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Function of Nup98 subtypes and their fusion proteins, Nup98-TopIIβ and Nup98-SETBP1 in nuclear-cytoplasmic transport

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ABSTRACT

Nup98 is a component of the nuclear pore complex. The *nup98*-fusion genes derived by chromosome translocations are involved in hematopoietic malignancies. Here, we investigated the functions of Nup98 isoforms and two unexamined Nup98-fusion proteins, Nup98-TopIIβ and Nup98-SETBP1. We first demonstrated that two Nup98 isoforms are expressed in various mouse tissues and similarly localized in the nucleus and the nuclear envelope. We also showed that Nup98-TopIIβ and Nup98-SETBP1 are localized in the nucleus and partially co-localized with full-length Nup98 and a nuclear export receptor XPO1. We demonstrated that Nup98-TopIIβ and Nup98-SETBP1 negatively regulate the XPO1-mediated protein export. Our results will contribute to the understanding of the molecular mechanism by which the Nup98-fusion proteins induce tumorigenesis.

1. Introduction

In eukaryote, the nucleus and the cytoplasm are separated by the nuclear envelope, and molecules are transported through the nuclear pore complex (NPC) consisting of about 30 different nucleoporins (nups) [1]. Nup98, a nucleoporin, located at the nuclear and cytoplasmic sides of the NPC [2], facilitates protein and RNA export [3–5]. It has been reported that chromosomal translocations occur at the *nup98* gene locus in some hematopoietic malignancies, and the translocated genes produce the Nup98-fusion proteins. About thirty Nup98-fusion proteins identified so far consist of the N-terminus of Nup98 and the C-terminus of partner proteins [6,7]. Some of the Nup98-fusion proteins have been revealed to induce leukemia in mice [8], indicating the *nup98*-fusion genes contribute to oncogenesis. About half of the partner proteins of the Nup98-fusion proteins are involved in transcriptional regulation and these Nup98-fusion proteins have been studied extensively. On the other hand, the other half partner proteins share no common motif and the functions of these Nup98-fusion proteins remain largely elusive.

As such proteins, Nup98-Topoisomerase (Top) IIβ and Nup98-SET binding protein 1 (SETBP1) are found in acute myeloid leukemia [9] and acute T cell lymphoblastic leukemia [10], respectively. TopIIβ, a DNA topoisomerase, interacts with DNA strands and converts DNA topology [11], while SETBP1 is implicated in the regulation of gene expression and protein phosphorylation [12,13]. The functions of these two fusion proteins have never been examined.

In this study, we first characterized the expression and localization pattern of the Nup98 variants. Two *nup98* transcripts that are expected to be generated by alternative splicing have been registered in the database, although the differences in the expression pattern and subcellular localization of the two variants have never been examined so far. In addition, we examined the cellular functions of two uncharacterized Nup98-fusion proteins, Nup98-TopIIβ and Nup98-SETBP1.

Abbreviations: NPC, nuclear pore complex; NES, nuclear export signal; NLS, nuclear localization signal.

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2. Materials and methods

2.1. Cell culture and transfection

HeLa and HEK293T cells were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin. Jurkat and FKH1 cells were maintained in RPMI1640 supplemented with 10% FBS, penicillin, and streptomycin. For transfection assays, GeneJuice (Merck KGaA) was used. LMB (L-6100) was purchased from LC Laboratories.

2.2. Plasmids

To make pCHA-Nup98(s)-TopIIβ and pCHA-Nup98(s)-SETBP1, cDNA fragments encoding the N-terminal region of Nup98(s) (1–514) and the C-terminal regions of TopIIβ and SETBP1 were obtained by RT-PCR and inserted into *Eco*RV-digested pBluescript II sk(+). RT-PCR was performed using total RNA derived from HeLa or HEK293T cells, ReverTra Ace (TOYOBO Co., Ltd), and KOD FX (TOYOBO Co., Ltd) or Phusion DNA polymerase (Finnzymes). The obtained vectors were used for overlap extension PCR to generate *nup98(s)-topIIβ* and *nup98(s)-setbp1* fragments. The PCR products encoding Nup98(s)-TopIIβ and Nup98(s)-SETBP1 were excised by *Bss*HII and ligated with *Mlu*I-digested pCHA. To obtain cDNA fragment encoding Nup98(s), *nup98(s)* fragments derived from pBluescript II-Nup98(s) (1–514) and pGEX-Nup98C [14] were used to perform overlap extension PCR. To obtain cDNA fragment encoding Nup98(l), RT-PCR products of Nup98(l) (1–576) and pGEX-Nup98C were used to perform overlap extension PCR. The PCR product encoding Nup98(s) or Nup98(l) was excised by *Bss*HII and cloned into *Mlu*I-digested pCHA. The cDNA for Nup98(s) (1–469) obtained by PCR was inserted into *Mlu*I-digested pCHA. To express EGFP-tagged Nup98 proteins, Nup98(s) and Nup98(l) cDNAs were inserted into *Xho*I and *Eco*RI-digested pEGFP-C1. Sequences of all fragments obtained by PCR were verified by sequencing analyses. Primers used for vector construction were listed in Supplemental Table 1.

2.3. RNA extraction and RT-qPCR

Total RNAs derived from mouse tissues and human cell lines were extracted using Sepasol-RNA I Super G (NACALAI TESQUE, INC.). Total RNA samples were reverse-transcribed by ReverTra Ace and oligo dT_{20}. For PCR, Paq5000 DNA polymerase (Agilent Technologies) was mixed with reverse-transcribed samples and primers. Sequences of the primers used in this experiment were as follows: 5′-TCATTTGGTACAACCAGTGGCG-3′ and 5′-TACTGGGGCCTGGGGG -3′.

2.4. Immunofluorescence assay

HeLa cells were fixed with 4% paraformaldehyde for 15 min or ice-cold methanol for 10 min. After permeabilization with 0.5% Triton X-100 in PBS for 10 min, the samples were incubated in 1% milk in PBS-T (0.1% Triton X-100 in PBS) for 30 min. Anti-HA (3F10, Sigma, 1:100), anti-Nup214 (ab70497, Abcam, 1:100), anti-p65 (ab7970, Abcam, 1:100), and anti-STAT2 (C-20, Santa Cruz, 1:100) antibodies were diluted with 1% milk in PBS-T and incubated with the samples for 30 min. After wash with PBS-T, the samples were incubated with Alexa Fluor 488 or 568 conjugated secondary antibodies (A11008, A21208, A11011, A11077, Thermo Fisher Scientific Inc., 1:100) for 30 min. The samples were observed by LSM5 Exciter confocal microscope with Plan-Apochromat 63× objective lens (Carl Zeiss Microscopy GmbH). Pictures were processed by ZEN software (Carl Zeiss Microimaging GmbH).

3. Results

3.1. Expression pattern of two Nup98 isoforms in mouse tissues and human cell lines

The Nup98 protein is translated from the *nup98-nup96* transcript or the alternatively spliced *nup98* transcript. On the database, two human nup98 transcripts are registered. The shorter and the longer isoforms (isoforms 2 and 3) encode 920 and 937 amino acids, respectively (Fig. 1A, hereafter referred to as Nup98(s) and Nup98(l)). Nup98(l) shares 920 amino acids with Nup98(s), and the additional 17 amino acids sequence of Nup98(l) is located between exons 10 and 11 of Nup98(s). Two isoforms, Nup98(s) with 967 amino acids and Nup98(l) with 984 amino acids, are also found in mouse. The sequence of 17 additional amino acids in Nup98(l) is identical between human and mouse. In hematopoietic malignancies associated with *nup98*, chromosomal translocations occur between intron 11 and 14 of the *nup98* gene in most cases, and the Nup98-fusion proteins contain the N-terminal half of Nup98 [8]. Although previous studies used either Nup98(s) or Nup98(l), differences in the expression patterns and functions of these two isoforms have never been elucidated. Thus, it is not clear whether two types of fusion proteins that contain either Nup98(s) or Nup98(l) are expressed from the translocated genomes or not. To examine the expression pattern of the two *nup98* isoforms, RT-PCR analyses using various mouse tissues were performed using a primer set to amplify the sequence harboring exons 10 to 12 (Fig. 1A). Two cDNA fragments corresponding to *nup98(l)* and *nup98(s)* with 315 and 264 base pairs, respectively, were expected to be amplified by this primer set. We found that both *nup98* isoforms were ubiquitously expressed in various mouse tissues including hematopoietic tissues (Fig. 1B). Two PCR products were sequenced and consistent with the sequences on the database. Interestingly, the expression ratio of *nup98(s)* to *nup98(l)* was different among tissues. We also examined the expression of both *nup98* isoforms in human cell lines by RT-PCR with RNAs extracted from HeLa, HEK293T, Jurkat, and FKH1 cells. Both isoforms were also detected in all human cell lines tested, although the expression level of two isoforms was different depending on cell lines (Fig. 1C).

3.2. Subcellular localization of Nup98 isoforms

Nup98 is accumulated in the nucleus with forming granular dots called GLFG bodies, in addition to the nuclear envelope [15]. First, we examined the subcellular localization of both Nup98 variants. EGFP-Nup98(s) and EGFP-Nup98(l) were clearly observed both in the nuclear envelope and the nuclear dots (Fig. 2A). The localization patterns of two isoforms were very similar to each other. These results indicate that the 17 amino acid region found in Nup98(l) is dispensable for the localization of the Nup98 proteins in the nuclear envelope and the nuclear dots. The similar localization patterns of both isoforms suggest that they are incorporated into the same NPCs and nuclear dots. To test whether both isoforms are co-localized, Nup98(s) and Nup98(l) with different tags were co-transfected in HeLa cells, and immunofluorescence assays were performed. When EGFP-Nup98(s) was expressed with HA-Nup98(l), the localization of both proteins was overlapped in the nuclear or cytoplasmic dots and the nuclear envelope (Fig. 2B, upper pictures). Similar observation was found in cells expressing EGFP-Nup98(l) and HA-Nup98(s) (Fig. 2B, lower pictures). These results support the idea that these two isoforms are localized to the same NPCs and nuclear dots.