FEBS Open Bio 5 (2015) 508–514

journal homepage: www.elsevier.com/locate/febsopenbio

Uncleavable Nup98–Nup96 is functional in the fission yeast *Schizosaccharomyces pombe*

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ARTICLE INFO

Article history:
Received 15 April 2015
Revised 18 May 2015
Accepted 7 June 2015

Keywords:
Nucleoporins
Nuclear pore complex
FG nup
Nup107-160 complex
Alternative splicing
Fission yeast

ABSTRACT

Essential nucleoporins Nup98 and Nup96 are coded by a single open reading frame, and produced by autopeptidase cleavage. The autocleavage site of Nup98–Nup96 is highly conserved in a wide range of organisms. To understand the importance of autocleavage, we examined a mutant that produces the Nup98–Nup96 joint molecule as a sole protein product of the *nup189*$^{+}$ gene in the fission yeast *Schizosaccharomyces pombe*. Cells expressing only the joint molecule were found to be viable. This result indicates that autocleavage of Nup98–Nup96 is dispensable for cell growth, at least under normal culture conditions in *S. pombe*.

1. Introduction

In eukaryotic cells, the nucleus is spatially and functionally separated from the cytoplasm by the nuclear envelope (NE). Macromolecule transport across the NE takes place through the nuclear pore embedded in the NE. The nuclear pore is formed by a gigantic protein complex named the nuclear pore complex (NPC), which is estimated to be 125 MDa in vertebrate cells [1] and 50 MDa in yeast cells [2,3]. The NPC is an eightfold symmetrical structure composed of multi-copies of approximately 30 different protein components called nucleoporins.

Among the NPC components, Nup98 and Nup96 are essential nucleoporins that are highly conserved in a wide range of eukaryotic organisms [[4–15]; reviewed in [16]]. Human nucleoporins Nup98 and Nup96 are encoded in a single open reading frame, and Nup98–Nup96 precursor protein is separated by autopeptidase cleavage to produce two proteins [6,17]. A tripeptide sequence is necessary as a target for autocleavage and is evolutionally conserved, suggesting that autocleavage production of Nup98 and Nup96 from a single polypeptide may have a significant role for their functions. In addition, Nup98 is translated from two types of transcripts generated by alternative splicing in humans: one is a large mRNA encoding a large Nup98–Nup96 protein, and another is a short mRNA encoding Nup98 but not Nup96 [6]. These manners of expression of Nup98 and Nup96 are also conserved among many eukaryotes. In human cells, a cleavage-deficient mutant Nup98-Nup96 protein is not localized at the NPC, indicating that autocleavage is necessary for accurate localization of Nup98 and Nup96 [6,18]. In budding yeast *Saccharomyces cerevisiae*, on the other hand, a cleavage-deficient mutant protein is localized at the NPC, indicating dispensability of autocleavage [4]. Thus the necessity of Nup98–Nup96 autocleavage has been contradictory in human and budding yeast cells.

In *S. pombe*, Nup98 and Nup96 are coded by a single gene, *nup189*$^{+}$. Deletion of the entire *nup189*$^{+}$ ORF, which therefore means abolishment of both Nup98 and Nup96, is lethal [7,10], and expression of either Nup98 or Nup96 cannot complement lethality [3]. In this paper, we demonstrate that Nup98 and Nup96, either as separate protein molecules or as a single joint protein molecule, can functionally compensate for the endogenous *nup189*$^{+}$ gene product.

Abbreviations: GLFG repeat, Gly-Leu-Phe-Gly repeat; NE, nuclear envelope; NPC, nuclear pore complex

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http://dx.doi.org/10.1016/j.fob.2015.06.004
2211-5463/

2. Materials and methods

2.1. Media and culture condition

Yeast extract with supplements (YES) and Edinburgh minimal medium 2 (EMM2) were used for growth media [19]. Cells were grown at 26 °C or 30 °C.

2.2. Preparation of total RNA

Total RNA was prepared from standard strains (972 and 968) as follows: Cells grown to log phase were harvested, and cell pellet was washed once with ice-cold water and suspended in TES buffer (10 mM Tris–Cl pH7.5, 10 mM EDTA, 0.5% SDS). The cell suspension was mixed with the same volume of acidic phenol–chloroform, and incubated at 65 °C for 1 h. After heating, the sample was placed on ice for 1 min, vortexed, and then centrifuged at 14,000 rpm for 15 min at 4 °C. The aqueous phase was isolated using Phase-Lock Gel Heavy (5 PRIME, Germany) according to the manufacturer's protocol.

2.3. Detection of splicing products by PCR

To detect spliced and unspliced transcripts, cDNA was synthesized from total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). The cDNA was subjected to PCR using oligonucleotide primers designed upstream and downstream of the intron: 5′-CGACGCGTATGTCTAGATACGGTTTG CTGG ATGATG-3′ (underlined region is an artificially added sequence) and 5′-CCTCGTCGACTTTTTTTGCATTATCGTCATAGTCACCTC-3′. The predicted length was 333 bp or 268 bp for DNA amplified from unspliced or spliced product, respectively.

2.4. Construction of mutant genes

To make a cleavage-deficient mutant gene and a splicing-deficient mutant gene, oligonucleotide-directed mutagenesis was applied using Site-Directed Mutagenesis Kit (Stratagene, USA) according to a manufacturer's protocol. *nup189*$^{+}$ ORF was amplified from genomic DNA by PCR and cloned to a conventional plasmid DNA. To make the cleavage-deficient mutant gene, oligonucleotide primers, 5′-GGTTCAGCATTTCGCTAGATACGGTTTG C-3′ and 5′-GCAAACCGTATCTAGCGAAATGCTGAACC-3′, were used. To make the splicing-deficient mutant gene, mutations were introduced at predicted splicing donor and acceptor sites. For the splicing donor mutation, oligonucleotide primers 5′-CAGGTAATTT A AAAAAATACGATCAACCAAACTTG-3′ and 5′-CAAGTTTGGTTGATC GTATTTTTTTAAATTACCTG-3′ were used. For the splicing acceptor mutation, oligonucleotide primers 5′-GTTACTCACCATACACCTGGT GCATTTCCCAAC-3′ and 5′-GTTGGGAAATGCACCAGGTGTATGGTGA GTAAC-3′ were used. For the splicing-deficient mutant, primers were designed to conserve the original amino acid sequence.

2.5. Strains and plasmid constructs

Strains used in this study are listed in Table 2. The construction of Nup98-GFP was described previously [3]. Briefly, we constructed a gene cassette, in which a GFP, a G418 resistance marker gene and the promoter sequence of *nup189*$^{+}$ were fused in tandem. This gene cassette was integrated in between the Nup98-coding region and the Nup96-coding region at the genomic *nup189*$^{+}$ gene locus by a two-step PCR method. To construct plasmids harboring other *nup189*$^{+}$ alleles, mutant genes or control wild type gene were cloned by PCR and inserted to a plasmid harboring GFP or mRFP and either the marker *lys1*$^{+}$ or the aureobasidin A-resistant allele of *aur1*$^{+}$ (Takara, Japan). All plasmid constructs express proteins with their C-termini fused to GFP. A 312 bp of 5′UTR region of *nup189*$^{+}$ was used as promoter. For construction of the mutant strains, endogenous *nup189*$^{+}$ was heterozygously deleted with the *ura4*$^{+}$ cassette in a diploid strain (namely, harboring one endogenous copy of *nup189*$^{+}$), and the diploid cells were transformed with each of plasmids carrying *nup189*$^{+}$ for integration at endogenous *lys1*$^{-}$ or *aur1*$^{+}$ loci. Transformants were selected by lysine prototrophy or aureobasidin A-resistance, and genomic integration was confirmed by PCR. After the transformation, diploid cells were induced to form spores. The spores were dissected and analyzed for auxotrophy. Clones that were positive for both uracil prototrophy and integration of the marker were used for experiments.

2.6. Complementation test

To test the *nup189* gene function for the splicing-deficient mutation or the cleavage-deficient mutation, or both, the mutant gene was introduced into the ectopic gene locus (*lys1*) of a diploid strain harboring a heterozygous deletion for *nup189* with a *ura4*$^{+}$ marker. Tetrad progenies (spores) were dissected from these diploid cells. Viable progenies were tested for uracil and lysine auxotrophy to determine their genotypes.

To test the cleavage-deficient Nup98-tail function, the mutant gene was integrated into the *aur1*$^{+}$ locus, and the gene corresponding to the Nup96-coding region was integrated into the *lys1*$^{-}$ locus of a diploid strain harboring a heterozygous deletion for *nup189* with a *ura4*$^{+}$ marker. Tetrad progenies were dissected from these diploid cells. Viable progenies were tested for uracil and lysine auxotrophy and aureobasidin A-resistance to determine their genotypes. The genotypes of inviable progenies were predicted from the segregation of these marker genes in viable progenies. Haploid progenies bearing the mutant Nup98-tail gene and the Nup96-coding region in the *nup189Δ* background, which are expected to be *lys1*$^{+}$, *ura4*$^{+}$ and aureobasidin A-resistant, were not obtained among viable progenies.

2.7. Western blot analysis

Whole cell extract was prepared as described previously [3], and 20 μg of whole cell extract was subjected to 7.5% SDS–PAGE. Proteins were transferred to a PVDF membrane in 48 mM Tris, 39 mM glycine, 0.025% SDS and 15% methanol by wet transfer. The membrane was blocked in 5% skim milk and incubated with a rabbit polyclonal anti-GFP antibody (final concentration 0.1 μg/mL; Rockland, USA) or a mouse monoclonal anti-Nup98 antibody, 13C2 (final concentration 0.3 μg/mL) [20]. HRP-conjugated goat anti rabbit IgG or HRP-conjugated goat anti mouse IgG (GE Healthcare, USA) was used as a secondary antibody. For a loading control, endogenous actin was detected on the same membranes after stripping using a mouse monoclonal anti-β-actin antibody, ab8224 (final concentration 0.1 μg/mL; Abcam, USA) and HRP-conjugated goat anti-mouse IgG as described above. Protein bands were detected by chemiluminescence using the ChemiDoc MP imaging system (Bio-Rad, USA).

2.8. Microscopy

Living cells were mounted between coverslips [21]. Images were obtained using a DeltaVision microscope system (GE Healthcare) equipped with a CoolSNAP HQ^{2} CCD camera (Photometrics, Tucson, USA) through an oil-immersion objective lens (PlanApoN60 × OSC, NA = 1.4, Olympus, Japan). Z-stack images were obtained at 0.4 μm intervals for 10 Z-steps, and