Monoclonal Antibodies Recognize Gly-Leu-Phe-Gly Repeat of Nucleoporin Nup98 of *Tetrahymena*, Yeasts, and Humans

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Nucleoporin Nup98, an essential component of the nuclear pore complex, has multifunctional roles in nuclear functions including transcriptional regulation and nucleocytoplasmic transport. These functions mostly depend on a Gly-Leu-Phe-Gly (GLFG) sequence appearing repetitively in the N-terminal region of Nup98. As the GLFG sequence is well conserved among Nup98s from a wide variety of species including humans, yeasts, and ciliates such as *Tetrahymena thermophila*, a specific antibody that recognizes the GLFG sequence is expected to detect various Nup98s from a wide-range of species. To generate monoclonal antibodies specific to the GLFG repeat of Nup98, we used two synthetic polypeptides derived from the macronuclear Nup98 of *T. thermophila* as an antigen. We obtained two monoclonal antibodies (MAbs), 13C2 and 21A10, that recognize Nup98s in indirect immunofluorescence staining and Western blot analysis of *T. thermophila*. Peptide array analysis of these monoclonal antibodies located the position of their epitopes at or near GLFG residues: the epitope recognized by the 13C2 MAb is FGxxN (x being any amino acid), and the epitope recognized by the 21A10 MAb is GLF. As expected by their epitopes, these monoclonal antibodies also recognize Nup98 homologs expressed by human cells and the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, indicating that 13C2 and 21A10 MAbs recognize Nup98 epitopes common to phylogenetically distinct organisms. Thus, these MAbs are useful in studying a wide variety of biological phenomena that involve Nup98, ranging from ciliate nuclear dimorphism to NUP98-related human leukemia.

Introduction

IN EUKARYOTIC CELLS, the cell nucleus is partitioned from the cytoplasm by the nuclear envelope. The nuclear envelope consists of the nuclear membrane, the nuclear lamina, and numerous nuclear pore complexes (NPCs). The nuclear membrane is composed of two lipid bilayers, and NPCs form passageways through the nuclear membrane, enabling molecular trafficking between the nucleus and the cytoplasm. The NPC is an enormous protein structure, about 60 MDa in yeast⁽¹⁾ and 125 MDa in vertebrates,⁽²⁾ composed of approximately 30 different protein components, called nucleoporins (Nups).⁽³⁻⁵⁾ Almost one third of the nucleoporins are FG-Nups.^(6,7) These nucleoporins possess a structurally disordered Phe-Gly (FG)-repeat region with binding sites for nuclear transport receptors. The central channel of the NPC is lined with FG-Nups.

Nucleoporin Nup98, an FG-Nup, is a mobile component of the NPC and locates to both the cytoplasmic and nuclear sides

of the NPC.⁽⁸⁻¹⁰⁾ It is a functionally essential NPC component, participating in nuclear export of mRNA and import of nuclear proteins.^(11,12) While the major fraction of this nucleoporin is located at the NPC, Nup98 is also present inside the nucleus. Although the function of Nup98 in the nucleoplasm is unknown, its intranuclear mobility is dependent on ongoing transcriptional activity.^(13,14)

The Nup98 FG-repeat region has a typical repeat signature of Gly-Leu-Phe-Gly (GLFG).^(11,15) This region may have the potential to activate transcription, in addition to acting as a binding site for nuclear transport receptors at the nuclear pore.⁽¹⁶⁾ When the GLFG-coding region of the *NUP98* gene is fused with other genes encoding chromatin-binding proteins, such as homeobox (HOX) proteins, by gene rearrangement, the chimeric gene products induce several types of hematopoietic malignancies by the activation of inappropriate transcription.⁽¹⁷⁻¹⁹⁾

GLFG is a typical signature of Nup98 in model organisms like yeast and metazoans belonging to Opisthokonta, and this

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motif is well conserved in the ciliate *Tetrahymena thermophila* belonging to Alveolata. In *Tetrahymena*, cellular nuclei are differentiated into a germ-line micronucleus (MIC) and a somatic macronucleus (MAC),⁽²⁰⁾ and MAC-specific Nup98 orthologs have GLFG repeats while MIC-specific Nup98 have NIFN repeats.^(21,22) Because this difference in the repeat signature is important for nucleus-selective nuclear transport in *T. thermophila*,⁽²¹⁾ Nup98 is thought to play a key role in the nuclear dimorphism of this organism.

In order to facilitate the study of the function of *T. thermophila* Nup98, we attempted to develop monoclonal antibodies (MAbs) against the GLFG-repeat region of MAC-specific Nup98. We obtained two hybridoma strains that produced MAbs that recognized the GLFG region of MacNup98A. This is the first monoclonal antibody that can detect *T. thermophila* nucleoporin and NPC. Furthermore, these MAbs react not only with *T. thermophila* MacNup98A, but also with Nup98 homologs from yeasts and humans. Accordingly, our MAbs can be used to study the role of Nup98 in nuclear function using *T. thermophila* and model organisms such as *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* and human cell lines; and since these antibodies recognize human Nup98, they can also be used in pathological studies of NUP98-related hematopoietic malignancies.

Materials and Methods

Immunization, selection, and yield of monoclonal antibodies

Two synthetic peptides were used as an antigen for immunization of BALB/c mice: 1-MFGNTGGGGLFGNTQTQQTGGGLFGQPQQ-29 (peptide 1) and 646-SNPTQGGGLFGAANPGLGG-664 (peptide 2). Numbers refer to the residue number in the *T. thermophila* MacNup98A (1105 aa) protein. Each peptide was coupled to keyhole limpet hemocyanin, and then injected into BALB/c mice with Freund's complete adjuvant. Hybridomas were generated by Sendai virus-mediated cell fusion of spleen cells from the immunized mice with mouse myeloma cells. An enzyme-linked immunosorbent assay (ELISA) was used for screening for positive hybridoma clones. The positive clones were then screened again using indirect immunofluorescence staining of *T. thermophila* cells to determine whether the fluorescent signal appeared in the macronuclear periphery of the cells. The subclass of monoclonal antibody from each positive clone was determined using an isotyping kit (Serotec, Oxford, United Kingdom). To concentrate the antibody, hybridoma clones were grown to confluence in GIT medium (Wako, Osaka, Japan), the culture medium was collected, and ammonium sulfate was added to a final concentration of 55%. Resulting precipitates were collected by centrifugation, dissolved in PBS, and desalted by centrifugation through a 100 kDa cutoff filter (Merck Millipore, Billerica, MA).

Cell cultures

T. thermophila Inbred B strain CU427 cells (wild-type phenotype) were obtained from the *Tetrahymena* Stock Center and maintained in shallow culture medium, containing 1.5% proteose peptone (BD Difco, Sparks, MD), 0.5% yeast extract, and 0.5% D-glucose, in a plastic dish at 30°C without shaking or agitation. GFP-MacNup98A-expressing cells were gener-

ated by transformation with a DNA plasmid constructed from the rDNA-based vector pIGF-1,⁽²³⁾ as described previously.⁽²¹⁾ Expression of GFP-MacNup98A was induced by addition of 0.5 µg/mL cadmium chloride to the culture medium.

HeLa cells were obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in DME medium (Gibco, Grand Island, NY), supplemented with 10% calf serum at 37°C with 5% CO₂, as described previously.⁽²⁴⁾

Schizosaccharomyces pombe wild-type strain AY160-14D was obtained as a derivative from standard strains.⁽²⁵⁾ To obtain a HA800-7A strain expressing Nup98-GFP under a native promoter, a GFP gene was inserted chromosomally into the 3' region of the gene in frame; this strain expresses Nup98-GFP instead of Nup98. The genotypes of these yeast cell strains were as follows: AY160-14D, *h⁹⁰ ade6-216 ura4-D18 leu1-32 lys1-131*; HA800-7A, *h⁹⁰ ade6-216 ura4-D18 leu1-32 lys1-131 nup189n-GFP-kanr*. The cell strains were cultured in YES medium.

Saccharomyces cerevisiae wild-type strain YPH499 was obtained from Elmar Schiebel (Universität Heidelberg, Germany). The genotype of the YPH499 strain was *MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*. This yeast cell strain was cultured in YPDA medium (Clontech, Palo Alto, CA).

Indirect immunofluorescence staining

For indirect immunofluorescence staining, *T. thermophila* cells were fixed with cold methanol (−30°C) for 30 min as described previously.⁽²¹⁾

HeLa cells were cultured in a glass-bottom dish (MatTek, Ashland, MA) for 2 days before fixation. The cells were fixed with cold methanol (−30°C) for 30 min as described previously.⁽²⁴⁾

S. pombe cells were fixed with 4% formaldehyde for 10 min, treated with 0.6 mg/mL Zymolyase 100T (Nakalai Tesque, Kyoto, Japan) at 36°C for 70 min, and permeabilized with 1% Triton X-100 for 1 min, as described previously.⁽²⁶⁾

For *S. cerevisiae*, cells were fixed with 4% formaldehyde and 10% methanol in 0.1 M sodium phosphate buffer (pH 6.8) at room temperature for 30 min, and treated at room temperature for 30 min with 0.1 mg/mL Zymolyase100T and 0.5 mg/mL lysing enzyme (Sigma-Aldrich, St. Louis, MO) in PEMS buffer (100 mM PIPES, 1 mM EGTA, 1 mM magnesium chloride, and 1.2 M sorbitol). The cells were placed on poly-L-lysine-coated coverslip using Cytopro (Wescor, Logan, UT) at 1000 rpm for 1 min and permeabilized for 1 min with PEMS buffer containing 1% Triton X-100.

After washing with PBS, all fixed samples were blocked with 1% BSA for 2 h at room temperature. MAbs 13C2 and 21A10 were diluted to 0.5 µg/mL in PBS. In some cases, diluted supernatants of hybridoma culture were used. A commercial anti-Nup98 rat MAb 2H10⁽²⁷⁾ was also diluted to 0.5 µg/mL in PBS. Blocked samples were treated with these primary antibody solutions overnight at 4°C. The secondary antibodies were 4 µg/mL of Alexa 488-labelled anti-mouse IgG for 13C2 and 21A10 or anti-rat IgG for 2H10. Finally, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Samples were washed three times with PBS between treatments.

Fluorescence images were obtained with an Olympus fluorescence microscope IX-70 (Tokyo, Japan) controlled by

DeltaVision microscope system (Applied Precision, Issaquah, WA). Olympus oil-immersion objective lenses UApo40A (NA=1.35) and PlanApo 60 (NA=1.42) were used for the observation of *T. thermophila* and HeLa cells and for the observation of the yeasts, respectively. Images with z-sections were deconvolved using the software equipped with the DeltaVision microscope system.⁽²⁸⁾

Western blot analysis

T. thermophila cells were fixed with cold methanol for 30 min, then washed with 0.1 M phosphate buffer (pH 6.8). The fixed cells were lysed by sonication in SDS-sample buffer.

HeLa cells were removed from the culture dish with a cell scraper and collected by low-speed centrifugation. After washing with PBS, the cells were lysed by sonication in SDS-sample buffer.

Yeast cells were cultured in culture medium containing 1 mM phenylmethanesulfonyl fluoride (PMSF) for 5 min before preparation of the extract. Cells were then harvested and heated in distilled water at 95°C for 5 min. The cells were suspended in 100 mM phosphate buffer (pH 6.8), 4 M urea, 2.5% SDS, 0.05 mM EDTA, and disrupted by glass beads using a Multi Beads Shocker (Yasui Kikai, Osaka, Japan). The sample was clarified by centrifugation, and the supernatant was used as the whole cell extract.

Dithiothreitol (DTT) was added to all samples to a final concentration of 20 mM, and the samples were heated at 70°C for 15 min in the SDS sample buffer (2% SDS, 10% glycerol, 0.025% bromophenol blue, 60 mM Tris-HCl [pH 6.8]) before loading on 10% acrylamide gels made with 0.13% bis-acrylamide. SDS-PAGE was performed using a constant current of 30 mA.

After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 0.8 mA/cm² for 2 h using a semi-dry blotting apparatus. Membranes were blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBS-T) for 3 h at room temperature. The membranes were then incubated with MAbs diluted to 0.4 µg/mL in PBS containing 1% skim milk overnight at 4°C. In some cases, diluted supernatants of hybridoma culture were used. Membranes were then washed three times for 10 min with PBS-T followed by incubation with 0.4 µg/mL horseradish peroxidase (HRP)-labeled anti-mouse IgG or anti-rat IgG (both from Cappel) for our MAbs or the commercial 2H10, respectively, for 2 h at room temperature. The membranes were then incubated with ImmunoStar reagent (Wako) for 1 min, and chemiluminescence signals were detected by an image analyzer.

Epitope-masking experiment

The supernatants of the hybridoma culture media were used as the monoclonal antibody containing fractions. The supernatants were mixed with equal volumes of each of the synthetic oligopeptides, GGGGLFGNTQ, TGGGLFGQPQ, or QGGGLFGAAN, dissolved in PBS at 1 mg/mL, and then incubated for 30 min at room temperature; these amino acid sequences were chosen from the sequences around the GLFG signatures present in immunizing peptides 1 and 2. After the 30 min incubation, the mixtures were diluted 5-fold with PBS, and then applied to fixed and blocked *Tetrahymena*. Subsequent indirect IF staining was performed according to the method described above.

Peptide array for epitope mapping

Synthetic peptide array on the membranes were purchased from JPT Peptide Technologies (Berlin, Germany): 5 nmol of each oligopeptide was covalently bound to the spots. The membranes were then treated with methanol for a few seconds, washed with PBS three times, blocked with 5% skim milk in PBS for 3 h, and treated for 3 h with supernatants of hybridoma culture medium diluted 1:5 with the blocking buffer. After washing with PBS three times, the membranes were incubated for 2 h with the secondary antibody HRP-labeled anti-mouse IgG (Cappel). All procedures were carried out at room temperature. Signals were detected using an image analyzer, as described above for Western blot analysis.

Results and Discussion

From the hybridoma library raised against two synthetic polypeptides containing GLFG sequences of the macronucleus-specific MacNup98A of *T. thermophila*, we selected the two hybridoma clones 13C2 and 21A10 based on positive screening results with ELISA (data not shown) and indirect immunofluorescence (IF) staining (Fig. 1A). Both 13C and 21A10 monoclonal antibodies stained the macronuclear periphery of *T. thermophila*, and the macronuclear localization corresponded well to that of MacNup98A.⁽²¹⁾ MAb 13C2 was highly specific to the macronucleus (see Fig. 1A, MAb 13C2 inset). In contrast, in addition to clear macronuclear staining, MAb 21A10 also stained the micronuclear periphery (see Fig. 1A, MAb 21A10 inset). This indicates that MAb 21A10 recognizes Nups localizing to the micronucleus in addition to MacNup98A.

We also tested two commercial anti-nucleoporin MAbs: the MAb 2H10 was raised against human Nup98⁽²⁷⁾ and the MAb 414 monoclonal antibody recognizes FG-Nups in human and yeasts.⁽²⁹⁾ Neither of the commercial antibodies stained the nuclear periphery of *T. thermophila* (Fig. 1A).

To specify protein components that can be recognized by these MAbs, Western blot (WB) analysis was performed (Fig. 1B). Both MAbs 13C2 and 21A10 detected a protein with an apparent molecular weight (MW) of 98 kDa (Fig. 1B, arrowheads in wild-type); this value approximates the MW of MacNup98A (112 kDa) deduced from the DNA sequence. To further verify that this protein band corresponds to MacNup98A, we performed WB analysis using cells ectopically expressing GFP-MacNup98A in addition to endogenous MacNup98A (Fig. 1B, far-right lane). MAb 13C2 detected proteins with MWs of 98 and 125 kDa in these samples (GFP-MacNup98A arrow). The 125-kDa band corresponds to GFP-MacNup98A and is approximately 28 kDa larger than the endogenous protein detected by 13C2. As the MW of GFP is 28 kDa, this result suggests that the 98 kDa protein band is endogenous MacNup98A. The commercial 2H10 MAb also detected a protein with the same MW as endogenous Nup98A, but the band was very faint (Fig. 1B, compare protein bands indicated by arrowheads).

To determine the position of epitopes, indirect IF staining for *Tetrahymena* cells was performed with and without pretreatment of the MAbs with masking peptides. MAbs were incubated with GGGGLFGNTQ (Fig. 2A, MP1; the position of GLFG is italicized), TGGGLFGQPQ (Fig. 2A, MP2), or QGGGLFGAAN (Fig. 2A, MP3) prior to incubation with *Tetrahymena*. These three sequences are present in the

FIG. 1. MAbs 13C2 and 21A10 detect MacNup98A of *Tetrahymena*. (A) IF staining of *T. thermophila* cells using MAbs 13C2, 21A10, 2H10, and 414. Black-and-white images were obtained with DAPI. Color images represent MAb (green) merged with DAPI (magenta). Dotted lines represent the outline of cells. Open arrow indicates the micronucleus. Insets are magnified images showing the position of the micronucleus. Bar, 20 μ m. (B) WB analysis of *T. thermophila* specimen from wild-type (left lanes) or GFP-MacNup98A expressing cells (far right lane). MAbs used are indicated above each lane. Signals were detected using the same staining conditions, except that the exposure time of the middle lane (21A10 long exposure) was about 10 times longer than that of the other lanes. Open arrowheads and closed arrow show the positions of MacNup98A and GFP-MacNup98A, respectively. Diamonds and asterisks identify uncharacterized proteins.

oligopeptides used for immunization of BALB/c mice. Pretreatment with MP1 or MP2 partially blocked recognition of the macronucleus by 13C2 MAb, while pretreatment with MP3 completely blocked recognition of the macronucleus by 13C2 MAb (Fig. 2B). This difference suggests that the epitope for 13C2 MAb exists in part in amino acid residues outside of the common GLFG sequence. In contrast, all three peptides completely blocked recognition of the macronucleus by MAb 21A10 (Fig. 2B), suggesting that the epitope of 21A10 exists within the common GGGLFG sequence shared by these three peptides.

It should be noted that cytoplasmic granular signals disappeared in both 13C2- and 21A10-treated cells when nuclear recognition was completely blocked by the masking peptides, suggesting that these cytoplasmic signals are specific. The biological significance of the granular localization of Nup98 in the cytoplasm is presently unclear.

To map the epitopes of 13C2 and 21A10 more precisely, MAbs were applied to a peptide array. A series of 10-residue peptides based on masking peptides MP1, MP2, and MP3 bound onto a membrane was incubated with MAbs 13C2 or 21A10 (Fig. 2C). MAb 13C2 reacted with spots commonly possessing the sequence FGAAN. However, among the five amino acid residues of FGAAN, the N residue is required for strong recognition because TQGGLFGAA, which does not have the N residue, was recognized very poorly by MAb 13C2 (Fig. 2C, lane 5). In addition, as this antibody recognized both human and yeast Nup98s, which do have FGxxN but not FGAAN (as described below), we conclude that the epitope for 13C2 MAb is FGxxN (x being any amino acid). This result is consistent with the results of indirect IF staining shown in Figure 2B, where MP1 and MP2 only partially blocked recognition of the macronucleus by 13C2 while MP3 completely blocked recognition.

A *T. thermophila* MacNup98A (1-700/ total 1105 aa)

MP1 MP2
 MEGNTGGGGLFGNTQTQTTGGGLFGQPOQTQFGQTGATGGGLFGGATNTFGGGGGGLFGGNNNQTNPTAGGGIFGQGTTLGGGAPAQTTGGGLFGAPQNN
 peptide1
 QGGGLFGGGTTTGGGMFGNQANTQTGGGGLFGGSPQPTTQPPAFSLNNPTTGGGGLFGQPANTMGNNNGGLFGGQTNSTFGANNMGLNNRNPQAGIFGG
 ATTTAPTNTGMFGGIGANNNGGLFGMNNNTNPTGGFGATNPTAGGGGLFGGATTTGGGGLFGGNTQGGGLGTANTTAGLLGGGFNMNNNTGGIL
 GQTNNQFGLGSFGTNNNAAPFQPKASANGVLTKEKNCYAISSNGTDFCIFEALTRKLVKAGQLKPGAQAGGMFGQPAQGGNGLFGGGGAATTP
 FGGAQNGNLFGGQNTQAQGGGLFGAPVNNATGAGGGLFGAKPAATTTGGGLFGQMPAQTTGGFLGNTATQAGGGLFGGATTTQAPGGGGGLFGGNTTA
 ATTTGGGLFGGNTQTGGATGGLFGGQPPNQGGLFLNTGNANNANTGGGLFGGATTTTATGGGLFGGSTNTQPLATGGGLFGNNQASQPAAGGLFGGAA
 MP3
 PQQNSLFGGATAGGQTGGGLFGGATGATQGGGGLFGQTASNPTGGGLFGAANPGLGGAAAGTQVAQPLNGFQSLIQLLEQTQNNNDKFALD-----
 peptide2

B **C**

FIG. 2. Determination of epitopes for 13C2 and 21A10 MAbs. (A) Amino acid sequence of MacNup98A from residue 1–700; the total length of MacNup98A is 1105 amino acids. The two regions (peptides 1 and 2) underlined are the sequences used for immunization of BALB/c mice. The sequences highlighted in yellow, designated MP1, MP2, and MP3, were synthesized for the antibody-masking experiments shown in B. Each GLFG is shown in red. (B) The epitope masking effect of none (w/o peptide), MP1 (GGGGLFGNTQ), MP2 (TGGGLFGQPQ), and MP3 (QGGGLFGAAN) was examined by IF staining using MAbs 13C2 (upper panel) and 21A10 (lower panel). MAbs were incubated with one of the oligopeptides for 30 min prior to incubation with fixed *T. thermophila* cells. Bar, 20 μ m. (C) Peptide array analysis using MAbs 13C2 (top) or 21A10 (bottom). Numbers at the top and bottom of the membrane correspond to those listed on the right; each of these 10-mer peptides was tested for binding to MAbs 13C2 and 21A10, as described in Methods section. Residues shown in red are common sequences among the peptides detected by each MAb.

MAb 21A10 reacted with all spots possessing GLF (Fig. 2C), suggesting that GLF is the epitope for MAb 21A10. This result is consistent with that of IF staining where all three masking peptides blocked the recognition of the macronucleus by 21A10 (Fig. 2B). The GLF motif appears frequently in Nup98 homologs (e.g., 28 times in macronucleus-specific MacNup98A in *Tetrahymena*), and it also occurs in other nucleoporins (nine times in binuclear Nup308). Nup308 is a *Tetrahymena*-specific nucleoporin possessing GLFG repeats in the N-terminal region and is localized to both nuclei.⁽²¹⁾ This is consistent with the results of indirect IF staining shown in Figure 1A where 21A10 primarily stains the macronucleus but also recognizes the micronucleus. If GLF is the major epitope for

21A10, this MAb should react with Nup98s of humans and yeasts since GLF is well conserved among species and appears frequently as a repetitive structure in human and yeast Nup98 homologs.

To test the idea that MAbs 21A10 and 13C2 may be able to recognize human Nup98, the cross-reactivity of 21A10 and 13C2 to human cervical cancer-derived HeLa cells was assessed using indirect IF staining and WB analysis (Fig. 3). Both MAbs 13C2 and 21A10 stained the nuclear periphery of the human cells in a punctate pattern similar to that of MAb 2H10 (Fig. 3A). As noted above, MAb 2H10 was raised against human Nup98.⁽²⁷⁾ Surprisingly, the signal at the nuclear periphery with MAb 21A10 was much higher and the

FIG. 3. MAbs 13C2 and 21A10 cross-react with Nup98 of HeLa cell. **(A)** IF staining of HeLa cells using MAbs 13C2, 21A10, or 2H10. Black-and-white images represent fluorescence images obtained with MAb (top) and DAPI (middle). Color images represent MAb (green) merged with DAPI (magenta). Bar, 20 μ m. **(B)** WB analysis of HeLa cells using 13C2, 21A10, or 2H10. The blots were treated identically; the exposure time for chemiluminescence detection was 10 min. Open arrows represent the position of Nup98. The lower bands appearing in the 2H10 lane were non-specific staining with the anti-rat IgG secondary antibody (data not shown).

TABLE 1. SUMMARY OF SUITABILITY OF MABs FOR IMMUNOLOGICAL APPLICATIONS

MAb	Isotype	T. thermophila		HeLa cell		S. cerevisiae		S. pombe		Epitope
		IF	WB	IF	WB	IF	WB	IF	WB	
13C2	Mouse IgG1	+++	+++	+	+++	+++	+++	+++	+++	FGxxN
21A10	Mouse IgG1	+++	+	+++	—	+++	++	+++	++	GLF
2H10	Rat IgG2c	—	++	+++	+	+++	+++	+++	++	Unidentified

IF, indirect immunofluorescence staining; WB, Western blot analysis.

background lower than that of 2H10. Staining the nuclear periphery of both *T. thermophila* and HeLa cells with MABs 13C2 and 21A10 is consistent with the epitope analysis described above. The conclusion that 13C2 MAb recognizes FGxxN is discussed in greater detail below.

MAb 13C2 detected a protein band with an apparent MW of approximately 95 kDa in WB analysis of HeLa cell speci-

mens (Fig. 3B). MAb 2H10 detected a similar band, suggesting that MAB 13C2 specifically recognizes human Nup98. Surprisingly, the detection of human Nup98 with 13C2 MAb was much better than detection with 2H10 (compare 13C2 and 2H10 lanes in Fig. 3B). MAb 21A10 did not react with any protein bands, even when exposure time was prolonged (Fig. 3B). Accordingly, in HeLa cells, 13C2 is a good

FIG. 4. MABs 13C2 and 21A10 cross-react with Nup98 of fission yeast. **(A)** IF staining of *S. pombe* cells using MABs 13C2, 21A10, and 2H10; 1:10 dilution of the supernatants of hybridoma cultures of 13C2 or 21A10 or 10 μ g/mL IgG solution of 2H10 were used. Black-and-white images represent fluorescence images obtained with MAb (top) and DAPI (middle). Color images represent merged images of MAb (green) with DAPI (magenta). Dotted lines represent the outline of cells. Bar, 5 μ m. **(B)** WB analysis of *S. pombe* cell extract using MABs 13C2 (left lanes), 21A10 (middle lanes), and 2H10 (right lanes); 1:10 dilutions of the supernatants of hybridoma culture medium of 13C2 or 21A10 or 1 μ g/mL IgG solution of 2H10 were used. Left and right lanes represent specimens from a wild-type strain and an *S. pombe* strain in which Nup98 was chromosomally replaced with Nup98-GFP,⁽³¹⁾ respectively.

FIG. 5. MABs 13C2 and 21A10 crossreact with multiple nucleoporins of budding yeast. **(A)** IF staining of *S. cerevisiae* cells using MABs 13C2, 21A10, and 2H10; 1:10 dilution of the supernatants of hybridoma cultures of 13C2 or 21A10 or 10 μ g/mL IgG solution of 2H10 were used. Black-and-white images represent fluorescence results obtained with MAB (top) and DAPI (middle). Color images represent MAB (green) merged with DAPI (magenta). Dotted lines represent the outline of cells. Bar, 5 μ m. **(B)** WB analysis of *S. cerevisiae* cell extract using MABs 13C2, 21A10, or 2H10; 1:10 dilution of the supernatants of hybridoma culture medium of 13C2 or 21A10 or 2 μ g/mL IgG solution of 2H10 were used. Arrows represent the positions of the indicated nucleoporins. Asterisks represent uncharacterized proteins.

MAB for WB analysis, while 21A10 is good for indirect IF staining (Table 1).

A Nup98 homolog is expressed in *S. pombe*⁽³⁰⁾ and it has a GLFG signature in its N-terminal region.^(22,31) Therefore, we examined the cross-reactivity of MABs 13C2 and 21A10 to fission yeast *S. pombe* using indirect IF staining and WB analysis (Fig. 4). Both 13C2 and 21A10 stained the nuclear periphery of *S. pombe* in a punctate pattern similar to that found with MAB 2H10 (Fig. 4A, compare 13C2 and 21A10 with 2H10). As noted above, MAB 2H10 was raised against human Nup98.⁽²⁷⁾ In addition, by WB analysis, these three MABs detected a protein with an apparent MW of 110 kDa (Fig. 4B, wild-type lanes). To further verify that these MABs recognize the Nup98 gene product, we utilized an *S. pombe* strain in which Nup98 was chromosomally replaced with Nup98-GFP.⁽³¹⁾ These cells express Nup98-GFP as a single gene product under its native promoter and do not express wild-type Nup98. These three MABs detected a single protein band with an apparent MW of $\sim$ 140 kDa by WB analysis (Fig. 4B, Nup98-GFP lanes). Since the GFP tag is $\sim$ 30 kDa, this result suggests that the 140 kDa band corresponds to Nup98-GFP and that both MABs 13C2 and 21A10 cross-react specifically with the *S. pombe* Nup98 homolog.

S. cerevisiae also expresses Nup98 homologs Nup116, Nup100, and Nup145N.^(32,33) As with the *S. pombe* Nup98 homolog, these proteins have numerous GLFG motifs in their N-terminal halves. Therefore, we also examined the cross-reactivity of MABs 13C2 and 21A10 to budding yeast *S. cerevisiae* using indirect IF staining and WB analysis. Indirect IF staining showed that MABs 13C2 and 21A10 stained the nuclear periphery of *S. cerevisiae* in a typical nucleoporin staining pattern, similar to that seen with commercial MAB 2H10 (Fig. 5A). Each of these MABs detected Nup116 (116 kDa) and Nup145N (65 kDa) by WB analysis (Fig. 5B, all lanes). In ad-

dition, MABs 13C2 and 2H10 detected Nup100 (100 kDa) (Fig. 5B). MABs 21A10 and 2H10 also detected two additional bands with apparent MWs of 57 kDa and 49 kDa, probably corresponding to Nup57 and Nup49, GLFG-rich nucleoporins (Fig. 5B).^(34–36) MAB 13C2 also detected two unknown proteins visualized as bands (Fig. 5B, asterisks).

The results from the peptide array analysis shown in Figure 2C indicate that the epitope of 21A10 is GLF. The results with *S. cerevisiae* strongly support this conclusion as both Nup57 and Nup49 are recognized by MAB 21A10 and both possess numerous GLF motifs.

The results from the peptide array analysis shown in Figure 2C indicate that the epitope of 13C2 is FGAAN. However, our results also demonstrate that 13C2 recognizes human and yeast Nup98 homologs (Figs. 4, 5). Since FGAAN appears only once in *T. thermophila* MacNup98A and is not present in human or yeast Nup98 homologs, the true epitope is likely to be FGxxN (where x is any residue), as FGxxN appears several times in all Nup98 homologs stained by 13C2: eight times in *T. thermophila* MacNup98A, four times in human Nup98, 16 times in *S. pombe* Nup98, 14 times in *S. cerevisiae* Nup116, 18 times in *S. cerevisiae* Nup100, and six times in *S. cerevisiae* Nup145N. This also suggests that the unidentified proteins recognized by MAB 13C2 in *S. cerevisiae* are likely to contain FGxxN sequences.

MABs 13C2 and 21A10 were highly specific to Nup98 homologs of phylogenetically distinct organisms—the protozoan *T. thermophila*, yeasts, and humans. Thus, these MABs recognize Nup98 homologs from species from both Alveolata and Opisthokonta. MAB 13C2 was an excellent antibody for use in WB analysis for all four organisms examined in this study. MAB 21A10, on the other hand, was better for indirect IF staining. These two MABs with distinct characteristics and defined epitopes will be useful for many aspects of research

related to Nup98 and the nuclear pore complex, including their functions, molecular dynamics, and relationship to leukemic diseases.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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