Identification of a structural and functional domain in xNAP1 involved in protein–protein interactions

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ABSTRACT

xNAP1 (Xenopus nucleosome assembly protein) belongs to the family of nucleosome assembly proteins (NAPs) and shares 92% identity with human and mouse NAP1. NAPs have been reported to have a role in nucleosome assembly, cell cycle regulation, cell proliferation and transcriptional control, although the precise function of NAP1 is still not clear. Here we report the identification of a putative domain of xNAP1 by limited proteolysis. This domain has been mapped in the xNAP1 protein sequence to residues 38–282 and thus lacks the acidic sequences at the N- and C-termini. We have studied this domain and related fragments in vitro and by a functional assay involving over-expression of the protein in *Xenopus laevis* embryos. Analytical ultracentrifugation shows that removal of the acidic N- and C-terminal regions does not prevent the formation of larger multimers, which are predominantly hexadecamers. Injection of mRNA encoding the full-length xNAP1 or the putative domain and other related constructs into Xenopus embryos gave identical phenotypes. These results are discussed in relation to protein–protein interactions between NAP1 octamers and a possible ‘squeezing’ mechanism.

INTRODUCTION

*Xenopus* nucleosome assembly protein (xNAP1) is a protein of 392 residues and a molecular mass of 45 258 Da, although it migrates anomalously as ~60 kDa species as assayed by SDS–PAGE. xNAP1 is a member of a family of proteins that share the ability to assemble histones into nucleosomes on DNA in vitro. NAP1 proteins are universally present in eukaryotic cells (1) and have homology with nucleosome assembly proteins (NAPs), nucleoplasmin and N1/N2 in the acidic C-terminus region (1). All these proteins possess negatively charged regions, which have been suggested to promote nucleosome assembly in vitro (2,3). xNAP1 is closely related to human and mouse NAP1, sharing 92% identity with these proteins.

Experiments on *Drosophila* NAP1 (4) revealed that dNAP1 associates with H2A and H2B and that the protein is found in the nucleus during S phase but is mainly cytoplasmic during G2 phase. NAP1 acts as a core histone shuttle, delivering histones from the cytoplasm to the nucleus where chromatin assembly takes place. Recent studies have indicated a novel role for yNAP1 as a mediator of chromatin fluidity, by incorporating histone variants into chromatin and assisting nucleosome sliding (5). In this model, NAP1 has a much more active role in shaping chromatin structure. Histone variants locally alter chromatin structure, with histone chaperones and other cellular factors promoting histone exchange and chromatin fluidity (6). It is believed that these processes can facilitate the interchange between different chromatin states, having varying degrees of transcriptional activity (6).

Other studies have also linked NAPs with gene regulation. NAP1 and NAP2 have been reported to form a complex with p300 (7). Histone acetylation by p300 promotes the transfer of H2A and H2B to NAP1 in vitro (8) potentially leading to transcriptional activation mediated by disruption of the histone octamer. In another study, xNAP1 was shown to have a transcriptional role in embryonic blood formation. xNAP1 is expressed tissue specifically in the ectoderm, precisely overlying the ventral blood island and the outer globin expressing cells (9). Depletion of xNAP1 in embryos showed that mRNA levels of haematopoietic marker genes SCL and AML decreased significantly but endothelial markers Hex and Fli-1 were unaffected. Since depletion of xNAP1 led to gene-specific changes, it was proposed that xNAP1 has a role in tissue restricted gene regulation (10).

There are a variety of conserved regions in NAP1 sequences. There are three negatively charged regions, including a large acidic region at the C-terminal and a smaller
one at the N-terminal. Studies on yNAP1 have revealed that the acidic C-terminal region is dispensable for nucleosome assembly in vitro (11), but that it is required for the dissociation of H2A/H2B dimers from nuclear core particles, whereby yNAP1 can subsequently facilitate nucleosome sliding along the DNA to a thermodynamically favourable position (5). dNAP1 has two PEST regions and a possible PEST region has also been identified in xNAP1 (9). Nuclear localization (NLS) and nuclear export signals (NES) also appear to be conserved in NAPs (4,9,12,13). Studies by Li et al. (12) identified a phosphorylation site adjacent to the NES sequence in dNAP1, and it has been suggested that this plays a role in the translocation of NAP1 between the cytoplasm and nucleus.

The yNAP1 NES sequence is necessary for shuttling the protein from the nucleus to the cytoplasm. Failure to do so was found to inhibit mitotic progression (13). The NES sequences have been shown to align in yeast, nematode, *Xenopus*, *Drosophila* and human NAP1 (13). A KGIPEFWLT and a SFFNFF sequence are also conserved in NAP1s across these species (1,4,11). Although the KGIPEFWLT region has been assigned no structural or functional role, the SFFNFF sequence is necessary for nucleosome assembly (11).

Secondary structure analysis of the yeast orthologue, yNAP1, by circular dichroism spectroscopy (14) indicates that the C-terminal domain (approximately one-third of the protein) is mostly unstructured with ~25% α-helix content. Removal of residues 354–365 (PRAVDWFTGALE) located adjacent to the C-terminal acidic region caused a substantial loss of α-helical structure. Recent studies (15,16) suggest that NAP1 forms dimers, octamers and hexadecamers in a concentration-dependent manner. Both the dimer and the octamer of NAP1 appear to bind histones H2A and H2B in a one NAP1 monomer per histone monomer ratio, but the hexadecamer appears not to be involved in histone interactions. Based on this, a model for cell cycle-dependent shift of the NAP1 dimer–octamer equilibrium has been proposed that reflects different biological functions of NAP1 (16). According to this model, the low concentrations of NAP1 in the nucleus at G1 lead to dimer formation and a role in gene regulation via chromatin rearrangement. Accumulation of NAP1 in the nucleus during S phase leads to formation of octamers capable of carrying multiple H2A.H2B dimers to replication sites.

In this article we define a structural domain of xNAP1 by limited proteolysis. We show that this domain multimerizes, forming predominantly hexadecameric complexes in vitro, and that over-expression of the domain affects axial patterning in *Xenopus*, despite the lack of several of the conserved regions believed to be important for activity.

**MATERIALS AND METHODS**

**Cloning of expression constructs**

Full-length and deletion constructs of xNAP1 were amplified by PCR using Vent DNA polymerase (NEB) before cloning. These NAP1 sequences were ligated into NcoI/XhoI restricted pET28b(+) (Novagen) and XbaI/XhoI restricted pBUT plasmids. Recombinants in each case were fully sequenced to confirm the presence of the insert.

**Protein expression and purification**

Expression of NAP1 and truncations thereof was carried out in 500 ml 2xYT broth with 50 μg ml⁻¹ kanamycin antibiotic inoculated with 10 ml of an overnight culture of *Escherichia coli* BL21(DE3) pLysS. The culture was incubated at 37°C by shaking until mid-log phase. Protein expression was induced by 1 mM IPTG, and the cells incubated for a further 3 h before harvesting. The sample was lysed by sonication (Virbracell™ VCX 500 Jencons-PLS) and the soluble fraction isolated by centrifugation. Recombinant protein was purified using a Hi-trap chelating column (Amersham Pharmacia) followed by preparative size exclusion chromatography on a superose 12 column.

**Limited proteolysis of xNAP1**

NAP1 was subjected to limited proteolysis with either chymotrypsin or trypsin (Sigma). The enzymes were dissolved in 1 mM HCl to make 5 mg ml⁻¹ stock solutions, and aliquots were frozen at −20°C. The enzymes were added to the xNAP1 (0.4 mg ml⁻¹) sample at an enzyme to substrate (w/w) ratio of 1:10000 in a buffer of 100 mM sodium phosphate at pH 7.0, 100 mM NaCl, 1 mM EDTA and 1 mM DTT. The reactions were incubated at 25°C. Aliquots were taken at various time points and the reaction was stopped by the addition of PMSF (1 mM final concentration). Samples were analysed on 10% SDS–PAGE gels, followed by staining with Coomassie blue.

**Embryo manipulation**

Embryos were obtained as described by Smith and Slack (17) and staged according to Nieuwkoop and Faber (18). In *vitro* transcription and microinjection of mRNA was carried out as described previously (19,20). Injections of mRNA were carried out at the 1-cell stage with 4 nl of RNA solution (37 pg RNA total).

**Mass spectroscopy**

The digested xNAP1 samples were sent for mass spectrometry to M-Scan Ltd, Wokingham. Salts were removed from the 1 ml sample by washing on a desalting column with 0.1% aqueous formic acid, and the retained analytes were eluted in a solvent of aqueous acetonitrile. The desalted sample was loaded into a nanospray needle (MDS Protana) and analysed using a Sciex Q-Star/Pulsar, ‘Q-TOF’ mass spectrometer (MDS Sciex) operating in positive electrospray mode. Data were collected by MCA acquisition and interpreted by manual inspection of the multiply charged species observed in the spectrum.

**Dynamic light scattering**

Dynamic light scattering (DLS) was performed on purified T2 protein at 20°C using a Protein Solutions DynaPro temperature-controlled microsampler. The samples were in gel filtration buffer containing 10% glycerol and centrifuged at 14 000 r.p.m. at 4°C for 10 min before DLS. From the resulting hydrodynamic radius, \( R_h \), an upper estimate of the molecular mass, \( M_r \), of the protein was obtained using the empirical relationship for typical globular proteins:

\[
M_r = (1.68 \times R_h)^{2.34}
\]

using the supplied software.
Analytical ultracentrifugation
A Beckman Optima XL-A analytical ultracentrifuge (Beckman-Coulter, Palo Alto, CA, USA) was used for sedimentation equilibrium experiments. All measurements were recorded at 20°C. T2 protein was purified by metal chelate affinity chromatography and size exclusion chromatography on a Superdex 75 column, using the T2 buffer with an additional 10% glycerol. The protein was dialysed overnight into AUC buffer (100 mM triethanolamine, 50 mM KCl, 1 mM EDTA and 10% glycerol, pH 8.5). The experiment was performed in six-channel cells of 12 mm optical path length, using 90 µl of solution at three protein concentrations (0.14, 0.07 and 0.035 mg ml⁻¹). AUC buffer (100 µl) was loaded into the corresponding channel. The rotor was accelerated to 3000 r.p.m. and scans of absorbance versus radial displacement were taken at 280 nm every 5 h for over 24 h and again after a total of 48 h; equilibrium was seen to be reached after 20 h. Scans were analysed using the Beckman XL-A software. However, the lower concentration samples could not be fitted with any degree of accuracy and were not subsequently used.

RESULTS
xNAP1 contains structural domains resistant to proteolytic digestion
Limited proteolysis is a sensitive technique that can be used to examine the tertiary structure of proteins, since tightly folded domains are more resistant to proteolysis. We therefore chose this approach to identify structural domains within xNAP1. In separate experiments, xNAP1 was digested with the proteases trypsin or α-chymotrypsin. Samples were taken at various time intervals over a 2 h period. Trypsin digestion (Figure 1a) produces a prominent fragment that has an apparent molecular mass of ~50 kDa (T1). Digestion with α-chymotrypsin (Figure 1b) also reveals a prominent fragment (C1) of ~50 kDa; in both the cases the band corresponding to the intact protein decreases in intensity. Digestion with trypsin when extended over 24 h resulted in the appearance of a major 35 kDa (T2) fragment (Figure 1c). In contrast, after a 24 h digestion of xNAP1 with α-chymotrypsin the C1 fragment is still the predominant band, although two faint additional bands, ~35–37 kDa, in size are visible. These faint bands are labelled C2 (i) and (ii) (Figure 1c). After 24 h of digestion the samples were passed down a nickel Hi-trap column (Amersham), and both the T2 and C1 fragments were found in the flow through. Since the divalent ion chelating His-tag is at the N-terminus of the purified recombinant xNAP1, and neither fragment is capable of chelating nickel, the T2 and C1 fragments must lack the N-terminus.

Proteolytic fragments C1 and T2 map to overlapping regions of the xNAP1 sequence
We next determined the molecular mass of the xNAP1 putative structural domains T2 and C1 by mass spectroscopy. Samples were digested with the respective enzymes under limiting conditions and sent lyophilized for ESI mass spectrometry. The mass spectrometry results of the xNAP1 sample digested with α-chymotrypsin showed that the largest fragment observed is 39 500 Da in size, while mass spectroscopy of the trypsin digested sample detected three major fragments between 27 826 and 28 970 Da. Since xNAP1 runs anomalously on SDS–PAGE, we used 2D IEF to compare the pI of both the T2 and C1 proteolytic fragments with that of the full-length protein. T2 and C1 were found to have a pI of ~4.8 and ~5.2, respectively, comparable with the full-length xNAP1 pI of ~5.2, and therefore both T2 and C1 can be expected also to run anomalously.

The T2 domain forms hexadecameric complexes in vitro
To facilitate analysis of the putative T2 domain the DNA sequence encoding the T2 fragment was subcloned into the pET-28b plasmid vector, allowing bacterial expression of the domain as a histidine tagged fusion. The recombinant T2 protein was expressed and purified before being assayed by analytical ultracentrifugation and dynamic light scattering (DLS). DLS shows the presence of any polydispersity in the sample and gives the hydrodynamic radius ($R_h$) of the components. A major component (>90%) was observed with a hydrodynamic radius of ~9 nm (Figure 3), indicating that the T2 domain forms discrete multimers. It is not possible to calculate a true molecular weight from the $R_h$ except for spherical particles, as significant asymmetry and/or unstructured tails lead to an overestimate of the $M_r$. In addition, the histidine tag, being unstructured and extended, will lead to a further increase in $R_h$ over and above that owing to asymmetry, and hence would further overestimate the molecular weight.

We therefore performed sedimentation equilibrium on the recombinant T2 domain in order to determine more precisely the size of the protein complexes. The concentration distribution at equilibrium depends only on molecular mass, and is independent of the shape of the molecule. Figure 4 shows the equilibrium distribution of the T2 sample at 0.14 mg ml⁻¹. When fitted to a single species model, the average molecular mass of the sample was 430 kDa (± 60 kDa). The T2 fragment has a molecular mass of 28 560 Da but the recombinant form used in this experiment
also includes a 2470 Da his-tag sequence, giving a total molecular weight of 31 030 Da. Thus AUC suggests that the T2 domain forms oligomeric complexes of 12–16 subunits, indicating a predominance of hexadecamers. The data could also be fitted to an octamer–hexadecamer equilibrium model, and $K_d$ values in the range 0.3–2.0 mM gave models that described the data equally well.

Expression of full-length xNAP1, C1 or T2 domains in *Xenopus laevis* embryos leads to identical phenotypic defects

NAP1 has a role in chromatin fluidity that could affect transcription and hence embryonic development. Several studies have shown that NAP1 has an important role in development. Inactivation of NAP1 in *Drosophila* is embryonic lethal (21) and in Xenopus, has also been shown to have a role in tissue specific gene regulation (9,10). xNAP1 has a number of conserved regions, which may be important in these functions, but several of these are missing in the structural domain T2. In particular, the T2 domain lacks the C-terminal acidic regions and hence the PEST sequence. We, therefore, tested how these truncations affected the *in vivo* activity of xNAP1.

To test the *in vivo* activity of the structural domains C1 and T2 we used gain of function analysis by expressing these domains in *Xenopus* embryos. Injection of *in vitro* transcribed mRNA encoding the protein of interest into *Xenopus* is a well-established developmental investigative tool, since over-expression of the target protein can lead to clues to its function *in vivo*. We subcloned the DNA encoding the T2 fragment into the pBUT-2 plasmid, which provides the 5'-UTR (5'-untranslated region) and 3'-UTRs of the globin gene that both stabilizes the mRNA post-injection and maximizes its translation, therefore increasing the levels of encoded protein. We also subcloned two extra constructs (Figure 5a), one based on the proteolytic fragment T2 but

![Figure 1](image-url)
without the N-terminal half of the bi-partite NLS and another
based on the full-length xNAP1 lacking only the PEST
sequence. These proteins were exogenously expressed in
Xenopus
embryos and assayed for any phenotypic changes
thereby induced.

The mRNA coding for either xNAP1 or its domains was
injected into 1-cell embryos, and these were cultured until
stage 36 when phenotypes were observed. Similar phenotypes
were observed in stage 36 embryos regardless of whether
xNAP1 or truncated xNAP1 mRNA was injected. The
affected embryos showed shortening of the embryo along
the anterior/posterior axis, microcephaly and reduced mobil-
ity (Figure 5b). The proportion of embryos that displayed
these effects with 100 pg of injected mRNA varied between
27 and 55% depending on the expressed protein (Table 1).
Western blotting showed equal levels of expression of the
proteins (data not shown). Uninjected and β-gal mRNA injec-
ted control embryos were unaffected.

In previous experiments, we have shown that antisense
morpholino-injected NAP knockout embryos are also par-
tially paralysed (10) and, at high morpholino concentrations,
have truncated axes (A. Abu-daya and M. J. Guille, unpub-
lished data), correlating with the phenotypes observed here.
The fact that gene knockout and over-expression experiments
lead to similar phenotypes suggests a ‘squelching’ mechan-
ism, as discussed below.

**DISCUSSION**

Here, we report the identification of a structural domain of
xNAP1 that retains a functional activity. Digestion of full-
length xNAP1 with trypsin or α-chymotrypsin gives rise to
two fragments (T1 and C1 respectively). Increased enzyme
concentration or longer digestion times with trypsin gener-
ated a smaller fragment with an apparent molecular weight
of 35 kDa (T2) as assayed by SDS–PAGE, whereas the
fragment arising from α-chymotrypsin digestion is largely
unaltered by longer digestion times. Therefore, the T2 frag-
ment is likely to represent the smallest stable proteolytic
domain of xNAP1, and it is located almost entirely within
the larger C1 fragment.

N-terminal sequencing of T2 and C1 successfully defined
the N-termini of the trypsin fragment T2 (red box) and the
N-terminus of chymotrypsin fragments C1 (green box) are 13 amino acids
apart. The predicted C-terminus of T2 and C1 are also shown (red and green,
respectively). Acidic regions are underlined and the bipartite nuclear
localization signal is shown in blue. Preceding the wild-type start methionine
(numbered 1) is the His-tag sequence present in the recombinant T2 protein.
SFFNFF and the PEST sequences, both of which have been proposed to have functional roles. Both T2 and C1 fragments contain the NES and the full bi-partite NLS of the complete protein, as well as the conserved KGIPEFWLT sequence.

Full-length NAP1 monomers readily multimerize into complexes varying in size from dimers to octamers and hexadecamers, in a concentration-dependent manner (16). The trypsin fragment T2 retains the ability to multimerize as assayed by dynamic light scattering and sedimentation equilibrium, forming predominantly hexadecameric complexes. It has been proposed that the NAP1 octamers form annular discs, with two discs stacking face to face to form hexadecamers (16). If the negatively charged acidic regions in the wild-type protein were near the octamer–octamer interface, their absence in the T2 domain could favour the hexadecamer by reducing charge repulsion between octamers.

Since these experiments were completed, the first crystal structure of NAP1 (from yeast) has become available (22). The protein crystallizes as a dimer, the dimer interface being maintained by two α-helices in the N-terminal domain of the protein. The remainder of the protein is formed from a core of two further helices (α3 and α4) and six β strands, plus a C-terminal domain consisting of three helices (α6–α8). If the structure of yeast and Xenopus NAP1s are similar, as seems likely from their conserved sequences, then we can locate the proteolytic sites for xNAP1 with reference to the structure of yNAP1 when the sequences are aligned.

The N-terminus of the chymotryptic (C1) fragment, Ala51 in xNAP1, corresponds to the start of the first α helix (Q38 in yNAP1), and thus to the first structural domain. The N-terminus of the T2 tryptic fragment (Gln38) is 13 amino acid residues away from this in the unstructured region, but is the nearest basic amino acid to the C1 site. The C-terminus of the T2 fragment (Gly282) corresponds to Lys298 in yNAP1, and lies within the loop between β5 and β6, which is disordered in the crystal structure and hence readily susceptible to proteolysis. Thus, it is clear that the T2 domain of xNAP1 is lacking β6 plus the three C-terminal helices, α6, α7 and α8. Our data suggest that these structural elements are not required for the formation of octamers and hexadecamers.

Considerable evidence now suggests that NAP1 proteins have a role in developmental regulation by controlling transcriptional activity, most likely at the level of chromatin assembly (10,23). We tested the ability of the putative domains C1 (51–392) and T2 (38–282) to affect development relative to the full-length xNAP1 by exogenous expression of these constructs. In addition to the full-length xNAP1 and the putative domains mapped by partial proteolysis, two other constructs were created for expression in Xenopus embryos. These consisted of the T2 fragment minus the C-terminal half of the bi-partite NLS (38–260), and a construct lacking much of the C-terminus up to and including the PEST sequence (1–336).

Table 1. Percentage of embryos effected after injection of 400 pg mRNA from various xNAP1 constructs

| Construct  | 1–392 (full-length) | 1–336 | 51–392 (C1) | 38–282 (T2) | 38–260 |
|------------|---------------------|-------|-------------|-------------|-------|
| Number of embryos injected | 180 | 180 | 180 | 140 | 140 |
| Percent embryos effected | 55 | 53 | 30 | 28 | 27 |

Figure 5. Injection of any of the xNAP1 constructs leads to axial defects. (a) Injected constructs are shown with conserved regions indicated. Basic regions marked by a black box, the PEST sequence is shown in green embedded in the C-terminal basic region. The NLS is marked in red and the SFFNFF shown in purple. (b) One-cell embryos were injected with 100 pg of either full-length or truncated versions of xNAP1. Un-injected embryos from the same fertilization were cultured in parallel as controls. Embryos were grown in modified bath serum at 18°C until stage 36, fixed in MEMFA (0.5 M MOPS, pH 7.4, 0.5 M EGTA, 0.5 M MgSO4 and 37% formaldehyde) and stored at –20°C in methanol before photographing. All forms of injected xNAP1 gave rise to a shorter A/P axis (lack of head and tail are arrowed) than the un-injected control embryos shown in (c).
concentration-dependent shift in equilibrium towards formation of hexadecameric NAP1 complexes that have previously been shown to be unable to bind histones (16). We have shown that the T2 domain retains the ability to multimerize in vitro. Indeed, the predominance of the hexadecameric form of truncated NAP1 suggests that this complex may be favoured by removal of the acidic tails. Presumably, truncated NAP1 can form mixed hexadecameric complexes with wild-type NAP1, thus transferring endogenous NAP1 into inactive complexes.

Our in vitro studies show that the ability to multimerize resides in the central region of the full-length NAP1 protein, and removal of the acidic N- and C-terminal regions may indeed favour formation of larger multimers. In vivo injection of a range of truncated xNAP1 constructs into *Xenopus* embryos leads to axial defects. The ability to produce this phenotype resides primarily in the central 51–260 amino acid sequence and may reflect the ability of this region to multimerize into a non-histone binding hexadecamer.

ACKNOWLEDGEMENTS

The authors thank Dr James Taylor and Dr Anastasia Callaghan for advice and assistance with the analysis of AUC data. The authors are grateful to BBSRC for a graduate student bursary. Funding to pay the Open Access publication charges for this article was provided by the University of Portsmouth.

Conflict of interest statement. None declared.

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