of (H2A-H2B) (Fig. 2D, lane 2).

Quantification of yNAP1 binding to the histone dimer and tetramer was performed by plotting the concentration of free yNAP1 (digitized pixels) versus the molar
ratio of histone to yNAP1. We performed a linear extrapolation of the data to the x-intercept, which indicated the molar ratio of histone to yNAP1 where the fraction of free yNAP1 is zero (Fig 2E, 2F). The value obtained for (H2A-H2B) (0.56, +/- 0.09, N=4), indicating that yNAP1 binds ~0.5 molar equivalents of the (H2A-H2B) dimer. Similarly, the value for (H3-H4)_2 (0.29, +/- 0.04, N=3) indicates that yNAP1 binds ~0.25 molar equivalents of the (H3-H4)_2 tetramer. Since yNAP1 itself is likely to be a dimer, we conclude that one yNAP1 dimer binds one histone dimer, whereas two NAP1 dimers can bind a histone tetramer. This data suggests that one yNAP1 molecule binds to each histone present in the respective histone complexes.

The N-terminal histone tails are not required for binding to yNAP1, but provide selectivity for the (H3-H4)_2 tetramer.

Previous approaches revealed little or no binding to histones in which the flexible N-terminal tails have been removed by treatment with trypsin (33). We directly examined the interaction of yNAP1 with tailless core histones using yNAP1 GST pull-down assays. Figure 3A shows that yNAP1 binds ‘tailless’ histone dimers and tetramers with approximately equal apparent affinity (compare lanes 2 and 6). Thus, the preference of yNAP1 for the (H3/H4)_2 tetramer might be due to additional interactions between yNAP1 and the N-terminal tails of either histone H3 or H4. To test this hypothesis, GST-fusion proteins with the N-terminal tails of *S. cerevisiae* core histones were tested for their ability to bind yNAP1 in a GST pull-down assay. Figure 3B shows that no yNAP1 binding was detected to the N-terminal tail of either histone H2A or H2B (lanes 3-6). In contrast, robust yNAP1 binding to the N-terminal tails of
histone H3 (lanes 7, 8), and modest binding to the tail of H4 (lanes 9, 10), was observed.

**Distinct domains of yNAP1 are required for histone binding and chromatin assembly.**

An earlier study utilized convenient restriction sites within the coding region to generate deletion mutants of yNAP1 (43). To investigate its domain structure, yNAP1 was subjected to limited trypsin proteolysis. This revealed two stable products with apparent molecular weights of 33 and 24 kDa (Fig. 4A). Subsequent mass spectrometry and N-terminal sequencing of the digestion products revealed that the larger peptide consisted of amino acid residues 74-293 (calculated molecular weight 25,099 kDa), while the smaller peptide comprised residues 302-417 (molecular weight 13,212 kDa). The C-terminal fragment contains a majority of acidic residues, accounting for its anomalously slow migration on SDS PAGE. Attempts to separate and purify the two cleavage products by conventional ion exchange, hydrophobic, and gel filtration chromatography failed (data not shown). Only denaturing, reverse phase HPLC resolved the two peptides. We therefore conclude that the cleavage site is located in a flexible surface loop of a single-domain protein encompassing amino acids 74 – 417.

Based on these results and on sequence comparisons, we generated a series of deletion mutants of yNAP1. Figure 4B shows these constructs schematically, including relevant features. The secondary structure content of all deletion mutants was analyzed by circular dichroism. The CD spectrum of full-length yNAP1 (Fig. 4C) indicates ~25 % α-helix content. We used this as a measure of the structural integrity of the deletion
mutants. Due to its presumably unstructured N-terminal region, full-length yNAP1 is less helical (on a per residue basis) than the smaller N- and C-terminally deleted constructs (amino acids 74-417 and 74-365, respectively). However, deletion of 12 more residues from the C-terminus (construct 74-353) generated a CD spectrum displaying significant loss of negative intensity at 222 nm, indicative of substantial loss of α-helical character. Thus, the residues spanning 354-365 contribute substantially to the structural integrity of the protein. This region harbors a stretch of amino acids that is extremely well conserved within the NAP1 family (Pro-Arg-Ala-Val-Asp-Trp-Phe-Thr-Gly, amino acids 354-362). The C-terminal construct (aa 302-417), that was intimately associated with the core region (aa 74-293) following limited proteolysis, appears unstructured when assayed in isolation.

All yNAP1 deletion mutants were tested for their ability to assemble nucleosomes onto plasmid DNA. Topological assays indicated that only amino acids 74-365 are required for robust chromatin assembly (Fig. 4D). Loss of the largest acidic region of yNAP1 (amino acids 365-417) had little effect on chromatin assembly, consistent with a previous report (43). As inferred from the essential role of amino acids 354–365 in maintaining structural integrity, the loss of these amino acids (fragment 74-353) resulted in significant loss of chromatin assembly activity (Fig. 4D). Similarly, a construct representing the larger of the trypsin proteolysis fragments (aa 74-293) failed to assemble chromatin. The trace amounts of supercoiled DNA observed for yNAP1 aa 74-293 and aa 74-353 are similar to that observed in the absence of yNAP1. As expected, the construct representing the smaller of the proteolysis fragments (aa 302-
417) also failed to assemble chromatin (data not shown). Results from CD and nucleosome assembly assays for each yNAP1 fragment are summarized in Table 1.

The unstructured, acidic C-terminus of yNAP1 binds basic proteins in a non-specific manner.

Irrespective of chromatin assembly activity, all of the constructs tested above retained the ability to bind both types of histone sub-complexes, as tested with GST-pulldown assays (Fig. 5A). Interestingly, the most C-terminal construct (yNAP1 aa 302-417), though lacking secondary structure and chromatin assembly activity, bound histone dimers and tetramers as strongly as full-length yNAP1 (Fig. 5A, compare lanes 3 and 6, and lanes 10 and 13). Figure 5B demonstrates that all the deletion constructs tested were also capable of binding both the full-length and tailless histones (Table 1). Interestingly, with the exception of GST-302-417 (lanes 11 and 12), all of these constructs showed a clear preference for H3 and H4 if incubated with full-length histones, and this preference disappeared upon incubation with tailless histones. GST-302-417 retained nearly equivalent ratios of dimer and tetramer, independent of the histone tails (Fig. 5B, compare lanes 11 and 12). Because of the highly acidic and unstructured nature of this C-terminal yNAP1 construct, it is likely that the binding results from an electrostatic, structure-independent interaction with the basic histone proteins.

We sought to determine which regions of yNAP1 were responsible for preferential binding to the H3 and H4 N-terminal tails, and thus for conferring selectivity for the (H3-H4)₂ tetramer. We tested yNAP1 constructs which either contain or lack the
acidic C-terminus for their ability to bind GST fusions with H3 and H4 N-terminal tails. Figure 5C shows that yNAP1 74-417, which functions in chromatin assembly and histone binding assays similarly to full-length yNAP1, interacts strongly with both the N-terminal tails of H3 and H4 (Fig. 5C, lanes 3, 4). This binding is comparable to the tail binding of full-length yNAP1 (see Fig. 3B, lanes 7-10). Carboxyl-terminal deletion of amino acids 366-417 (yNAP1 74-365) resulted in significant loss of histone H3 binding, and completely abrogated H4 tail binding (Fig. 5C, lanes 7, 8).

To investigate whether any of these interactions were based simply on electrostatic attractions, we compared the abilities of full-length and truncated yNAP1 to interact with purified native Drosophila histone H1 in a GST pull-down assay (Fig. 5D, Table 1). Histone H1 is highly basic, but does not contain the histone fold, and there is no sequence homology with the core histones. Full-length GST-yNAP1 bound well to H1, although deletion of the C-terminal acidic region (aa 366-417) completely abrogated H1 binding. A fragment containing the acidic region (aa 302-417) bound H1 as well as full-length yNAP1. This suggests that the histone binding activity observed with the acidic domain of yNAP1 (aa 366-417) is based primarily on structure-independent electrostatic interactions. Taken together, these data suggest that yNAP1 contains two distinct binding domains. The first, which is contained within the central region of the protein (aa74-365), specifically recognizes the histone fold motif. The second, located at the extreme C-terminus of yNAP1 (aa 365-417), is responsible for nonspecific binding to positively charged proteins.
DISCUSSION

Here, we demonstrate through direct binding and competition assays that yNAP1 preferentially binds the (H3-H4)$_2$ tetramer over the (H2A-H2B) dimer. We establish the stoichiometry of binding by gel shift assay, and determined that one yNAP1 dimer binds one histone-fold dimer. The preference for (H3-H4)$_2$ is conferred by the N-terminal tails of H3 and H4, which interact directly with yNAP1. No binding of yNAP1 to the N-terminal tails of H2A and H2B is observed. We further demonstrate that the acidic C-terminal region of yNAP1 participates in binding via electrostatic interactions in a structure-independent manner, and that at least in vitro, this region also binds other basic proteins.

The observation that yNAP1 prefers recombinant (H3-H4)$_2$ over (H2A-H2B) contradicts previous studies showing preferential binding of ‘native’ H2A and H2B (32,33). We showed that the preference towards the (H3-H4)$_2$ tetramer occurs with both recombinant and native histones, and thus is unlikely to be a consequence of posttranslational modifications that are only found in native histones. Discrepancies with earlier studies may stem from differences in the assays used to demonstrate binding, and in the folding and aggregation state of the histones. Here, we studied the binding of refolded and highly defined histone (H2A-H2B) dimers and (H3-H4)$_2$ tetramers to yNAP1 using gel filtration, native gel electrophoresis, and direct binding assays. To our knowledge, these complexes are structurally equivalent to the in vivo substrate. In contrast, the two previous studies either used far-western analysis to detect NAP1 binding to individual denatured core histones that have been separated by SDS-PAGE and blotted to a membrane (33), or utilized a modified ELISA to determine
the binding preference of the human homolog AP-1 (32). The first assay might not properly reflect binding to a structural motif such as the histone fold. Thus, the observed differences may well be a result of the integrity of the histone substrate used. Perhaps yNAP1 only displays its preference for the (H3-H4)$_2$ tetramer when presented with homogeneous, folded histone-pairs. Regarding the experiments described in (32), the modified ELISA relied on antibody recognition of fixed, immobilized AP-1 / histone complexes, which may have sterically hindered the AP-1 epitope, particularly with the larger (H3-H4)$_2$ complex. In addition, though the NAP1 homologs share considerable amino acid similarities, some differences, including a long N-terminal acidic region in the case of human AP-1, do exist.

In vivo, only histones H2A and H2B have been isolated in complex with NAP1 (30,31,55). In light of our demonstration that in vitro yNAP1 preferentially binds (H3-H4)$_2$, a mechanism must exist in the living cell to preclude the binding of histones H3 and H4 to NAP1. The binding sites on (H3-H4)$_2$ necessary for the NAP1 interaction may be occluded by other factor, for example CAF1 (21) and/or RCAF (24). However, independent of its exact in vivo function, the importance of NAP1 as an in vitro chromatin assembly tool warrants a more thorough understanding of its in vitro attributes, as described in this study.

We establish a binding stoichiometry of 0.5 molar equivalents of (H2A-H2B) dimer, and 0.25 molar equivalents of (H3-H4)$_2$ tetramer per mole of yNAP1. Due to the internal symmetry of the histone fold, each (H2A-H2B) dimer exposes two structurally similar histone-fold faces on the surface, whereas the tetramer exposes four histone-fold faces. In addition, the histone fold dimers of (H2A-H2B) and (H3-H4) are
superimposable with an rmsd of ~ 2 Å, (Fig. 7) (3,25). We thus conclude that each
yNAP1 monomer is capable of binding one histone fold motif within the context of a
histone fold dimer. Attempts to determine binding constants were limited by the
sensitivity of Coomassie staining, which mandated the use of NAP1 amounts well above
the likely dissociation constant (Kd) for these complexes. Both fluorescent and radio-
isotopically labeled yNAP1 showed aberrant native gel mobility and, in most cases,
affected histone binding. This precluded rigorous binding constant determinations.

In direct competition assays, yNAP1 shows a marked preference for the tetramer
over the dimer, which appears to be a consequence of the preferential binding of
yNAP1 to the N-terminal tails of H3 (and, to an extent, H4) over those of H2A and H2B.
Thus, we propose that the histone fold domains as well as the N-terminal tails of the
histones contribute to binding, and that the relative affinities of the two histone
complexes may be modulated by posttranslational modification of histone tails. The
acidic C-terminus of yNAP1 (aa 365-417) likely contributes to the overall affinity due to
its acidic nature, whereas the central portion of yNAP1 (amino acids 74-365) specifically
recognizes the structural properties of the histone fold dimer (either H3-H4 or H2A-
H2B). The acidic domain possibly contributes to tail binding, as the loss of these
residues significantly abrogates H3 and H4 tail binding. The protein binding
characteristics of yNAP1 domains are summarized in Table 1.

An additional observation arises from careful inspection of gel shift experiments
where increasing concentrations of histones were added to yNAP1. With concentrated
solutions of yNAP1 we observe an additional band that is also shifted upon the addition
of histones. yNAP1 appears to undergo concentration-dependent oligomerization,
consistent with the high MWapp observed by analytical gel filtration, as well as by hydrodynamic measurements of yNAP1 (S.J. McBryant and Peersen, O.B., manuscript in preparation). An early study with the *Drosophila* homolog reported that dNAP1 elutes from gel filtration and sediments in a glycerol gradient as a much larger species (600 kDa and 120 kDa, respectively) (30). The *Xenopus* histone binding protein nucleoplasmin exists as a pentamer (56,57), as does the *Drosophila* nucleoplasmin like protein dNLP (58). Our own experiments using gel filtration also showed a large increase in Mw upon addition of (H3-H4)$_2$ tetramer. Ligand- or concentration- induced changes in yNAP1 oligomerization state, and the expected symmetries resulting from self-association likely play a role in histone-fold recognition, binding and release by yNAP1 *in vivo* (25).

The acidic C-terminus of yNAP1 has been shown to be dispensable for chromatin assembly (Fig. 4D, and (43)). How might this domain function in nucleosome assembly *in vitro*? We propose that this region acts through an electrostatic mechanism to enhance the histone binding activity of yNAP1. As our chromatin assembly reactions contain significant amounts of yNAP1, and were allowed to proceed for 16 hours, the requirement for this ‘ancillary’ function of the acidic C-terminus may be negated. It is possible that a comparative, kinetic analysis of yNAP1 and select deletion mutants could test the plausibility of this hypothesis.

What is the implication of our results for nucleosome assembly? The fact that the (H3-H4)$_2$ tetramer organizes the central turn of the DNA supercoil (1,9) mandates its deposition onto the DNA prior to the deposition of the two dimers. The (H3-H4)$_2$ tetramer acquires a unique set of lysine acetylations, and is then deposited onto the
DNA by CAF1, RCAF, N1/N2, or other factors (23,31,59). However, we and others have found that yNAP1 assembles nucleosomes onto plasmid DNA in the absence of other chaperone factors, and that these nucleosomes are closely and regularly spaced (33,60,61).

Further, it has been shown *in vitro* by an elegant set of experiments (34) that the tetramer can be bound by NAP1 and transferred to a supercoiled plasmid to form a looped DNA / (H3-H4)\(_2\) complex, a ‘tetrasome’. When a NAP1 / (H2A-H2B) complex is added, (H2A-H2B) dimers are transferred to this ‘tetrasome’ in proportion to the amount of tetramer present. No such deposition is observed in the absence of tetramer, indicating that dimer deposition depends on the protein – protein interactions between the DNA-bound (H3-H4)\(_2\) tetramer and (H2A-H2B). Ito and collaborators conclude that the ordered deposition of (H3-H4)\(_2\) and then (H2A-H2B) requires a distinct set of preferential affinities, most importantly that the affinity of (H2A-H2B) for NAP1 is greater than for free DNA. This work, however, did not address how ordered deposition occurs when other reports have concluded that NAP1 preferentially binds (H2A-H2B) over (H3-H4)\(_2\) *in vitro* (29,31,32). Our results using direct competition assays demonstrating an (H3-H4)\(_2\) preference clarify this discrepancy. We have also observed that (H2A-H2B) dimers can be exchanged from a folded nucleosome in a yNAP1 – dependent manner, but that under the same conditions the exchange of (H3-H4)\(_2\) tetramer is virtually non-existent (Y.J. Park and K.L., unpublished results). Taken together, it appears that it is both the difference in affinity between the dimer and tetramer for NAP1, as described here, as well as the higher affinity of the (H2A-H2B) dimer for the (H3-H4)\(_2\) tetramer /
DNA complex as compared to free DNA (33), which ensures the ordered deposition of histones in vitro.

Our results shed light on the mode of recognition of histone sub-complexes by NAP1. However, many questions remain. For example, what is the mechanism of yNAP1-dependent histone deposition onto DNA? How are histone sub-complexes released from the interaction with yNAP1 in the presence of DNA? How can yNAP1 bind and remove a histone dimer from intact chromatin? And finally, what is the true function of NAP1 in vivo? One attractive hypothesis is that NAP1 not only participates in the replication-dependent assembly of chromatin, but also plays a role in the exchange of (H2A-H2B) dimers (possibly also containing histone H2A variants) during interphase, for example during transcription, recombination, or repair. Indeed, (H2A-H2B) dimers have been shown to exchange at a more rapid rate than the (H3-H4)2 tetramer in vivo (62). yNAP1 has also been shown to be receptive to (H2A-H2B) which has been released from nucleosomes subsequent to remodeling and histone acetylation (36). Finally, it was recently proposed that transcription by RNA polymerase II through nucleosomes result in the transient formation of a ‘hexasome’, a nucleosome from which one (H2A-H2B) dimer has been removed (63,64). The potential of NAP1 to participate in and facilitate all of these vital processes warrants further investigation.

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REFERENCES


FIGURE LEGENDS

Figure 1. yNAP1 preferentially binds the histone tetramer. (A) yNAP1 GST pull-down assays on (H2A-H2B) dimers, (H3-H4)\textsubscript{2} tetramers, and ‘octamer’ (= two (H2A-H2B) dimers and one (H3-H4)\textsubscript{2} tetramer under the conditions used). Purified recombinant *Xenopus laevis* histone dimers (90 pmoles), tetramers (47 pmoles), or ‘octamers’ (50 pmoles), were incubated with 100 pmoles GST alone, or GST-yNAP1 fusion protein bound to glutathione agarose beads. Bound proteins were separated by 18% SDS-PAGE and visualized by Coomassie staining. Forty percent of each output is shown (lanes 1, 4, and 7). Non-specific protein-protein interactions were monitored using GST alone (lanes 2, 5). Binding of GST-yNAP1 (labeled as yNAP1) to the dimer, tetramer and ‘octamer’ is shown in lanes 3, 6, and 8, respectively. The position of bound histones, GST, GST-yNAP1 and protein standards are indicated. (B) Comparison of recombinant and native *Drosophila* core histone ‘octamer’ binding to yNAP1. Purified recombinant or native *Drosophila* histone octamers (50 pmoles) were incubated with 100 pmoles GST alone, or GST-yNAP1 (labeled as yNAP1), as indicated. Bound proteins were identified as described above. Thirty-six percent of each output is shown (lanes 1, 4). The four core histones are indicated. (C) Gel filtration chromatography of yNAP1/histone complexes. Gel filtration was performed on 15 µM yNAP1 alone, or
mixed with equimolar amounts of histone dimer, tetramer, or octamer, as indicated. A chromatographic absorbance trace at 280 nm is shown for yNAP1 (solid), yNAP1 and histone dimer (dashed), and yNAP1 and histone tetramer (dotted). The fractions evaluated in (D) are indicated at the top. The yNAP1-octamer trace nearly overlapped with that observed with the yNAP1-tetramer, and is thus omitted for clarity. The void volume for the column is 8.16 ml (fraction 4). (D) SDS-PAGE analysis of the yNAP1-histone chromatographic fractions. Precipitated proteins from the indicated column fractions and chromatographic analyses were analyzed by 18% SDS-PAGE followed by Coomassie staining. The positions of the relevant proteins are indicated. Inset, a region of the SDS gel showing the histone proteins present in fractions 5 and 6 was enlarged to show the relative amounts of dimer and tetramer that elute with yNAP1 following gel filtration of the yNAP1-octamer mixture.

**Figure 2. Visualization and quantitation of the stoichiometries for yNAP1-histone complexes by quantitative EMSA.** yNAP1 binding to increasing concentrations of the histone pairs by native gel electrophoresis. (A) Increasing concentrations of the histone (H3-H4)$_2$ tetramer (0.025 to 0.25 molar equivalents) were incubated with a constant amount of yNAP1 (10 µM). Protein-protein complexes were analyzed by EMSA and visualized by Coomassie staining. The double banding is a gel artifact of this particular experiment, which otherwise best typifies the binding pattern. (B) Increasing concentrations of the histone (H2A-H2B) dimer (0.1 to 1.0 molar equivalents) were incubated with a constant amount of yNAP1 (10 µM). Analysis and visualization was as described above. (C) To visualize higher-order complexes, increasing ratios of (H3-H4)$_2$
tetramer (0.1 to 0.5 molar equivalents) were incubated with a constant amount of 
yNAP1. Analysis and visualization was as described for panel A. (D) 150 µM yNAP1 
was diluted to 10 µM and incubated in the absence and presence of the histone (H2A-
H2B) dimer (0.25 and 0.5 molar equivalents). Analysis and visualization was as 
described for panel A. (E) Representative plot of tetramer titration onto constant yNAP1 
used for stoichiometric quantitation. The abscissa shows the amount of free yNAP1 (as 
digitized pixels), while the ordinate shows molar ratios. Linear curve fit and extrapolation 
to the X-intercept yields the stoichiometry of histone to yNAP1. (F) Representative plot 
of dimer titration onto constant yNAP1 used for stoichiometric quantitation. Axes and 
analysis are similar to (E).

Figure 3. Direct binding of yNAP1 to tailless histones, and to isolated H3 and H4 
histone tails. (A) yNAP1 GST pull-down assays on the wild type and tailless histone 
‘octamer’. Purified full-length histone ‘octamer’ (wt octamer) and N-terminally deleted 
octamer (‘tailless octamer’) (50 pmoles each) were incubated with 100 pmoles GST 
alone, or GST-yNAP1 fusion protein bound to glutathione agarose beads. Bound 
proteins were identified by 18% SDS-PAGE followed by Coomassie staining. Forty 
percent of each onput is shown. Non-specific protein-protein interactions were 
monitored using GST alone (lanes 3,4). The positions of the intact and tailless histones 
are indicated, with a “t” prefix denoting tailless. (B) Analysis of yNAP1 binding to the 
histone N-terminal tails by GST pull-down. Two concentrations of yNAP1 (50 and 100 
pmoles; left and right lane within each bracket, respectively) were incubated with GST 
alone, or GST-histone tail fusion proteins (GST-H2A tail, GST-H2B tail, GST-H3 tail,
and GST-H4 tail; 100 pmoles each) bound to glutathione agarose beads. Bound proteins were separated by 15% SDS-PAGE and visualized by Coomassie staining. Onput (lane 1) contains 20 pmoles of yNAP1. The GST control was performed in the presence of 100 pmoles yNAP1 (lane 2). Bound yNAP1, the GST-histone tails and protein standards are indicated.

Figure 4. Proteolytic cleavage of yNAP1 reveals distinct domains. (A) Proteolysis of yNAP1 reveals two stable polypeptides. Purified yNAP1 (60 µg) was incubated with trypsin (0.06 µg) for the indicated times. Aliquots from the time course were separated on 15% SDS-PAGE, and visualized by Coomassie staining. Lane 1 shows 10 µg undigested yNAP1. The two yNAP1 proteolytic digestion products are denoted by symbols, and protein standards are indicated. (B) Schematic illustration of yNAP1 truncation mutants constructed based on the proteolysis described in A. The coordinates and significant structural features of yNAP1 are shown. (C) Circular dichroism (CD) analysis of the structural integrity of the truncated yNAP1 proteins. Spectra are buffer corrected, and normalized for concentration and number of residues to allow direct comparison of secondary structure content between proteins (● Full-length yNAP1; ♦ aa74-471; Δ aa74-365; o aa74-353; + aa74-293; x aa301-417). (D) Chromatin assembly properties of the yNAP1 deletion mutants. One-dimensional DNA topological assay comparing chromatin templates assembled with recombinant core histones in the presence of the indicated yNAP1 deletion mutant. The DNA topoisomers were resolved on an agarose gel, and the DNA stained with SYBR-Gold. The supercoiled (SC) and topoisomerase-1 relaxed (R) DNA, and the increasing
octamer: DNA ratios (0.14:1, 0.43:1, 0.71:1, 1:1) are indicated. Assembly function of the yNAP1 mutants was assessed by the ability of the proteins to induce supercoils (reflective of nucleosome deposition) into the relaxed, circular DNA plasmid.

**Figure 5. Characterization of deletion mutants reveals two distinct binding domains within yNAP1.** (A) yNAP1 deletion mutants bind the dimer and tetramer. GST pull-down assay with yNAP1 deletion mutants and the (H2A-H2B) dimers and (H3-H4)_2 tetramers. Purified histone dimers (90 pmoles) (lanes 2-6), or tetramers (47 pmoles) (lanes 9-13) were incubated with GST alone, or the indicated GST-yNAP1 fusion proteins (GST-NAP [fl], GST-74-417, GST-74-365, GST-302-417) (100 pmoles each) bound to glutathione agarose beads. Bound proteins were separated by 18% SDS-PAGE and visualized by Coomassie staining. Forty percent of the dimer and tetramer output is shown in lanes 1 and 8. The 16 kDa molecular weight marker is shown in lane 7. Non-specific protein interactions were monitored using GST alone (lanes 2, 9). (B) yNAP1 deletion mutants bind to the both full-length (wt) and tailless octamer. Purified full-length and N-terminally deleted octamers (50 pmoles each) were incubated with GST alone, or the indicated GST-yNAP1 fusion proteins (100 pmoles each), as described in panel A. Forty percent of the wt and tailless octamer output is shown in lanes 1 and 2. Bound histone proteins are indicated to the right and left of the figure. The tailless histones are denoted with a “t” prefix. (C) Deletion of the yNAP1 C-terminus reduces histone tail binding. Purified His6-tagged yNAP1 deletion mutants (aa74-417, lanes 1-4) or aa74-365, lanes 5-8) (20 pmoles each) were incubated with GST alone, or the indicated GST-H3 or -H4 tail (10 pmoles each). Bound yNAP1 deletion mutants
were separated by 12% SDS-PAGE and probed with an anti-His\textsubscript{6} antibody for Western blot analysis. Twenty five percent output of each yNAP1 mutant is shown in lanes 1 and 5. Note: the smaller polypeptides also recognized by the anti-His6 antibody are minor impurities or C-terminal degradation products of yNAP1. (D) The acidic C-terminus mediates non-specific binding to basic proteins. Purified \textit{Drosophila} histone H1 (100 pmoles) was incubated with GST alone, or the indicated GST-yNAP1 deletion mutant (100 pmoles). Bound H1 was separated on 15% SDS-PAGE and visualized by Coomassie staining. Twenty five percent H1 output is shown in lane 1.

\textbf{Fig. 6. Structurally similar motifs in the (H2A-H2B) and the (H3-H4) histone fold dimers are accessible for molecular recognition from two sides.} The two dimers were structurally aligned using lsqman, and are shown in two different orientations that are related by a 180 ° rotation around the y axis as indicated. Histone fold domains for H2A, H2B, H3, and H4 are shown in yellow, red, blue and green respectively, the histone fold extensions and tails are shown in wheat.
Table 1. Properties of yNAP1 and Derivatives.

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<th>yNAP1 construct</th>
<th>Assembly</th>
<th>Solubility</th>
<th>(H3-H4)$_2$ Preference</th>
<th>H2A/H2B</th>
<th>H3/H4</th>
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* The indicated His6-tagged yNAP1-truncated proteins were soluble only following urea denaturation / renaturation. The renatured protein was used in topological and tail-binding assays. The GST-tagged proteins were insoluble, and therefore could not be used in the GST pull-down assays (histone binding assays).
A. tailless octamer: wt octamer:  

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H3  
H2A/H2B  
H4

B. yNAP 1

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GST-tails

McBryant, et al., Figure 3
A. 

B. 

C. 

D. 

McBryant, et al., Figure 4
A.  

H2A-H2B dimer  (H3-H4)2 tetramer

B.

wt octamer +  t-octamer  

H3 ➔  H2A/H2B ➔  H4 ➔

C.

yNAP1 (74-417)  yNAP1 (74-365)

D.

Drosophila H1

McBryant, et al., Figure 5
McBryant, et al., Figure 6

H2A-H2B histone fold dimer

H3-H4 histone fold dimer

'Inside view'

'Outside view'