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NAP-2 Is Part of Multi-Protein Complexes in HeLa Cells

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Abstract We previously reported that a complex of nuclear proteins from HeLa cells, among them histone H1 and casein kinase 2 co-eluted from immobilized nucleosome assembly protein 2 (NAP-2)-Sepharose. Here, using HeLa cell nuclear extracts, we found NAP-2 migrates in a blue-native polyacrylamide gel with an apparent molecular weight of 300 kDa. HeLa cell NAP-2, labeled in vivo with radioactive orthophosphate, co-precipitated with at least two phosphoproteins, with an apparent mass of 100 and 175 kDa, respectively, as determined by SDS–PAGE. NAP-2 from total HeLa cell extract co-purified with other proteins through two sequential chromatographic steps: first, a positively charged resin, Q-Sepharose, was used, which purified NAP-2 more easily with other proteins that eluted as a single peak at 0.5 M NaCl. This fraction possessed both relaxing and supercoiling activities, and it was able to assemble regularly spaced nucleosomes onto naked DNA in an ATP-dependent manner. Second, a negatively charged resin (heparin) was used, which retained small amounts of NAP-2 (a very acidic polypeptide) and topoisomerase I. This fraction, although able to supercoil relaxed DNA, did so to a lesser extent than the Q-Sepharose fraction. The data suggest that NAP-2 is in complex(es) with other proteins, which are distinct from histones. J. Cell. Biochem. 93: 398–408, 2004.
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In eukaryotes, it is thought that the first level of chromatin folding is the nucleosome, wherein the DNA is wrapped around a central histone octamer, comprising two molecules each of the core histones (H2A, H2B, H3, H4) [Arents and Moudrianakis, 1993; Luger et al., 1997; Luger and Richmond, 1998] and one of the linker histone, H1. The process of the DNA wrapping around the core histones is coupled to DNA-replication [Shibahara and Stillman, 1999] and DNA-repair [Senshu et al., 1978; Worcel et al., 1978; Gaillard et al., 1996]. It is thought that the assembly mechanism occurs in at least two steps [Jackson and Chalkley, 1981; Jackson et al., 1981; Kleinschmidt et al., 1990], in which the deposition of H3/H4 is independent of H2A/H2B. This two-step mechanistic model of nucleosome assembly did not contemplate the loading of histone H1 which remains elusive so far.

Several factors known as histone chaperones have been identified in vitro, which are involved in nucleosome deposition onto naked DNA [Ito et al., 1997]. In humans, two related nucleosome assembly proteins NAP-1 and NAP-2, have been isolated [Ishimi et al., 1987; Hu et al., 1996; Rodriguez et al., 1997]. Biochemical characterization of NAP-1 suggests that it functions in a manner similar to nucleoplasmin, i.e. it deposits histones onto DNA [Ishimi and Kikuchi, 1991; Fujii-Nakata et al., 1992]. Unlike p48, a component of chromatin assembly factor 1 (CAF-1), which binds preferentially to histones H3/H4 [Verreault et al., 1996], NAP-1 possesses higher affinity for histones H2A and H2B, and can stimulate nucleosome assembly in vitro [Ishimi et al., 1984, 1987; Ishimi and Kikuchi, 1991].

In vivo and in vitro evidence supports the concept that the histones H3/H4 and H2A/H2B exist as independent complexes with specialized chaperones. For example, NAP-1 co-precipitates with histones H2A/H2B from *Drosophila* embryos [Ito et al., 1996]. Similarly, human NAP-1 co-precipitates from HeLa cytoplasmic extract with H2A, but it does not interact with H3/H4 [Chang et al., 1997]. In *Xenopus*, the

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histone chaperone nucleoplasmin is in a complex with H2A/H2B, whereas the chaperone N1 complexes with H3/H4 [Kleinschmidt et al., 1985, 1990]. Moreover, the non-nucleosomal dimer H2A/H2B and the tetramer H3/H4 are not associated in solution [Eickbush and Moudrianakis, 1978]. Furthermore, the dimer H2A/H2B elutes from chromatin arrays at a lower salt concentration than the tetramer H3/H4 [Simon and Felsenfeld, 1979]. The existence of the H3/H4 tetramer and the H2A/H2B dimer as independent complexes has been confirmed in experiments in yeast, which also suggested that H2A and H2B are co-transported into the cell nucleus as heterodimers [Moreland et al., 1987].

At present, there is little evidence suggesting that NAP-2 interacts with other proteins, except for core histones, histone 1 (H1), and casein kinase 2 (CK2) [Rodriguez et al., 2000]. Similarly, *Drosophila* NAP-1 binds to both the alpha and beta subunits of CK2 [Li et al., 1999], while recombinant yeast NAP-1 binds to purified H1 and high mobility group (HMG) proteins [McQuibban et al., 1998]. Chromatin assembly factors, such as CAF-1, are recruited to the replicating DNA templates by specific proteins, like PCNA [Shibahara and Stillman, 1999]. In this regard, we previously proposed that mammalian NAP-1 and NAP-2 might also be targeted to the assembly sites through specific protein factor(s) [Rodriguez et al., 2000]. In this study, we show that the HeLa cell NAP-2 interacts with high molecular mass phosphoproteins that are distinct from histones. Moreover, NAP-2 co-eluted with several other proteins, from two different resins, and the fraction possessed both relaxing and supercoiling activities.

MATERIALS AND METHODS

Native Polyacrylamide Gel Electrophoresis

HeLa nuclear extract (0.3 mg) was resolved by blue-native-charge-shift polyacrylamide gel electrophoresis (BN-PAGE) (6–20%) in the presence of Coomassie blue G250 dye, as described in [Schagger and von Jagow, 1991; Schagger et al., 1996]. The advantage of the blue-native method for preserving intact protein complexes, while introducing a net negative charge onto such complexes by the negatively charged Coomassie blue dye, has been previously documented [Schagger and von Jagow, 1991; Schagger et al., 1996]. Thus, in this type of electrophoresis, similarly to SDS–PAGE, the proteins migrate by their relative masses and not by their intrinsic charges, since all the proteins have been negatively charge-shifted by the presence of Coomassie blue. Remarkably, Coomassie blue, unlike SDS, does not dissociate the native interaction between proteins hence the name blue-native-charge-shift electrophoresis. Following either BN- or SDS–PAGE, NAP-2 was detected by Western blot with the anti-N-NAP-2 antibody. Recombinant human NAP-2 (40 ng), purified from *E. coli* [Rodriguez et al., 1997], was used as a control for the migration pattern of NAP-2 alone in BN-PAGE.

Immunoprecipitation of In Vivo Phosphorylated NAP-2

Log phase HeLa cells (50% confluence, 100 mm plates, Nunc) were grown in complete Alpha MEM medium (GIBCO) and labeled with [^{32}P]-orthophosphate, as follows: the cells were incubated in 3 ml Alpha MEM phosphate-free medium, containing 10% dialyzed fetal bovine serum (FBS, GIBCO), for 30 min, and then 300 μCi of ^{32}P-orthophosphate/ml (Amersham Pharmacia Biotech) was added to the medium. The cells were further incubated for 3 h in a humidified CO_2 incubator at 37°C, and then washed with the same medium lacking serum. The cells were then scraped into 0.5 ml PBS, containing 1% NP40, 2 mM $NaVO_4$, 50 mM NaF, antiprotease cocktail (TPCK, TLCK, leupeptin, and aprotinin, at 10 μg/ml, each and 1 mM PMSF) 50 mM EDTA and incubated on ice for 30 min. The extract was centrifuged in a microfuge at 14,000 rpm for 30 min and precleared with 20 μl each of preimmune serum and protein A-Sepharose. NAP-2 was immunoprecipitated by the addition of 5 μg of rabbit anti-NAP-2 antibody. To avoid cross-reaction of the antibody with other proteins with similar acidic tails, as present in the extract, the anti-NAP-2 antibody was first purified on a Δ(330–375)-NAP-2-Sepharose column (a NAP-2 mutant missing the acidic C-terminal) [Rodriguez et al., 1997, 2000]. Similarly, to avoid cross-reaction with NAP-1, the antibody eluted from Δ(330–375)-NAP-2-Sepharose column was also passed through a NAP-1-Sepharose column. The anti-NAP-2 antibody present in the flow-through of the NAP-1-Sepharose column was used in the immunoprecipitation assays. Alternatively,

100 μl of a rabbit polyclonal antibody, generated against a conserved region at the N-terminus (amino acids 1 to 34) of NAP-2, was used with identical results. As a control, preimmune serum was added to the supernatant for 1 h at 4°C. Then, 20 μl protein A was added for 1 h, and the samples were centrifuged for 10 min at 4°C. The pellets were washed five times with 1 ml of the same extracting buffer supplemented with 0.1% SDS and 1% sodium deoxycholate or 1% NP40. The samples were boiled for 5 min in sample buffer (100 mM DTT, 50 mM Tris-HCl, pH 8, 1% SDS, 1% glycerol) and resolved by 7.5% SDS–PAGE. After electrophoresis, the gel was dried and exposed for autoradiography at −80°C with an intensifying screen. Typically, the radiolabeled NAP-2 was detected after 4–5 h of exposure.

Nucleosome Assembly Activity

Fractions eluted from Q-Sepharose at 250 and 500 mM NaCl (QS250 and QS500, respectively) (8 mg/ml) and fractions eluted from heparin-Affigel (1 mg/ml), were treated with RNAase A (10 μg/sample) for 30 min at 37°C, and then incubated with 1 μg of purified HeLa core histones [Simon and Felsenfeld, 1979] for 20 min, on ice. The reaction mixture contained PBS, 5% glycerol, 0.5 mM PMSF (buffer B), 10 mM ATP (some experiments were performed without ATP, as indicated in Fig. 3 and figure legend) and 0.2 mM PMSF. One microlitre (0.2 μg) of closed circular plasmid ($pKSII^+$) DNA, relaxed with 1 unit of topoisomerase I (topo I; Promega), was then added to the mixture and placed at 37°C for 1 h. The reaction was terminated with the addition of stop buffer (100 mM Tris-HCl, pH 7.5, 12.5 mM EDTA, 0.15 M NaCl (final concentration), 1% SDS, and 1 μg of proteinase K) and further incubated at 37°C for 1 h. The DNA was extracted with phenol/chloroform and subjected to electrophoresis in a 1% agarose gel, (in Tris-Borate/EDTA buffer, pH 8) to determine the gain in negative supercoiling by the histone transfer reaction [Germond et al., 1975]. Alternatively, the nucleosome assembly activity was monitored by DNAase I digestion [Prunell et al., 1979; Kurumizaka and Wolffe, 1997]. Briefly, DNA assembled into nucleosomes was treated with 10 IU of DNase I (Boehringer) per μg of plasmid DNA, in 10 mM $MgCl_2$, at 37°C. Samples (1 μg) were taken at increasing intervals between 0 and 4 min. The reaction was terminated with the addition of stop buffer and extracted with phenol/chloroform (1:1). The resulting ladder of nucleosomal DNA was resolved by electrophoresis in 1.5% agarose and visualized by ethidium bromide staining.

Co-Purification of NAP-2

Total HeLa cell extract (1,200 mg), was passed through Q-Sepharose (20 ml column, 2 cm × 10 cm), equilibrated with 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 5 mM DTT, 10 μg/ml pepstatin and aprotinin, 0.5 mM PMSF and 5% glycerol (buffer A). The resin was washed stepwise (see Table I) with (i) buffer A, (ii) buffer A containing 0.250 M NaCl, final concentration and (iii) buffer A containing 0.5 M NaCl final concentration. The eluants (the first 100 ml) were dialyzed against PBS, 5% glycerol and 0.5 mM PMSF (buffer B). The dialyzed fractions eluted at 500 mM NaCl (QS500) were loaded

TABLE I. Co-Fractionation of NAP-2 From HeLa Cell Extracts by Q-Sepharose Total HeLa Cell Extracts Were Loaded Onto Q-Sepharose, After the Regin was Washed, With 0.1 M NaCl, the Proteins Were Eluted Stepwise at Increasing Ionic Strength (see Materials and Methods)

Purification steps	Volume (ml)	Total protein (mg)	Supercoiling activity	Topoisomerase I activity	NAP-2 detection by Western blot (Fold-purification and recovery %)
Crude total HeLa cell extract	400	1,200	−	+	+
Flow-through	400	600	−	−	−
Wash buffer A	2,000	400	−	−	−
Wash, buffer A, containing 250-mMNaC1	25	100	+	+	−
Wash buffer A, containing 500-mMNaC1	33	80	+	+	+(15): (100%)

The presence (+) or absence (−) of supercoiling activity, Topo I activity and NAP-2 protein are indicated for every step. Fold-purification (15) and recovery (100%) are indicated in the last column.

onto a heparin-Affigel column (Bio-Rad) (25 ml, 2 cm × 12 cm), equilibrated with buffer B. The resin was treated stepwise with i) buffer B, ii) buffer B containing 0.25 M NaCl, and iii) buffer B containing 2 M NaCl. The eluants were monitored by a mini-Bradford protein assay (Pierce) and the washes were continued until no protein was detected in the eluants. All the fractions were then dialyzed against buffer B and stored at −80°C.

RESULTS

Presence of NAP-2 in High Molecular Weight Complex

To investigate whether or not NAP-2 is present both as free protein and in complex with other proteins, HeLa cell nuclear extracts were subjected to electrophoresis on a blue-native-charge-shift gel (BN-PAGE) [Schagger and von Jagow, 1991; Schagger et al., 1996; Ruiz et al., 2002], together with highly purified recombinant NAP-2 protein [Rodriguez et al., 1997, 2000], and the gel was then blot-transferred and the membrane subjected to Western blot analysis with anti-NAP-2 antibody (Fig. 1A). Since BN-PAGE is a native-charge-shift method, where the electrophoretic mobility of the proteins is mainly determined by the negative charges of the bound Coomassie blue dye, the proteins and protein complexes are separated by their relative masses and not by their intrinsic electric charges. Under these conditions, recombinant NAP-2 protein migrated with an apparent molecular weight (MW) of approximately 90 kDa (Fig. 1A, lane 1), suggesting the formation of a NAP-2 homodimer and not of multimers, while NAP-2 from nuclear extracts was clearly detected as migrating both at the position of recombinant NAP-2 protein (dimer), and at 300 kDa (Fig. 1A, lane 2) (note also a less resolved signal at the top of the gel), indicating that it is in complex with other protein(s).

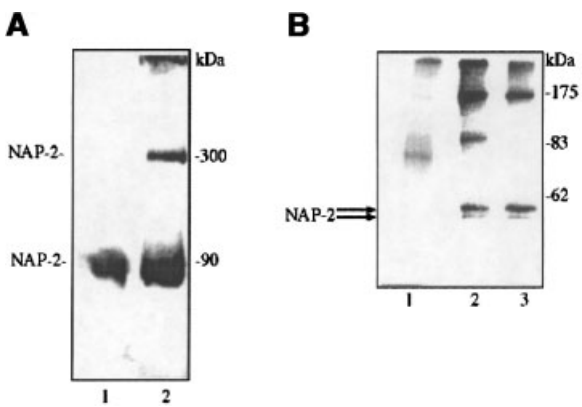

Fig. 1. Blue native electrophoresis and immunoprecipitation of NAP-2. **A**: Anti-NAP-2 Western blot analyses of HeLa cell nuclear extracts subjected to electrophoresis on a native acrylamide gel containing Coomassie blue dye (see Materials and Methods); recombinant NAP-2 (**lane 1**), HeLa cell nuclear extracts (**lane 2**). **B**: Autoradiogram of the anti-NAP-2 immunoprecipitation. Immunoprecipitated NAP-2 was incubated with pre-immune serum (**lane 1**) or washed at low (**lane 2**) or high (**lane 3**) stringency conditions, using buffers containing NP-40 and SDS, respectively.

NAP-2 Is Associated With Phosphoproteins

To complement the finding using the BN-PAGE, HeLa cells were labeled in vivo with [^{32}P]-orthophosphate (see Materials and Methods) and the proteins were extracted and immunoprecipitated with anti-NAP-2 antibody. The immunoprecipitated proteins were subsequently resolved by SDS–PAGE, the gel was dried and exposed to autoradiography (Fig. 1B). Two stringency conditions were used, (i) a less stringent one, in which the immunoprecipitate was washed in a buffer containing NP-40 (Fig. 1B, lane 2), and (ii) a more stringent one, in which 0.1% SDS was included in the wash buffer (Fig. 1B, lane 3); under both conditions a phosphoprotein of approximately 175 kDa, was detected, in addition to free NAP-2, which migrated as a doublet (Fig. 1B, lanes 2 and 3). Under the less stringent wash, an additional phosphoprotein band of approximately 100 kDa was detectable (Fig. 1B, lane 2). None of these bands were present in the sample that was immunoprecipitated with pre-immune sera (Fig. 1B, lane 1). These data indicate that two phosphoproteins of approximately 100 and 175 kDa associate with NAP-2 in vivo. The resulting total MW of the precipitated polypeptides (~322 kDa, including NAP-2, 47 kDa, according to its reported amino acid sequence) is in agreement with that obtained by BN-gel electrophoresis, in which NAP-2 migrated at around 300 kDa.

Purification of NAP-2 Through Q-Sepharose

In an effort to identify the proteins associated with NAP-2, total HeLa cell extracts were passed through a column of Q-Sepharose (QS), a high-salt-equilibrated anion exchange resin used for retaining only the very acidic proteins and excluding the less acidic and positively

charged ones, at pH 7.5 (Fig. 2). Under these conditions, the positively charged proteins can be retained only if they are in complex with acidic proteins. Due to the large amount of protein (1,200 mg) in the input material, the bound protein was eluted from the resin in a stepwise manner, using increasing salt concentration, rather than a continuous salt gradient. Although this strategy may result in some loss in resolution of the protein complexes, it avoids cross-contamination of proteins that will elute specifically at either 100, 250, or 500 mM, due to either insufficient elution volume or the normal (bell-shaped) distribution of the protein fractions. Moreover, stepwise elution permitted extensive washing of the columns with large volumes of solvent (see Table I, wash with buffer A), ensuring that the eluted protein fractions were not contaminated with proteins from the previous peak. As it can be seen in Figure 2A (lane 5), washing of the QS resin with 2 L of

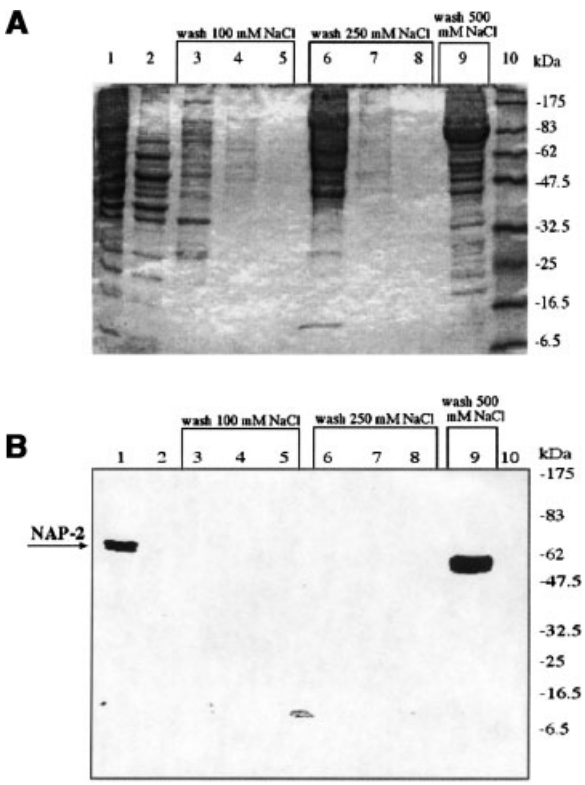

Fig. 2. Purification profile of NAP-2 eluted from a Q-Sepharose column. Fractions eluted from a Q-Sepharose column were subjected to SDS–PAGE on a 12% gel. **A**: Coomassie blue staining and (**B**) Western blot, using an anti-NAP-2 antibody. Total HeLa cell extract (**lane 1**); flow-through from Q-Sepharose column (**lane 2**); washes with 100 mM NaCl (**lanes 3–5**) and 250 mM NaCl (**lanes 6–8**); wash with 500 mM NaCl (**lane 9**); molecular weight marker in kDa (**lane 10**).

100 mM NaCl revealed no protein bands that could be detected by either Coomassie blue staining (sensitivity ~20–50 ng per protein band) or by mini-Bradford reagent measurements (not shown). Thus, the possibility that the proteins specifically eluted at 250 mM NaCl (see Fig. 2A, lane 6) might be cross-contaminated with proteins from the 100 mM fractions, and so on, can be excluded. Although several proteins were eluted from the column (Fig. 2A, lanes 2–9), NAP-2 could not be detected by Western blot in either the flow-through fractions (Fig. 2B, lane 2) or in the fractions of the washes performed with 100 mM NaCl (QS100 fraction; Fig. 2B, lanes 3–5) and 250 mM NaCl (QS250 fraction; Fig. 2B, lanes 6–8). NAP-2, was eluted from the QS column at 500 mM NaCl (QS500 fraction) in a single peak (Fig. 3A and B, lane 9). Since NAP-2 was detectable by Western blot only in the 500 mM NaCl fraction, it can be assumed that throughout the chromatographic steps there was 100% recovery for it. Although NAP-2 possesses supercoiling activity, it was considered inappropriate to calculate the fold of its purification, using this activity, for the following reasons: (1) the supercoiling activity was undetectable in the input crude extract, as is often the case with other biological activities, possibly due to the presence of inhibitors in the input mixture, since the nucleosome assembly was displayed only in the fractions QS250 and QS500; and (2) as shown by us (Fig. 3A, lane 8) and others (see Introduction and Discussion), a variety of proteins and protein complexes may be responsible for nucleosome assembly. These facts would complicate the calculation of purification-fold of NAP-2 on the basis of a general supercoiling or nucleosome assembly activity, attributes that are not exclusively inherent to this protein. Instead, the fold of purification of NAP-2 was calculated by dividing the input amount of total protein (1,200 mg) by the final amount of total protein obtained in the QS500 fraction (80 mg) (assuming a 100% recovery in this fraction for NAP-2). Based on this calculation, NAP-2 was purified by approximately 15-fold with nearly 100% recovery (Table I).

Characterization of the Eluants From Q-Sepharose

The fractions eluted from the QS column were tested for the presence of supercoiling and/or relaxing activities (Fig. 3). Fractions QS250 and

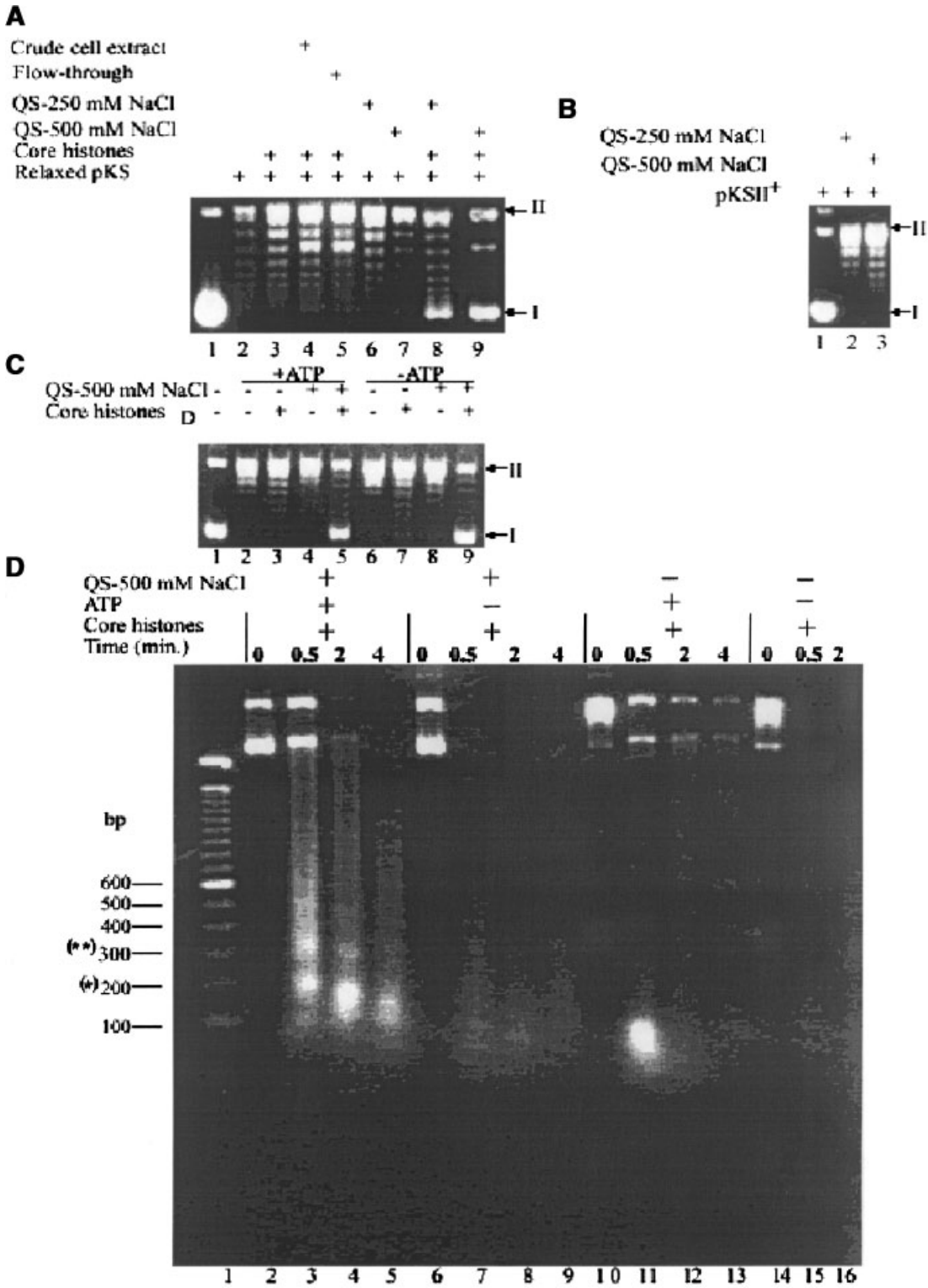
A
Crude cell extract
Flow-through
QS-250 mM NaCl
QS-500 mM NaCl
Core histones
Relaxed pKS
II
I
1 2 3 4 5 6 7 8 9
B
QS-250 mM NaCl
QS-500 mM NaCl
pKSII+
II
I
1 2 3
C
+ATP
-ATP
QS-500 mM NaCl
Core histones
D
II
I
1 2 3 4 5 6 7 8 9
D
QS-500 mM NaCl
ATP
Core histones
Time (min.)
0 0.5 2 4 0 0.5 2 4 0 0.5 2 4 0 0.5 2
bp
600
500
400
(**) 300
(*) 200
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 3.

QS500 possessed both supercoiling (Fig. 3A, lanes 8 and 9) and relaxing (Fig. 3B, lanes 2 and 3) activities. The supercoiling activity depended on the addition of exogenous HeLa core histones (Fig. 3A, lanes 8 and 9), whereas the addition of core histones or the QS (QS250 and QS500) fractions alone was not sufficient to supercoil the relaxed plasmid DNA to form I (Fig. 3A, lanes 3, 6, and 7). Interestingly, the QS250 fraction possessed supercoiling activity (Fig. 3A, lane 8), despite the absence of NAP-2 protein from it. Note that the total amount of protein from fraction QS250 loaded in lane 6 (Fig. 2A) is higher than the protein in fraction QS500 (see lane 9, Fig. 2A), further supporting the notion that fraction QS250 is depleted from NAP-2, since the anti-NAP-2 antibody was unable to detect it (the antibody can detect 1 ng of NAP-2 on blots). Taking into account that NAP-1 and NAP-2 by themselves are inefficient histone deposition factors [Rodriguez et al., 1997, 2000], it can be concluded that undetectable traces of NAP-2 would not be responsible for the supercoiling activity generated by fraction QS250, which is at least 50% of that generated by fraction QS500 (compare lane 8 with lane 9 in Fig. 3A). Furthermore, crude HeLa cell extracts were unable to supercoil the relaxed pKSII$^+$ plasmid DNA in the presence of core histones (Fig. 3A, lane 4).

Since QS500 contained NAP-2, we tested the ability of this fraction to supercoil relaxed plasmid DNA, in the presence of HeLa cell core histones and in the presence and absence of ATP (Fig. 3C). QS500 was able to supercoil the relaxed plasmid DNA in the presence of core histones, regardless of the presence or absence of ATP (Fig. 3C, lanes 5 and 9), whereas core histones or QS500 alone, either in the presence or absence of ATP, were not (Fig. 3C, lanes 3, 4, 7 and 8).

The extent of histone deposition onto naked plasmid (pKSII$^+$) DNA can be assessed by using the supercoiling assays, while the periodicity of histone deposition and the length of the DNA wrapped around the core histones can be determined by nuclease digestion [Rodriguez et al., 1997]. We used DNase I to digest the assembled DNA, in order to assess the stability of the assembled nucleosome in the presence and absence of ATP. In the presence of both ATP and core histones, mono and dinucleosomes were detected (Fig. 3D, lanes 3 and 4), indicating that the nucleosome assembly reaction requires ATP for the regular spacing of nucleosomes. The assembled histones protected DNA of approximately 170–190 bp in length. In contrast, the DNA assembled in the absence of ATP did not show nucleosomal organization and exhibited almost no resistance to digestion by DNase I (Fig. 3D, lanes 6–9). Similarly, core histones alone, either in the presence (Fig. 3D, lanes 10–13) or absence (Fig. 3D, lanes 14–16) of ATP failed to protect the DNA template as mono and dinucleosomes from digestion by DNase I. Although some resistance to DNase I digestion was apparent (lane 11), the size of the protected DNA fragment (<50 bp) was smaller than would be expected even for a subnucleosomal particle containing H3/H4 histones (130 bp) [Ruberti and Worcel, 1986; Zucker and Worcel, 1990]. Moreover, this DNA

Fig. 3. Functional characterization of fractions QS250 and QS500 eluted from Q-Sepharose. **A**: The supercoiling activity was assayed as described in Materials and Methods [added "+" and omitted "−" components are indicated]. The analyzed Q-Sepharose fractions were mixed with purified HeLa core histones and relaxed pKSII$^+$ plasmid (form II) DNA. To determine the gain in supercoiling, resulting from the histone-transfer reaction, the samples were deproteinized, subjected to electrophoresis through an agarose gel (1%) and stained with ethidium bromide. **Lane 1**: supercoiled pKSII$^+$ (form I); **lane 2**, relaxed pKSII$^+$ plasmid DNA (form II); **lanes 3–9**, relaxed pKSII$^+$ plasmid DNA incubated with core histones (lane 3); crude HeLa cell extract and core histone (lane 4); QS flow-through and core histone (lane 5); QS250 (lane 6); QS500 (lane 7); QS250 and core histones (lane 8); QS500 and core histones (lane 9). **B**: QS250 and QS500 fractions possess relaxing activity. Five microlitres QS250 and QS500 eluants were mixed with closed-circular supercoiled pKSII$^+$ plasmid DNA [0.2 μg in buffer B, at 37°C for 1 h]. After deproteinization, the relaxed closed circular DNA was resolved by agarose (0.9%) gel electrophoresis and stained with ethidium bromide. **Lane 1**: supercoiled pKSII$^+$; **lanes 2** and **3**, supercoiled pKSII$^+$ in the presence of QS250 and QS500 fractions, respectively. **C**: Supercoiling activity of QS500 in the presence (+) and absence (−) of ATP. Relaxed closed circular DNA was incubated with QS500 and core histones in the presence (**lanes 2–5**) or absence (**lanes 6–9**) of ATP. After 1 h (see Materials and Methods), the DNA was extracted from proteins and separated by agarose gel electrophoresis. **Lane 1**, supercoiled pKSII$^+$; **lane 2**, relaxed pKSII$^+$ with topo I; **lane 3**, same incubated with core histones; **lane 4**, same incubated with QS500 fraction; **lane 5**, same incubated with QS500/core histones; **lanes 6–9** are the same as lanes 2–5, but without ATP. **D**: Nucleosome assembly by the QS500 fraction, in the presence or absence of ATP, was monitored by DNase I digestion. The samples were run in a 1.5% agarose gel and stained with ethidium bromide (mono (*) and dinucleosomes (**) are indicated).

fragment was completely digested at 2 min (compare lane 12 to lane 4), suggesting a non-specific interaction of core histone with DNA.

Purification of NAP-2 by Heparin-Affigel

The QS500 fraction (eluted from Q-Sepharose) was purified further by heparin-Affigel chromatography and the fractions eluted from the heparin-Affigel column (equilibrated with PBS), were analyzed by SDS–PAGE (Fig. 4A). Some protein bands were recovered in the flow-through (Fig. 4A, lane 3) and in the washes with PBS (Fig. 4A, lanes 4–7). Additional proteins were eluted with PBS containing 250 mM NaCl (Fig. 4A, lanes 8–13), peaking at fraction 11, and with PBS containing 2 M NaCl (Fig. 4A, lanes 14–17), peaking at fraction 15. Western blot analysis of this gel using anti-topo I antibody (Fig. 4B) revealed that topo I eluted at 2 M NaCl in a single fraction (lane 15). Western blot analysis of the same gel using anti-NAP-2 antibody (Fig. 4C) revealed that some NAP-2 eluted in the flow-through (Fig. 4C, lane 3), but the bulk of it was eluted with PBS (Fig. 4C, lanes 4–7) and PBS containing 250 mM NaCl (Fig. 4C, lanes 8–13). NAP-2 was also co-eluted with topo I with 2 M NaCl (Fig. 4C, lane 15).

Characterization of the Eluants From a Heparin-Affigel Column

Functional assays performed for topo I activity showed that although relaxing activity was present in the input material to the heparin-Affigel column (Fig. 4D, lane 2; compare with lane 1, supercoiled DNA), it could not be detected in either the flow-through (Fig. 4D, lane 3), or in the fractions eluted with PBS (Fig. 4D, lanes 4–7) and PBS containing 250 mM NaCl (Fig. 4D, lanes 9–13); relaxing activity was present only in fraction 15 eluted with 2 M NaCl (Fig. 4C, lane 15), which contained topo I (Fig. 4B, lane 15). Finally, we also assessed the supercoiling activity of the fractions eluted with 2 M NaCl (Fig. 4E). Relaxed pKSII$^+$ plasmid DNA (Fig. 4E, lane 2) when mixed with core histones (Fig. 4E, lane 3) was not able to be supercoiled. Some supercoiling activity was observed when fraction 15, containing topo I and NAP-2 (Fig. 4B and C, lane 15, respectively), was added together with the core histones (Fig. 4E, lane 4), which, however, was less than the supercoiling activity of the QS500 fraction (compare with Fig. 3A, lane 9). These results suggest that either the amount of NAP-2

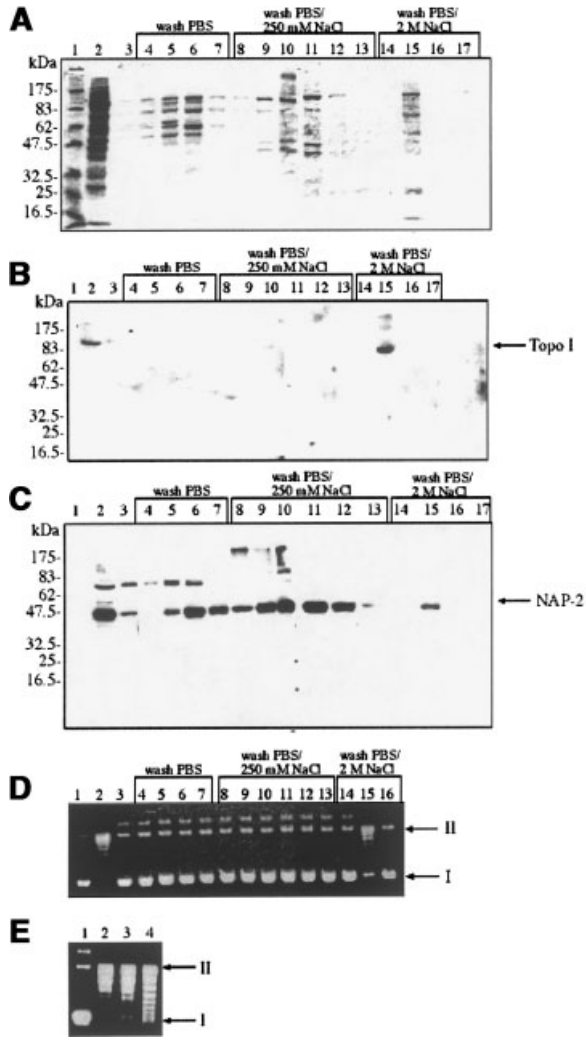

Fig. 4. **A**, **B**, and **C**: Purification profile of NAP-2 eluted from a heparin-Affigel column. Fractions (40 μl) eluted from a heparin-Affigel column that was equilibrated with PBS were analyzed by (A) 12% SDS–PAGE followed by Coomassie blue staining; (B) Western blotting using an anti-topoisomerase I antibody (Topogen). The position of topo I is indicated; (C) Western blotting using an anti-NAP-2 antibody (see Materials and Methods). **Lane 1**: molecular weight marker (in kDa); **lane 2**, input HeLa cell total extract; **lane 3**, flow-through; **lanes 4–7**, washes with PBS; **lanes 8–13**, washes with PBS containing 250 mM NaCl; **lanes 14–17**, washes with PBS containing 2 M NaCl. **D**: Relaxing activity of the eluants from the heparin-Affigel column [the experimental conditions are described in Figure 3B]. **Lane 1**: closed circular supercoiled pKSII$^+$ plasmid; **lanes 2–16**, the same heparin-Affigel fractions as in A, B, and C. **E**: Supercoiling activity of fraction 15, eluted from the heparin-Affigel column. **Lane 1**: supercoiled pKSII$^+$ plasmid DNA; **lane 2**, pKSII$^+$ relaxed with topo I; **lane 3**, relaxed pKSII$^+$ plasmid DNA, mixed with purified core histones; **lane 4**, relaxed pKSII$^+$ plasmid DNA, incubated in the presence of fraction 15 and core histones.

may be limiting in fraction 15 or that some of the assembly components may be lost in the course of the purification.

DISCUSSION

We have demonstrated that the nucleosome assembly protein 2 (NAP-2), in HeLa cells, is associated with other proteins, distinct from histones. Several lines of evidences support this view. First, NAP-2 co-precipitated with multiple phosphoproteins from HeLa cells as determined by SDS–PAGE (total mass estimated to be equal to 322 kDa). Second, use of blue-native polyacrylamide gel electrophoresis (BN-PAGE) confirmed that NAP-2 protein is present in high MW complexes of approximately 300 kDa in HeLa nuclear extracts, consistent with the data obtained by co-immunoprecipitation, mentioned above. The possibility that the 300 kDa complex is a multimeric form of NAP-2 aggregates can be excluded, since the migration of purified recombinant NAP-2 migrates as a dimer (~90 kDa); thus, additional proteins, besides NAP-2, must be present in the nuclear extract, causing NAP-2 to shift. Third, NAP-2 co-eluted with topo I and several other polypeptides in two consecutive chromatographic resins (Q-Sepharose and heparin-Affigel). Finally, fourth, since NAP-2 (estimated isoelectric point of ~3.5) and heparin are highly negatively charged polymers at pH 7, recombinant NAP-2, purified from *E. coli* may not interact with a heparin-Affigel column at physiologic pH, as indeed was found (data not shown), suggesting that the small amount of NAP-2 from HeLa cell extracts was most likely retained on the heparin column via other interacting factors. We also report a rapid and simple purification method to obtain protein complexes, from mammalian cells with nucleosome assembly activity.

The fact that NAP-2 was found in high MW complexes in HeLa cell extracts suggests that this protein functions in concert with other factors. NAP-2 is complexed with histones throughout the cell cycle [Rodriguez et al., 2000]. Similarly, NAP-1 co-precipitates with histone H2A/H2B from *Drosophila* embryos [Ito et al., 1996], while it co-precipitates with H2A only from HeLa cytoplasmic extracts and it does not interact with H3/H4 [Chang et al., 1997]. Moreover, the non-nucleosomal dimer H2A/H2B is not associated with the H3/H4 tetramer in HeLa cell extracts. The H3/H4 histone complex appears to be associated with p48, a subunit of the chromatin assembly factor 1 (CAF-1) [Verreault et al., 1996]. This complex exhibits sedimentation behavior consistent with a molecular mass of 100 kDa, of which 50 kDa are being contributed by the H3/H4 tetramer and an additional 48 kDa by p48 [Chang et al., 1997]. In oocytes of *Xenopus laevis*, the H2A/H2B dimer is associated with the chaperone nucleoplasmin, whereas the H3/H4 tetramer is found in complex with N1/N2 [Kleinschmidt et al., 1985]. Both complexes are needed for optimal nucleosome assembly activity [Dilworth et al., 1987; Kleinschmidt et al., 1990; Zucker and Worcel, 1990]. Experiments in yeast have suggested that H2A and H2B are co-transported into the cell nucleus as heterodimers [Moreland et al., 1987]. The sum of these data indicates that the cellular pool of core histones is not pre-organized as a histone octamer. Rather, H3/H4 and H2A/H2B complexes exist as independent structures, which are associated with different chaperones. This scheme supports the notion that the mechanism of nucleosome assembly occurs in at least two steps, in which the H3/H4 tetramer is loaded first, followed by the loading of the H2A/H2B dimer [Kleinschmidt et al., 1990; Smith and Stillman, 1991]. In this process, the mechanism of deposition of H1 has not been investigated. The selective binding of NAP-1 to H2A/H2B [Ishimi et al., 1987] and of NAP-2 to H1 [Rodriguez et al., 1997] suggests the involvement of these chaperones in additional step(s) of nucleosome formation. It is reasonable to assume that other factor(s) are responsible for recruiting the NAP(s), with their histone cargo, to effect nucleosome assembly. Such an assumption is supported by the finding that NAP-2 is detected in 300 kDa MW complexes (Fig. 1), which cannot be attributed solely to its interaction with the core histones, whose sum of molecular mass is approximately 106 kDa. This notion is further supported by the results reported herein, which show phosphorylated NAP-2 co-precipitating with two phosphoproteins of 100 kDa and 175 kDa, respectively. Furthermore, NAP-2 co-eluted from Q-Sepharose with several other proteins. The NAP-2-containing fraction QS500 possessed relaxing and supercoiling activity and was able to load histones onto naked DNA in a regular array in an ATP-dependent fashion (Fig. 3). Interestingly, the supercoiling activity is not dependent

on the presence of ATP, but requires the addition of both core histones and the QS500 fraction. Similarly, the relaxing activity (Fig. 3B, lane 2) is independent of ATP and Mg^{2+}, suggesting that it is due to topoisomerase I (topo I) [Wang, 1996]. The requirement of ATP for nucleosome assembly has previously been described [Almouzni and Mechali, 1988; Kmiec et al., 1989; Banerjee et al., 1991; Ito et al., 1996; Ito et al., 1997]. It is interesting to note that fraction QS250 possessed supercoiling activity (Fig. 3A, lane 8), despite the absence of NAP-2 protein from it, suggesting perhaps the existence of an independent nucleosome assembly mechanism. Finally, the inability of the crude HeLa cell extracts to supercoil the relaxed $pKSII^+$ plasmid DNA in the presence of core histones (Fig. 3A, lane 4) suggests the presence of inhibitory factors of nucleosome assembly in the extracts. A single-chromatographic step, using Q-Sepharose, appears to be sufficient for obtaining fully active preparation of nucleosome assembly components. Interestingly, even though the supercoiling activity was independent of ATP, the stability of the assembled nucleosomes depended on it. Further purification of Q-Sepharose-eluted NAP-2 through a heparin-Affigel column showed that a small but detectable amount of NAP-2 was retained on this resin, co-eluting in a single fraction with topo I and several other polypeptides (Fig. 4). This fraction also possessed some supercoiling activity. The data suggest that the two opposed processes of nucleosome assembly and DNA relaxing activity are apparently linked.

Since the NAPs were found in complexes with histones in vivo [Rodriguez et al., 2000] and shuttled from the cytoplasm to the nucleus at the G1/S boundary [Ito et al., 1996; Rodriguez et al., 1997, 2000], it might be expected that they participate in the second step of nucleosome assembly by loading the H2A/H2B dimer and histone H1. The data reported here suggest that NAP-2 may be recruited to the site of nucleosome assembly via other factor(s), such as the 100- and 175-kDa phosphoproteins that were found associated with it.

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