Structures of the autoproteolytic domain from the Saccharomyces cerevisiae nuclear pore complex component, Nup145

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Key words: nuclear pore complex; Nup145; Nup145N; structural genomics; autoproteolysis.

INTRODUCTION

Nuclear pore complexes (NPCs) are large, octagonally symmetric dynamic macromolecular assemblies responsible for exchange of proteins and RNAs between the nucleus and cytoplasm. NPCs are made up of at least 456 polypeptides from ~30 distinct nucleoporins.1,2 Several of these components, sharing similar structural motifs, form stable subcomplexes that form a coaxial structure containing two outer rings (the nuclear and cytoplasmic rings), two inner rings, and a membrane ring.3,4 The yeast (Saccharomyces cerevisiae) Nup145 and its human counterpart are unique among the nucleoporins, in that they undergo autoproteolysis to generate functionally distinct proteins.5–9 The human counterpart of Nup145 is expressed as two alternatively spliced mRNA transcripts. The larger 190 kDa precursor undergoes post-translational autoproteolysis at the Phe863-Ser864 peptide bond yielding the 92 kDa Nup98 and the 96 kDa Nup96.8,9 The smaller 98 kDa precursor is also autoproteolysed at an analogous site giving 92 kDa Nup98-N and a 6 kDa C-terminal fragment, which may form a noncovalent complex.10,11

The yeast Nup145 precursor [Fig. 1(A)] contains twelve repeats of a “GLFG” peptide motif (FG repeats) at its N-terminus, an internal autoproteolytic domain (a


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Received 4 January 2010; Revised 28 January 2010; Accepted 9 February 2010
Published online 1 March 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/prot.22707
region of high conservation with the homologous yeast nucleoporins Nup110 and Nup116, neither of which undergo autoproteolysis), followed by the C-terminal domain. Various forms of the FG repeats are present in nearly half of all nucleoporins; they form intrinsically disordered regions implicated in gating mechanisms that control passage of macromolecules through NPCs. Nup145 undergoes autoproteolysis at the Phe605-Ser606 peptide bond to generate two functionally distinct proteins, Nup145N and Nup145C. Subsequently, Nup145C associates with six other proteins to form the heptameric Y-complex, a component of the outer rings of the NPC. Nup145, on the other hand, can shuttle between the NPC and the nuclear interior. It has been suggested that Nup145N, by analogy with Nup98, carries RNA between the nucleus and the NPC. Nup145 belongs to a highly conserved family of homologs found throughout eukaryota. Curiously, the Phe-Ser autoproteolytic site is not always conserved, resulting in the absence of autoproteolysis for some of these closely related proteins.

Our structural understanding of NPC function has benefited recently from X-ray crystal structures of segments of individual proteins. As a part of ongoing efforts at the New York SGX Research Center for Structural Genomics (www.nysgxc.org) to determine crystal structures of distinct components of the yeast NPC, we report herein structures of Nup145N residues 443–605 [Nup145N(443–605)] from the larger autoproteolytic segment. The monomeric architecture of Nup145N(443–605) is similar to that of the autoproteolytic domain of human Nup98 and the homologous domain of Nup116. In contrast, Nup145N(443–605) and Nup98-N show different modes of association in the crystalline state. Results of Small Angle X-ray Scattering (SAXS) in solution are consistent with the head-to-tail dimer of Nup145N(443–605), observed in two different crystal forms.

**EXPERIMENTAL PROCEDURES**

**Cloning, expression, and purification of Nup145N(443–605)**

The gene encoding Nup145N from *Saccharomyces cerevisiae* was cloned from the genomic DNA of the yeast strain Sc2601D-5 (American Type Culture Collection). The desired truncation (encoding residues 443–605) was PCR amplified using AATAAAACAGAGCGCGAAATAC and CAAATGATTGACTTGGAAAATCC as forward and reverse primers, respectively. The purified PCR product was subsequently TOPO® (Invitrogen) cloned into pSGX3, a derivative of pET26b (+), giving rise to a protein with a noncleavable C-terminal hexa-histidine tag. The resulting plasmid was transformed into BL21(DE3)-Condon + RIL (Invitrogen) cells for overex-
pression. Production of Se-Met protein was carried out in 1L of HY media at 22°C containing 50 μg/mL of kanamycin and 35 μg/mL of chloramphenicol. Protein expression was induced by addition of 0.4 mM IPTG. Cells were harvested after 21 h by centrifugation at 4°C.

For purification, the E. coli cell pellet was resuspended in 30 mL of cold buffer containing 20 mM Tris HCl pH 8.0, 500 mM NaCl, 25 mM imidazole, and 0.1% (v/v) Tween 20 and cells were lysed via sonication. Debris was removed by centrifugation at 4°C. The decanted supernatant was applied to a 5 mL HisTrap HP column (GE Health Care) charged with nickel and pre-equilibrated with 20 mM Tris HCl (pH 8.0), 500 mM NaCl, 10% (v/v) glycerol, and 25 mM imidazole. The sample was washed with 5 column volumes (CV) of 20 mM Tris HCl (pH 8.0), 500 mM NaCl, 10% (v/v) glycerol, and 40 mM imidazole, and subsequently eluted with 2 CV of same buffer with an imidazole concentration of 250 mM. Eluted protein was passed over a 120 mL Superdex 200 size exclusion column equilibrated with 10 mM HEPES pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, and 5 mM DTT (protein storage buffer). SDS-PAGE analysis showed greater than 95% purity and protein fractions corresponding to the symmetric portion of the size exclusion chromatography profile were pooled for concentration with AMICON spin filters. Concentrated protein aliquots were frozen in liquid nitrogen and stored at −80°C.

### Crystallization, data collection, and structure determination

Nup145N(443–605) was subjected to crystallization screening with the Classics, Classics II, and PEGs kits (Qiagen) using a Phoenix Liquid Handling System (Art Robins) via sitting drop vapor diffusion at 21°C (11.8 mg/ml; 0.3 μL protein + 0.3 μL reservoir solution). Tetragonal crystals grew in 100 mM Hepes pH 7.5, 25% PEG 3350, and 200 mM sodium chloride (Classics II suite; condition number 72) and were cryoprotected by addition of ethylene glycol [final concentration ~20% (v/v)] and flash frozen by immersion in liquid nitrogen. Hexagonal crystals were obtained in 1600 mM tri-sodium citrate dehydrate (Classics suite; condition number 45) and cryoprotected by addition of ethylene glycol [final concentration ~20% (v/v)]. Diffraction data were recorded using the LRL-CAT 31-ID beamline at the Advanced Photon Source (APS) and processed with MOSFLM[20] and SCALA (CCP4).[21] Structures were determined independently following similar procedures. All eight expected Se atoms were located with SHELXD[22] and phases were calculated using SHELXE as implemented in HKL2MAP.[23] Initial model building was carried out with ARP/wARP[24] followed by manual rebuilding with COOT.[25] Atomic models of Nup145N(443–605) refined to convergence using REFMAC5[26] show excellent stereochemistry.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Crystallographic Statistics</th>
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<td><strong>Data collection</strong></td>
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<td>Rmerge (%)</td>
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<tr>
<td>Multiplicity</td>
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<tr>
<td>I/σ(I)</td>
<td>22.9 (8.8)</td>
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*Values in parenthesis correspond to the highest-resolution shell

(Table I). Structural analyses were carried out using COOT and CCP4 and illustrations were prepared using PyMol.[27]

### Size-exclusion chromatography with multiangle laser light scattering (SEC-MALLS)

SEC-MALLS was used to determine molecular weight in solution with an analytical gel filtration column (Wyatt Technologies-055S; 7.8 mm × 300 mm; pore size = 150Å) connected to a binary LC pumping system (Agilent 1100), temperature controlled auto-sampler, a UV-visible detector, DAWN®-TREOS™ three-angle static light scattering detector (Wyatt Technology), and an Optilab® REX differential refractometer equipped with a Peltier temperature-regulated flow cell, maintained at 25°C (Wyatt Technology, USA). The LC system and light scattering detector (LSD) was controlled by ChemStation (Agilent Technologies, USA) and Astra V software (Wyatt Technology), respectively. The column and LSD was pre-equilibrated with the protein storage buffer. The experiment was performed at room temperature at a flow rate of 0.5 mL/min for 40 min. Approximately 100 μg of Nup145N(443–605), at concentrations of 11.8, 22.7, and 46.0 mg/mL, were injected and monitored at a wavelength of 280 nm. The molecular mass from light scattering data was calculated using a specific refractive index increment (dn/dc) value of 0.183 mL/g that is universal for proteins.
Small angle x-ray scattering (SAXS)

SAXS measurements of Nup145N(443–605) were carried out with Beamline 4–2 at the Stanford Synchrotron Radiation Lightsource (SSRL) and the SIBYLS Beamline 12.3.1 at the Advanced Light Source (ALS) yielding essentially identical results. At SSRL, we used an automatic sample delivery system equipped with a 1.5 mm diameter thin-wall quartz capillary within which a sample aliquot oscillated in the X-ray beam to minimize radiation damage. It was placed at 1.7 m from a Rayonix225 (MAR-USA) CCD detector with a binned pixel size of 293 μm × 293 μm. Ten 3-sec exposures were made for each protein sample maintained at 15°C. Each of the 10 diffraction images were scaled using the transmitted beam intensity, azimuthally integrated, and averaged to obtain fully processed data in the form of intensity versus \( q \cdot q = 4\pi\sin(\theta)/\lambda \), \( \theta = \) one-half of the scattering angle; \( \lambda = \) X-ray wavelength. The buffer profile was obtained in the same manner and subtracted from a protein profile. SAXS profiles of Nup145N(443–605) were recorded at protein concentrations of 0.5, 1.0, 2.0, 5.0, and 11.8 mg/mL in protein storage buffer. Moreover, mild concentration dependence was removed by extrapolating to the zero concentration. We merged the average of the lower scattering angle parts \( q < 0.15\AA^{-1} \) of the lower concentration profiles \( (0.5–2.0 \text{ mg/mL}) \) and the average of the higher scattering angle parts \( q > 0.12\AA^{-1} \) of the higher concentration \( (5.0–11.8 \text{ mg/mL}) \) profiles, to obtain the final, merged experimental SAXS profile. The shape of Nup145N(443–605) was calculated from this merged experimental SAXS profile by running DAMMIF30 10 times individually, followed by superposition and averaging with DAMAVER.31 The oligomeric state of Nup145N(443–605) was calculated from the merged experimental SAXS profile using OLIGOMER.32 The merged experimental SAXS profile was also compared with profiles calculated for the monomer (Chain B) and the crystallographic dimer of Nup145N(443–605) with IMP33 and CRYSOLO.34

RESULTS AND DISCUSSION

Structure of Nup145N(443–605)

The crystal structure of Nup145N(443–605) has been determined at 1.82Å resolution [Fig. 1(B), Table I]. The tetragonal crystal form \((P_{4}2_{1}2_{1})\) has two molecules in the asymmetric unit. The B chain could be traced continuously from Phe459 to Phe605. In the A chain, residues 557–560 appear disordered. Otherwise, the A and B chains are very similar with a root mean square deviation (r.m.s.d.) of 0.4Å for 144 Ca atomic pairs. The structure of Nup145N(443–605) contains two antiparallel β-sheets capped by α-helices [Fig. 1(B)], as predicted based on its sequence similarity with Nup98.35 A six stranded β-sheet is formed by β1-β2-β3-β6-β8-β7 and a two stranded β-sheet is formed by β4-β5. Helices α1, α2, and α3 form a cap near the N-terminus and α4 caps the six stranded β-sheet near the C-terminus. Nup145N(443–605) possesses three long loops: L1 (between β3-β4), L2 (between β5-β6), and L3 (between β6-α4). Autoproteolyis of Nup145 occurs at the Phe605-Ser606 peptide bond.6,7 Autoproteolytic events result from N—O or N—S acyl shift catalyzed by Ser/Thr or Cys side chains.36 In our structure, the Phe605-Ser606 bond is replaced by Phe605-Glu606 (Glu606 is a cloning artifact derived from the expression plasmid) and, thus, the electron density between Phe605 and Glu606 is continuous in both A and B chains.

Nup145N(443–605) forms a tight dimer in the crystal [Fig. 1(C)]. An identical dimer of Nup145N(443–605) was observed in our hexagonal \((P_{6}{_1})\) crystal form, the structure of which was independently determined with Se-Met experimental phases (Table I and [Fig. 1(D)])]. Monomers of the tetragonal and hexagonal crystal forms superimposed well with r.m.s.d. = 0.37Å for 145 Ca atom pairs. The dimers are also very similar (r.m.s.d. = 0.45 Å for 288 Ca atom pairs, [Fig. 1(D)])]. The total buried surface area on complex formation estimated with PISA37 is ~1900Å². This head-to-tail dimer is stabilized in part by extensive interactions between the β6-strand of one protomer and the α4 helix of its dimer counterpart. In addition, all strands of the six stranded β-sheet contribute to the dimer interface [Fig. 1(C)]. The geometry and amino acid composition of protein–protein interfaces can be helpful in distinguishing specific from nonspecific complexes.38 NOXclass39 analysis predicted Nup145N(443–605) to be a “biological” dimer with 99% confidence and further classified it as an obligate dimer with 71% confidence. The gap volume index (an indicator of shape complementarity within a protein–protein interface) for the Nup145N(443–605) dimer in the asymmetric unit is 3.26Å, which is less than the average value of 4.0Å observed for known obligate dimers.39

These findings prompted us to investigate the oligomerization state of Nup145N(443–605) in solution using SEC-MALLS and SAXS. Although Nup145N(443–605) eluted as an apparent monomer on the gel filtration column [Fig. 2(A)], light scattering measurements revealed a mixture of monomer and dimer species (data not shown). SAXS analyses showed: (i) the merged experimental SAXS profile matches the SAXS profile calculated for the crystallographic dimer of Nup145N(443–605) [Fig. 2(B)]; (ii) a measured radius of gyration \( (R_g) \) of 22.1 ± 0.01Å, obtained with AutoRg40 is close to the value of 21.5Å calculated from the structure of crystallographic dimer (for reference, the calculated \( R_g \) for the Chain B monomer is 18.0Å); (iii) the “ab initio shape” reconstructed from the merged experimental SAXS profile with DAMMIF30 and GASBOR41 matches the overall
shape of the Nup145N(443–605) head-to-tail dimer observed in the crystal [Fig. 2(C)]; and, (iv) based on the merged experimental SAXS profile, OLIGOMER\(^3\) estimates a dimer fraction of \(\sim98\%\). Thus, our SAXS analyses of the solution behavior of Nup145N(443–605) are consistent with the head-to-tail dimer observed in both crystal forms.

Comparison of Nup145N(443–605) with yeast Nup116 and human Nup98

The yeast nucleoporins Nup100 and Nup116 arose by duplication and divergence events from a gene ancestral to Nup145, with both gene products lacking the regions homologous to Nup145C.\(^5\). The solution NMR structure of yeast Nup116 (PDB Code 2AIV)\(^4\) revealed a similar overall structure to that of Nup145N(443–605) [Fig. 3(A); r.m.s.d. = 2.5Å for 117 C\(_\alpha\) atom pairs with 32% sequence identity], although it does not appear to be a dimer in solution. The Nup116 structure revealed a remarkable conformational flexibility of the loop regions and the \(\alpha\)-helix (along the helical axis).\(^4\) Differences between the two structures include the conformation of loop L3, which projects toward the six stranded \(\beta\)-sheet in Nup145N(443–605). Loop L3 of Nup116 adopts a conformation that is intermediate between those seen in Nup145N and human Nup98 (see below). Similarly, loop L2 of Nup145N projects toward the core of the protein as compared to L2 of Nup116. These conformational differences in the loops of Nup145N and Nup116 may explain, at least in part, their differing positions within the NPC. Nup116 is found mainly on the cytoplasmic face of the NPC, whereas Nup145N is found mainly on the nuclear face.\(^1\),\(^3\)

Structures for the autoproteolytic domain of human Nup98 have been determined both as a Nup98-N:C-terminal fragment complex (PDB Code 2Q5Y) and as a noncleavable Ser864Ala mutant (PDB Code 2Q5X).\(^7\),\(^8\) The structures of the Nup98-N and Nup145N(443–605) monomers are similar [Fig. 3(B); r.m.s.d. = 2.1Å for 128 C\(_\alpha\) atom pairs with 39% sequence identity]. Noticeable differences between the two structures include some longer \(\beta\)-strands in the case of Nup98-N and conformations of the loops connecting the secondary structural elements. In particular, the L3 loop between \(\beta\)-strand and \(\alpha\)-helix [residues 554–566 of Nup145N(443–605)] projects toward the protein core. Conversely, structurally equivalent residues of this loop in Nup98 project away from the protein core. The structure of the Nup98-N:C-terminal fragment complex contains two copies of the complex in the asymmetric unit. The surface area buried by association of the A and C chains of Nup98-N:C-terminal fragment complex [Fig. 2(C)] is 1147Å\(^2\) [compared with \(\sim1900\)Å\(^2\) buried within the Nup145N(443–605) dimer]. The two molecules of Nup98-N in the Nup98-N:C-terminal fragment complex associate quite differently from that seen for the Nup145N(443–605) head-to-tail dimer [Fig. 1(C)]. The \(\alpha\)-helix is involved in mediating extensive interactions within the dimeric interface of Nup145N(443–605). However, the \(\alpha\)-helix of the Nup98-N:C-terminal fragment complex, which is involved in binding to the tail peptide, contributes much less to the protein–protein interaction [Fig. 2(C)]. NOXclass\(^3\) analysis suggests that the Nup98-N dimer observed in the Nup98-N:C-terminal

![Figure 2](image-url)

**Figure 2**

A: Analytical gel filtration profile of Nup145N(443–605) at concentrations of 11.8 (black), 22.7 (blue), and 46.0 mg/ml (red) with elution times 21.5, 21.3, and 21.1 min, respectively. B: Comparison of the merged experimental SAXS profile (red) of Nup145N(443–605) with SAXS profiles computed by IM pobl930 for the crystallographic dimer (blue) and monomer (green) structures (inset shows the SAXS profiles across the entire measured resolution range). C: The shape of Nup145N(443–605) represented as mesh derived from the experimental SAXS profile.
The observed Nup145N dimer to NPC biogenesis and function is not yet clear, although SAXS analyses of Nup145N (443-605), presented herein, provide indirect support for dimer formation within the NPC and/or in the nucleus of yeast. Further biophysical and cell biological characterization of Nup145N is required to determine whether or not Nup145N and/or Nup98-N function as a dimer in vivo.

**Protein data bank codes**

Atomic coordinates and structure factors of the tetragonal and hexagonal crystal forms of Nup145N (443–605) were deposited to the PDB on 26 October 2009 with accession codes 1KEP and 1KES, respectively. The NYSGXRC target identifier for yeast Nup145 in TargetDB (http://targetdb.pdb.org) is “NYSGXRC-15145a”. Expression clone sequences and selected interim experimental results are available in PepcDB (http://pepcdb.pdb.org/).

**ACKNOWLEDGMENTS**

The authors thank the members of the Rout and Sali laboratories for their help and advice and also grateful to Drs. John Tainer and Michal Hammel for help in using the SIBYLS beamline 12.3.1 at ALS. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Basic Energy Sciences. Access to the LRL-CAT beam line facilities at Sector 31 of the APS was provided by Eli Lilly, which operates the facility.

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