Cell, Vol. 81, 215–222, April 21, 1995, Copyright © 1995 by Cell Press

The Peptide Repeat Domain of Nucleoporin Nup98 Functions as a Docking Site in Transport across the Nuclear Pore Complex

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Summary

We report the cDNA deduced primary structure of a wheat germ agglutinin–reactive nuclear pore complex (NPC) protein of rat. The protein, termed Nup98 (for nucleoporin of 98 kDa), contains numerous GLFG and FG repeats and some FXFG repeats and is thus a vertebrate member of a family of GLFG nucleoporins that were previously discovered in yeast. Immunoelectron microscopy showed Nup98 to be asymmetrically located at the nucleoplasmic side of the NPC. Nup98 functions as one of several docking site nucleoporins in a cytosolic docking activity–mediated binding of a model transport substrate. The docking site of Nup98 was mapped to its N-terminal half, which contains all of the peptide repeats. A recombinant segment of this region depleted the docking activity of cytosol. We suggest that the peptide repeat domain of Nup98, together with peptide repeat domains of other nucleoporins, forms an array of sites for mediated docking of transport substrate, and that bidirectional transport across the NPC proceeds by repeated docking and undocking reactions.

Introduction

There are now several lines of circumstantial evidence that implicate a subgroup of the nucleoporins (nups; a collective term for nuclear pore complex [NPC] proteins) to function in the nuclear import of proteins. This subgroup consists of those nucleoporins that share repetitive peptide motifs and therefore reactivity with certain monoclonal antibodies (MAbs). Some of these also share modification (except, so far, in yeast) of Ser/Thr by single N-acetylglucosamine residues, making them reactive with wheat germ agglutinin (WGA; for review, see Rout and Wente, 1994). Thus, WGA or MAbs, polyspecifically reacting with several of these nucleoporins, have been shown to inhibit nuclear import (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988a; Featherstone et al., 1988; Dabauvalle et al., 1988b). Also, depletion of WGA-reactive proteins from a cell-free nuclear reconstitution system derived from Xenopus laevis eggs has been shown to yield transport-defective nuclei. Transport competence was restored when the WGA-reactive glycoproteins were added back to the nuclear reconstitution system (Finlay and Forbes, 1990). In similar experiments, depletion by immunoaffinity of a subset of the WGA-reactive proteins also yielded transport-defective nuclei (Finlay et al., 1991). Moreover, nuclear import appears to be blocked in temperature-sensitive mutants of NSP1 and NUP49 in yeast (Mutvei et al., 1992; Schlenstedt et al., 1992; Nehrbass et al., 1993). Finally, immobilized MAb- or WGA-reactive nucleoporins have been reported to deplete 80% of the cytosol's import activity (Sterne-Marr et al., 1992). But, as this activity could not be recovered, it has not been characterized further. Moreover, it was not established which of the nucleoporins depleted the cytosol's import activity.

Several MAb- and WGA-reactive vertebrate nucleoporins have now been characterized at the molecular level. These are p62 (Starr et al., 1990; Carmo-Fonseca et al., 1991; Cordes et al., 1991), Nup153 (Sukegawa and Blobel, 1993), and Nup214 (CAN; Kraemer et al., 1994). All these proteins share numerous FXFG motifs. In Saccharomyces cerevisiae, NUP1 (Davis and Fink, 1990), NUP2 (Loeb et al., 1993), and NSP1 (Hurt, 1988; Nehrbass et al., 1990) also share FXFG motifs. So far, however, it is not known whether any of the vertebrate FXFG nucleoporins can complement any of the yeast FXFG nucleoporins. In yeast, another family of nucleoporins that contain numerous GLFG motifs has been discovered and characterized (Wente et al., 1992; Wimmer et al., 1992; Wente and Blobel, 1994; Fabre et al., 1994).

In this paper, we report the identification and molecular characterization of a mammalian GLFG nucleoporin, which we termed Nup98 (for nucleoporin of 98 kDa). By immunogold electron microscopy, we have localized Nup98 to the nucleoplasmic side of the NPC. Ligand blot assays suggest that Nup98 functions as a docking protein for cytosol-mediated docking of a model import substrate. The docking function was localized to the N-terminal half of Nup98, which contains all its GLFG repeats. Moreover, we found that a recombinant segment representing this region of Nup98 depleted the docking activity of the cytosol.

Results

cDNA Sequence and Deduced Primary Structure of Nup98

Many NPC proteins can be quantitatively extracted from isolated rat liver nuclear envelope by 2.0 M urea/EDTA (Radu et al., 1993). The extracted nucleoporins can then be further fractionated into those that are reactive or nonreactive with WGA (Radu et al., 1993). Among the WGA-reactive nucleoporins is a protein of 98 kDa, as estimated from its mobility on SDS–polyacrylamide gel electrophoresis (SDS–PAGE; see Figure 2, lane 3 in Radu et al., 1993). This protein also reacts with MAb192 (Kraemer and Blobel, unpublished data) and with MAb350 (Sukegawa and Blobel, 1993). To characterize this protein further, we subjected it to proteolysis and obtained partial amino acid sequence for some of the separated peptides. Polymerase

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I
   1      MFNKSFGTPFGGTGGFGTTSTFGQNTGFGTTSG
  34 GAFG TSAFGSSSNTG
  49 GLFG NSQTKPG
  60 GLFG TSSLSQPATSTSTGFGFGTSTGTSN
  89 SLFG TANTGTSLFSSQNNAFAQNKPTGFGNFGTSTSSG
 127 GLFG TTNTTSNPFGNTSG
 145 SLFG PSSFTAAPTGTTIKFNPPTGT

II
 170 DTMVKAGVSTNISTKHQCITAM
 192 KEYESKSLEELRLEDYQANRK

III
 213      GPQNQVGAGTTT
 225 GLFG SSPATSSAT
 238 GLFS SSTTNSAFSYGQNKTAFGTSTTGFGTNPG
 271 GLFG QQNQQTTSLFSKPFGQATTTPNTGFSFGNTSTLG
 309      QPSTNTM
 316 GLFG VTQASQPG
 328 GLFG TATNTSTGTAFGTGT
 347 GLFG QPNTGFGAVGS
 362 TLFG NNKLTTFGTSTTSAPSFGTTSG
 388 GLFG NKPTLTLGTNTNTSNFGFGTNNSGSSIFG
 421      SKPAAGTLGTGLGTGFGTALGAGQA
 446 SLFG NNQPKIGGPLGT
 462 GAFG APGFNTSTAILGFGAPQAPVALTDPNASAAQQAVLQQ
 503      HLNSLTYSPFG

IV
 514 DSPLFRNPMSDPKKKEERLKPTNPAAQKALTTPTHYKLTPRPA
 557 TRVRPKALQTTGTAKSHLFDGLDDDEPSLANGAFMPKKSIKKL
 600 VLKNLNNSNLFSPVNHDSEDLASPSEYPENGERFSFLSKPVDE
 643 NHQQDGDDDSLVSRFYTNPIAKPIPQTPESAGNKNNSSSNVED
 686 TFIALNMRAALRNGLEGSSEETSFHDESLQDDRDEIENSAFQI
 729 HPAGIVLTKVGYYTIPSMDDLAKITNEKGECIVSDFTIGRKGY
 772 GSIYFEGDVNLTNLNLDDIVHIRRKEVIVYVDDNQKPPVGEGL
 815 NRKAEVTLDGVWPTDKTSRCLIKSPDRLADINYEGRLEAVSRK
 858 QGAQFKEYRPETGSWVFKVSHFSKYGLQDSDEEEEEHPPKTTS
 901 KKLKTAPLPPAGQATTFQMTLNGKPAPPTSGREGQRM
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Figure 1. Deduced Amino Acid Sequence of Nup98

The stretches of amino acid sequence obtained by peptide sequencing are marked by thin underlines. Four structural domains can be distinguished. Domains I and III contain GLFG motifs (in boldface and aligned on the vertical), FG motifs (in boldface italics), and FXFG motifs (heavy underlines). Domains II and IV do not contain any of these motifs and are characterized by an abundance of the charged amino acid residues R, K, H, D, and E (35% and 30%, respectively) compared with only 2% and 3%, respectively, in domains I and III. The GenBank accession number for the Nup98 sequence is L39991.

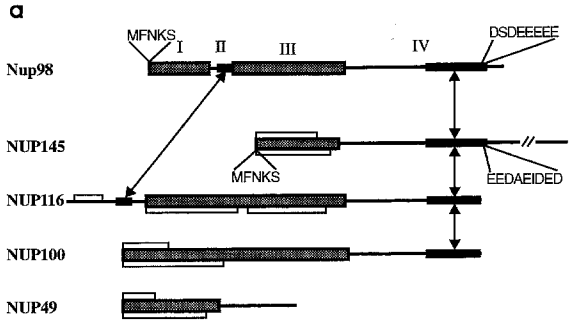
a
MFNKS
I
II
III
IV
DSDEEEEE
Nup98
NUP145
MFNKS
EEDAEIDED
NUP116
NUP100
NUP49
b
Nup98 186 QCITAMKEYESKSLEELRLEDYQANRK
Q IT M EY : S:EELR::DYQA RK
NUP116 140 QSITCMPEYRNFSFEELRFQDYQAGRK
c
CONSENSUS YY sPSmD L Sl gi Vsn vIG K YG I F VDL l
Nup98 737 KVGYYTIPSMDDLAKITNEKGECIVSDFTIGRKGYGSIYFEGDVNLTNLN-------LD--
NUP145 458 SFGYWCSPSPEQLERLSLKQLA-AVSNFVIGRRGYQCITFQHDVDLTAFTKSFREELFG--
NUP116 967 NENYYISPSLDTLSSYSLLQLR-KVPHLVVGHKSYGKIEFLEPVDLAGIP-------LTYL
NUP100 814 KNNYYISPSIETLGNKSLIELR-KINNLVIGHRNYGKVEFLEPVDLLNTP-------LDTL
CONSENSUS iV f Kt VY D KP GeGLNvra VTl n YPvDK TRkpIKd
Nup98 786 --DIVHIR-RKEVIVYVDDNQKPPVGEGLNRKAEVTLDGVWPTDKTSRCLIKSPDRLADIN
NUP145 514 --KIVIFRSSKTVEVYPDEATKPMIGHGLNVPAIITLENVYPVDKKTKKPMKDTTKFAEFQ
NUP116 1020 GGVYITFE-PKTCIIYANLPNRPKRGEGINVRARITCFNCYPVDKSTRKPIKDPNH----Q
NUP100 867 CGDLVTFG-PKSCSIYENCSIKPEKGEGINVRCRVTLYSCFPIDKETRKPIKNITH----P
CONSENSUS l rrL !k kF sYdP tGTw F VnH
Nup98 846 -YEGRLEAVSRKQGAQFKEYRPETGSWVFKVSHFSKYGL-QDSDE-E-EEE
NUP145 572 VFDRKLRSMR---EMNYISYNPFGGTWTFKVNHFSIWGLVNE-EDAEIDED
NUP116 1076 LVKRHIERLKKNPNSKFESYDADSGTYVFIVNH
NUP100 923 LLKRSIAKLKENPVYKFESYDPVTGTYSYTIDH
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Figure 2. Similarity between Nup98 and the GLFG-Containing Yeast Nucleoporins NUP145, NUP116, NUP100, and NUP49

(a) Schematic representation and alignment of similar structural domains of the five nucleoporins. The hashed boxes represent the GLFG-containing domains. The open boxes above the yeast sequences depict regions homologous to domain I of Nup98, whereas the open boxes below represent regions similar to domain III. Some regions of the yeast sequences present homology with domains I and III of Nup98, as indicated by open boxes both above and below the hashed boxes. The heavy double-arrowed lines depict segments similar to domains II and IV in Nup98. Note that both NUP145 and Nup98 start with an identical sequence of 5 amino acids, and only NUP145 contains the cluster of negatively charged amino acid residues near the C-terminal end of Nup98. Nevertheless, NUP145 does not contain any segment similar to domain II in Nup98, but such a segment is present in NUP116.

(b) Comparison of the sequence corresponding to the heavy line in domain II of Nup98 and a similar region of NUP116. A colon denotes similar amino acid residues; i.e., having a score equal or higher than 1 in the BLOSUM 62 similarity matrix (Henikoff and Henikoff, 1992).

(c) Comparison of the sequences corresponding to the heavy line in domain IV of Nup98 and similar sequences of the yeast nucleoporins. The consensus amino acids are presented in the upper line of each set: a capital letter denotes 3 identical amino acids with a fourth identical or similar, and a lower case letter denotes 3 identical or homologous amino acids. In bold at the end of the alignment are the negatively charged segments present only in Nup98 and NUP145.

chain reaction (PCR) was then used to generate an exact probe for screening a rat liver cDNA library. A cDNA sequence of 3264 nucleotides was obtained from overlapping clones. Because the N-terminal of the protein was found to be blocked, we have no information on its N-terminal sequence. However, the assignment of the ATG initiation codon is consistent with the following facts. First, the open reading frame encodes a protein of 97,821 Da, close to the $M_r$ estimated from its mobility on SDS–PAGE (Figure 1). Second, an A that is found in position −3 is the most conserved nucleotide preceding the eukaryotic initiation consensus site (Kozak, 1984). Third, there is an in-frame upstream stop codon at positions −78 to −76; a further downstream ATG codon at positions 514–516 is unlikely to be the initiation codon, as it would code for a protein that is only 81 kDa. The 3264 nucleotides long cDNA thus contains a 155 bp 5′ untranslated region. The 298 bp 3′ untranslated region contains the poly A tail.

The identity of the cDNA deduced primary structure was confirmed by protein sequence data obtained from eight endoproteolytic peptides (see thin underlines in Figure 1). Because the protein is located in the NPC (see Figure 5 and Figure 6), it will be referred to as Nup98 (nucleoporin of 98 kDa), in agreement with previously proposed nomenclature. The most striking feature of the primary structure of Nup98 is the presence of numerous GLFG motifs. Nup98 is thus a mammalian representative of a family of GLFG nucleoporins that was first discovered in yeast
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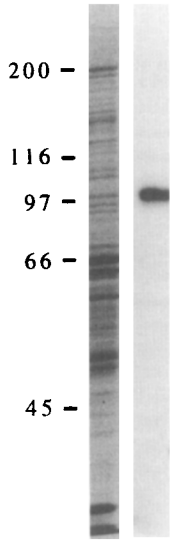

Figure 3. The Antibodies against the Nup98-2 Segment of Nup98 React with a Single Band of 98 kDa in Immunoblots of Rat Liver Nuclear Envelopes

Nup98-2 (residues 519–869) lacks the GLFG and FXFG repetitive motifs of Nup98 that also occur in rat nucleoporins p62, Nup153, Nup214, and the pore membrane protein POM121. (Left lane) SDS–PAGE-separated and Coomassie Blue–stained polypeptides of rat liver nuclear envelopes; (right lane) the separated polypeptides were transferred to nitrocellulose and incubated with affinity-purified anti-Nup98 antibodies followed by ^{125}I-labeled protein A. The M_r markers in kilodaltons are indicated on the left.

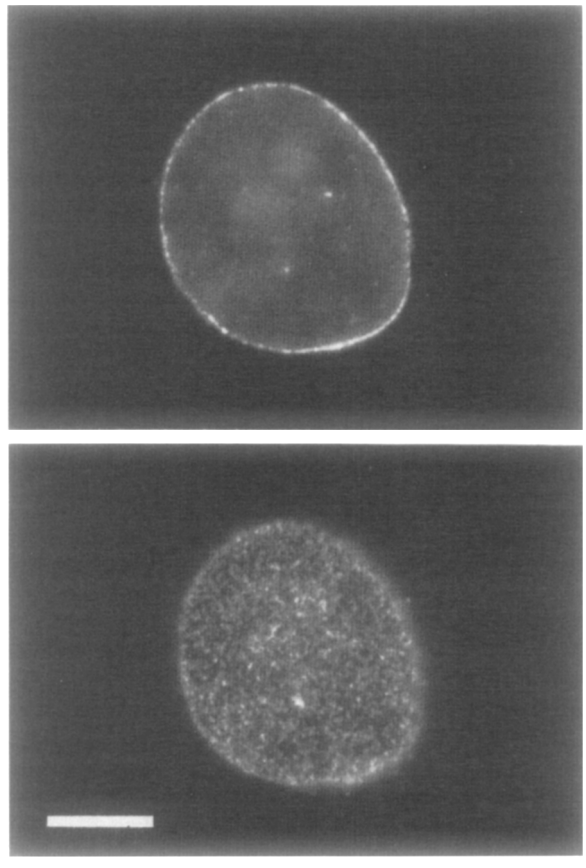

Figure 4. Localization of Nup98 by Indirect Immunofluorescence

BRL cells in culture were permeabilized and fixed with methanol, and then probed with affinity-purified anti-Nup98 antibodies followed by FITC-labeled goat anti-rabbit antibodies. The fluorescent signal shows the typical pattern for NPC staining: a discontinuous ring when the focal plane passes through the center of the nucleus (upper panel) and a punctate pattern when the image is tangential to the pore membrane (lower panel). Bar, 10 μm.

(Wente et al., 1992; Wimmer et al., 1992) (Figure 1; Figure 2). Based on the distribution of the repetitive GLFG motif and other structural features, Nup98 can be divided into four domains (Figure 1).

Domain I (residues 1–169) contains six GLFG repeats. These are separated from each other by spacer regions rich in serine, threonine, and asparagine, that contain nine FG motifs and a single FXFG motif, and that lack acidic residues. Moreover, the N-terminal residues MFNKS of Nup98 are identical to those of yeast GLFG NUP145 (Figure 2a).

Domain II (residues 170–212; Figure 1) contains a stretch of charged residues. A segment of this domain (residues 186–212) is 63% identical to a charged stretch in the yeast GLFG NUP116 (residues 140–166; Figure 2b).

Domain III (residues 213–513; Figure 1) contains ten GLFG motifs. As in domain I, the spacers between the GLFG motifs lack acidic residues. In addition, the spacers contain two FXFG and eleven FG motifs and are rich in serine, threonine, asparagine, and glutamine.

Domain IV (residues 514–937) lacks the repetitive GLFG, FXFG, and FG domains characteristic of some nucleoporins. However, it contains a sequence (residues 737–923) similar to that of some yeast GLFG nucleoporins (NUP145, NUP116, and NUP100) but absent in yeast GLFG NUP49 (Figures 2a and 2c). Near the C-terminal end of domain IV of Nup98, there is a segment of acidic residues (residues 886–893) that also occurs in a corresponding position in the yeast GLFG NUP145 (Figure 2a).

In addition, the octapeptide KGYGSIYF (residues 769–776) partially matches the RNP-1 binding motif K/RGF/YG/AFVXF/Y (Burd and Dreyfuss, 1994). This motif is also present in NUP145 and has been suggested to bind RNA (Fabre et al., 1994).

Localization of Nup98

Rabbit antibodies were raised against a segment of Nup98 (residues 519–869) that was expressed in Escherichia coli. Affinity-purified antibodies were monospecific, decorating only one protein of 98 kDa on a nitrocellulose blot of SDS–PAGE-separated polypeptides of isolated rat liver nuclear envelopes (Figure 3).

Immunofluorescence microscopy was done with cultured BRL cells that had been permeabilized and fixed with methanol. The fixed cells were probed with affinity-purified anti-Nup98 antibodies, and binding was visualized with fluorescein-labeled goat anti–rabbit IgG. A punctate nuclear rim staining (Figure 4, top) or a finely punctate nuclear surface staining (Figure 4, bottom) was observed. These staining patterns are characteristic for nucleoporins

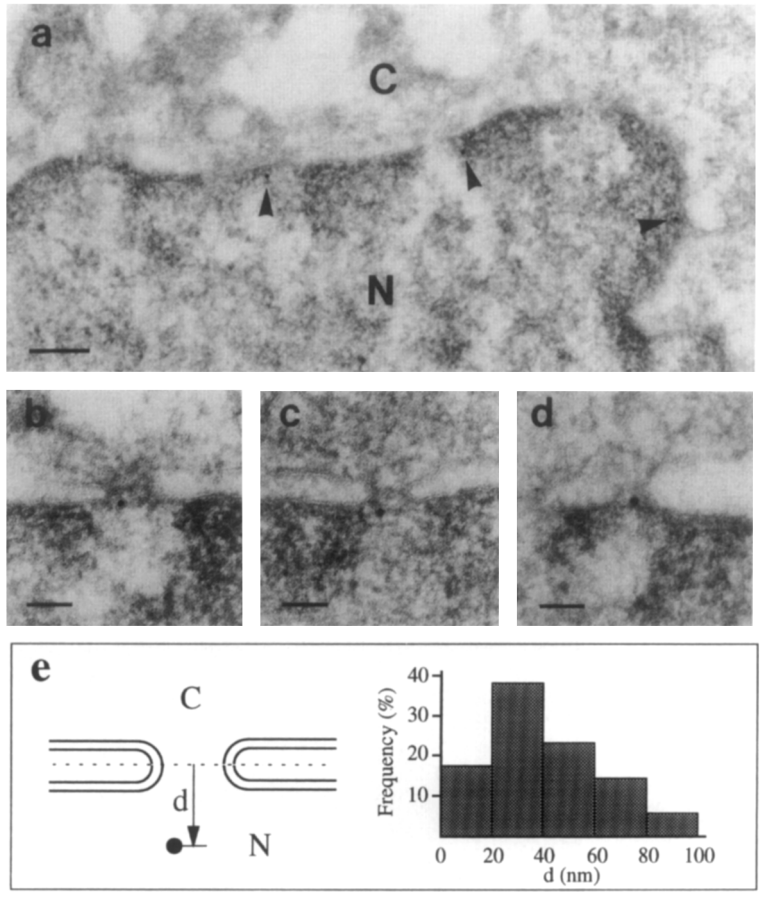

Figure 5. Immunoelectron Microscopy of BRL Cells Using Anti-Nup98 Antibodies

(a–d) BRL cells in culture were fixed with 2% paraformaldehyde, 0.05% glutaraldehyde, infused with sucrose, and frozen. Sections of the frozen cells were incubated with affinity-purified anti-Nup98 antibodies detected by gold-coupled goat anti-rabbit IgG. (a) Several NPCs; (b–d) single NPCs at higher magnification. Arrowheads point to gold particles. The gold particles are present on the nucleoplasmic side of the NPC. N, nucleus; C, cytoplasm. Bars, 200 nm (a), 100 nm (b–d).

(e) With respect to an imaginary surface passing at equal distance between the inner and outer nuclear membranes, the gold particles are located at a distance (d) ranging from 0 to 97 nm (average, 39 nm; n = 48). Particles are most frequently encountered in the range of 20–40 nm (see histogram at right).

(Davis and Blobel, 1986) and for pore membrane proteins (Wozniak and Blobel, 1992; Hallberg et al., 1993).

For immunoelectron microscopy, frozen ultrathin sections of BRL cells were incubated with affinity-purified anti-Nup98 antibodies and then with 10 nm gold–conjugated secondary antibodies. The gold particles decorated the nucleoplasmic side of NPCs (Figure 5). Nup98 appears to be a constituent of the basket structure and/or the nucleoplasmic ring of the NPC (Ris, 1991).

Function of Nup98

To determine whether Nup98 would be among the WGA-reactive nucleoporins that play a role in nuclear transport and to define this role in more detail, we developed a ligand blot assay. In this assay, various segments of Nup98 (Nup98-1, Nup98-2, and Nup98-3; Figure 6a) were expressed in E. coli, separated by SDS–PAGE (Figure 6b, lanes 1–4), transferred to nitrocellulose, and probed with ligand (nuclear localization signal–human serum albumin [NLS–HSA]) in the presence of Xenopus cytosolic fraction A, which was previously shown to function in docking NLS–HSA at the nuclear envelope (Moore and Blobel, 1992) (Figure 6b, lanes 5–8). We found that the near full-length Nup98-1 (Figure 6b, lane 6) and the N-terminal fragment Nup98-3 (Figure 6b, lane 7) bound NLS–HSA, whereas the C-terminal fragment Nup98-2 did not (Figure 6b, lane 8).

We also probed purified (see Figure 7a, lane 1) Nup98-3 (Figure 6c) and showed that NLS–HSA binding occurred only in the presence of Xenopus cytosolic fraction A (Figure 6c, lane 1 versus 5). Moreover, binding was specific. It could be competed by wild-type NLS peptide (Figure 6c, lane 2) but not by import-incompetent mutant NLS peptide (lane 3), and binding of HSA without conjugated NLS did not occur (lane 4). Together, these data suggested that Xenopus cytosolic fraction A mediates docking of the NLS substrate to the N-terminal domain of Nup98. As the N-terminal domain of Nup98 contains all of the the repetitive peptide motifs in Nup98, the former are likely to constitute the Nup98 docking site.

Because docking of NLS–HSA to Nup98-3 was dependent on Xenopus cytosolic fraction A, we used immobilized Nup98-3 to determine whether the docking activity of fraction A could be depleted. Indeed, incubation of fraction A with purified, immobilized Nup98-3 (Figure 7a) and subsequent testing of the nonbound material in a docking assay using digitonin-permeabilized cells and NLS–HSA showed complete depletion of fraction A's docking activity (Figure 7b, compare panels 1 and 2). In this assay, however, soluble Nup98-3 is potentially a competitor of NPC-bound Nup98, and if sufficient amounts of Nup98-3 leached off the column during incubation with fraction A, inhibition of docking might be due to leached off Nup98-3 rather than to depletion of docking activity from fraction

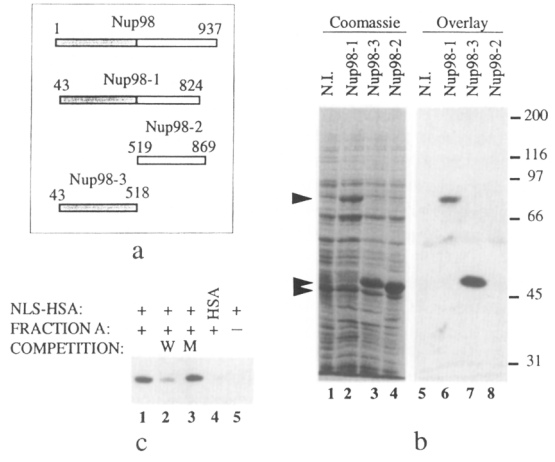

Figure 6. The Docking Site of Nup98 Is Located at Its Repetitive GLFG Motif–Containing N-Terminal Region

(a) The three segments of Nup98 termed Nup98-1, Nup98-2, and Nup98-3 that were expressed in E. coli. The shaded regions contain the repetitive GLFG motifs. The numbers indicate the terminal amino acid positions.

(b) Proteins of whole bacterial lysates were separated by SDS–PAGE and either stained with Coomassie Blue or transferred to nitrocellulose and probed for Xenopus cytosolic fraction A–dependent binding of NLS–HSA (see Experimental Procedures). Arrowheads indicate expressed proteins in lanes 2, 3, and 4. M_r standards in kilodaltons are indicated on the right. N. I., lysate of Nup98-1-transformed bacteria that were not induced.

(c) Purified Nup98-3 transferred to nitrocellulose after SDS–PAGE was probed as indicated. W, wild-type NLS; M, mutant NLS (Moore and Blobel, 1992); HSA, human serum albumin without conjugated NLS. W and M were used in 100:1 excess over NLS conjugated to HSA.

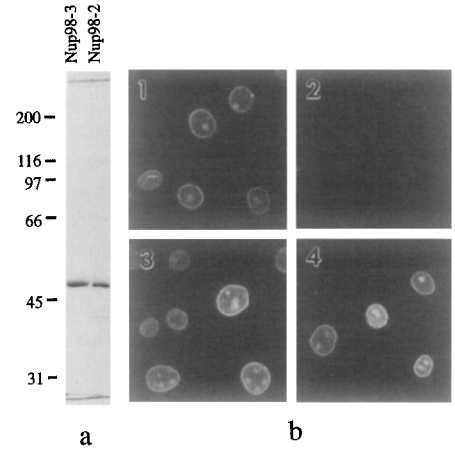

Figure 7. Immobilized GLFG-Containing Segment of Nup98 Depletes Docking Activity of Xenopus Fraction A

(a) Coomassie-stained SDS–PAGE gel of two His-tagged Nup98 segments, Nup98-3 and Nup98-2, after purification on Ni–NTA columns. M_r standards in kilodaltons are indicated on left.

(b) The purified proteins were reimmobilized on Ni–NTA colums and incubated with Xenopus fraction A for 1 hr at room temperature. The ability of fraction A to mediate docking of NLS–HSA in a digitonin-permeabilized cell assay (1) is lost after incubation with immobilized GLFG-containing Nup98-3 (2), but not after incubation with Nup98-2 (3). Buffer incubated with a Nup98-3 column does not inhibit fraction A–mediated docking (4), ruling out the possibility that the inhibition is caused by Nup98-3 leached from the column.

A. However, this scenario is unlikely, as incubation of immobilized Nup98-3 with buffer instead of cytosol did not yield inhibition of docking (Figure 7b, panel 3). A further control showed that Nup98-2, as expected, did not deplete the docking activity of fraction A (Figure 7b, panel 4).

Discussion

We report here the molecular characterization of a novel mammalian nucleoporin that contains numerous GLFG repeats and that we termed Nup98. By immunoelectron microscopy Nup98 was localized to the nucleoplasmic side and is therefore a likely component of the NPC's basket structure and/or nucleoplasmic ring. A blot assay using various immobilized segments of the recombinant Nup98 and the model substrate NLS-HSA as ligand showed cytosol-dependent ligand binding to the GLFG-containing N-terminal region of Nup98. Moreover, the immobilized N-terminal segment of Nup98 depleted the cytosol's NLS recognition and docking activity.

Nup98 is a mammalian member of a family of nucleoporins that contains numerous GLFG repeats. This family of nucleoporins was first discovered in yeast (Wente et al., 1992; Wimmer et al., 1992), where four of potentially five members have been molecularly characterized (Wente et al., 1992; Wimmer et al., 1992; Wente and Blobel, 1994; Fabre et al., 1994). The mammalian Nup98 shares primary structure homologies with all four characterized yeast GLFG nucleoporins (see Figure 2). It is therefore difficult to predict which, if any, of the yeast GLFG nucleoporin null mutants Nup98 might be able to complement.

Of particular interest is the immunoelectron microscopic localization of Nup98 to the nucleoplasmic side of the NPC, suggesting that it is a constituent of NPC's basket structure and/or nucleoplasmic ring (Ris, 1991). Nup98 is thus in the vicinity of Nup153, which has also been localized to the nuclear basket structure (Sukegawa and Blobel, 1993; Cordes et al., 1993; Panté et al., 1994). Like Nup98, Nup153 is a WGA-reactive nucleoporin that contains numerous peptide motifs, albeit of FXFG rather than of GLFG (Sukegawa and Blobel, 1993).

How could a nucleoporin that is located on the nucleoplasmic side of the NPC function as a docking protein for cytosolic factor–mediated nuclear import? We suggest that Nup98 is one of several docking site nucleoporins for bidirectional transport across the NPC. The existence of such multiple docking sites, successively extending over a distance of ~250 nm from the cytoplasmically exposed fibers to the nucleoplasmic baskets of the NPC, might be inferred from their decoration by nucleoplasmin-coated gold particles injected into amphibian oocytes (see Figure 5c of Richardson et al., 1988; Figure 3 and Figure 4 of Feldherr et al., 1984). Each of these gold particles might represent docking to one of these sites.

Moreover, using SDS–PAGE-separated nuclear envelope proteins transferred to nitrocellulose and a nuclear transport substrate (NLS–HSA) as a ligand, we previously showed Xenopus cytosolic fraction A–dependent binding

to at least four nucleoporins (p270, Nup214, Nup153, and Nup98; Radu et al., 1995; and unpublished data). These nucleoporins are located on both the cytoplasmic and nucleoplasmic side of the NPC (for review, see Rout and Wente, 1994). Moreover, these nucleoporins contain repetitive peptide motifs (for review, see Rout and Wente, 1994). Our mapping here of the docking site to the peptide repeat domain of Nup98 suggests that the repeat domains of the other nucleoporins may function in docking as well. We propose that a single repetitive peptide motif may constitute a low affinity binding site and that the strength of the binding would be proportional to the number of repetitive motifs.

Although the peptide repeat–containing nucleoporins have previously been implicated in nuclear transport (see Introduction), their precise function in this process had not been determined. Our demonstration here that the peptide repeat domain of Nup98 functions as a docking site constitutes biochemical evidence for a direct role in transport.

Binding of the transport substrate to the docking site nucleoporins is mediated by a cytosolic subfraction (fraction A; Figure 6c) (Radu et al., 1995). The repeat domain of Nup98 was shown to deplete the docking activity of fraction A (Figure 7) and, in one approach, was used for its purification (Radu et al., 1995). The docking activity of fraction A was shown to consist of a complex of two subunits, α and β, and termed karyopherin (Radu et al., 1995). Karyopherin α was found to function as an NLS receptor (Moroianu et al., 1995), whereas karyopherin β appears to function in docking the transport substrate–karyopherin α complex to the docking site nucleoporins (Moroianu et al., 1995; and unpublished data). Karyopherin α corresponds to Xenopus importin (Görlich et al., 1994) and most likely to p54/56 of bovine erythrocyte cytosol (Adam and Adam, 1994; Powers and Forbes, 1994), whereas karyopherin β is likely to correspond to p97 (Adam and Adam, 1994).

We propose that bidirectional transport across the NPC occurs via guided diffusion involving docking to and undocking from multiple docking sites that extend from the cytoplasmic to the nucleoplasmic ends of the NPC.

Serially arranged docking sites across the NPC may also facilitate the transport of very large substrates, such as large mRNPs (Mehlin et al., 1992). As such mRNPs contain numerous proteins, each with an NLS, it is conceivable that, for export, large "globular" mRNPs could be straightened into a millipede (each foot contributing an NLS) to cross the narrows of the NPC (Mehlin and Daneholt, 1993).

Experimental Procedures

Nup98 Isolation

WGA-binding proteins from rat liver nuclear envelopes were isolated as described (Radu et al., 1993). Briefly, nuclear envelopes were prepared by nuclease treatment (Dwyer and Blobel, 1976; Wozniak et al., 1989) of rat liver nuclei (Blobel and Potter, 1966). The nuclear envelopes were incubated with 2 M urea, 1 mM EDTA, and the extracted proteins were separated from the nuclear envelopes by 20,000 × g centrifugation. The trichloroacetic acid (TCA)–precipitated proteins were solubilized in 2% SDS, 20 mM dithiothreitol (DTT), diluted to 0.1% SDS, 1 mM DTT, and loaded batchwise on a WGA–Sepharose column. The retained proteins were eluted with 0.5 M N-acetylglucosamine (Aldrich, Milwaukee, WI) and 0.38 mM N,N′,N″-triacetylchitotriose (Sigma, St. Louis, MO). The eluate was TCA-precipitated and solubilized in SDS sample buffer; the proteins were separated by SDS–PAGE. Polypeptides were electrophoretically transferred to nitrocellulose membranes and visualized with Ponceau S. A band of 98 kDa was excised and digested with endoproteinase Lys-C, as described by Fernandez et al. (1992). The resulting peptides were separated by reverse-phase high performance liquid chromatography and subjected to N-terminal sequence analysis (Fernandez et al., 1992).

Cloning and Sequencing

Two degenerate PCR primers were synthesized based on the peptide sequence information (sense primer, amino acid positions 802–807; antisense, positions 819–824; Figure 1). The exact nucleotide sequence between these primers was determined by sequencing the PCR product generated from a rat liver cDNA template, as described (Radu et al., 1993). Based on this sequence information, a single-stranded DNA probe of 35 bases was synthesized and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. 200,000 plaque-forming units of a λgt10 cDNA library derived from BRL cells (Sukegawa and Blobel, 1993) were screened as described (Radu et al., 1993, 1994), and five partial, overlapping clones were selected. The phage DNA was subcloned in pBluescript SK(−) and sequenced in both directions. Software from DNASTAR, Inc. (Madison, WI) was used for sequence analysis, and searches in the protein data bases were based on the algorithm developed by Altschul et al. (1990).

Expression of Recombinant Proteins

Three segments of Nup98 (Nup98-1, amino acid residues 43–824; Nup98-2, positions 519–869, and Nup98-3, positions 43–518) were inserted between the BamHI and EcoRI sites of the pET21-b vector (Novagen, Madison, WI) containing a six His tag. The constructs were used to transform E. coli BL21(DE3) cells. The recombinant proteins were purified from inclusion bodies on Ni-nitrilotriacetic acid-agarose columns (Qiagen, Chatsworth, CA) under denaturating conditions as described in the Qiagen QIAexpress system instructions.

Raising Antibodies

The purified peptide Nup98-2 was subjected to SDS–PAGE; the Nup98-2 band was cut from the dried gel, rehydrated, homogenized, and used to immunize rabbits. For affinity purification, the antiserum was dialyzed against 50 mM Tris–HCl (pH 7.4) and centrifuged for 30 min at 120,000 × g. The supernatant was incubated batchwise for 2 hr at room temperature with Ni-nitrilotriacetic acid-agarose containinig immobilized Nup98-2. The column was washed with 1 M NaCl in 50 mM Tris–HCl (pH 7.4) and eluted with the ImmunoPure gentle Ag/Ab elution buffer from Pierce (Rockford , IL). The elution buffer was removed by three cycles of concentration in a Centricon 100 concentrator (Amicon, Beverly, MA), followed by dilution in 50 mM Tris–HCl (pH 7.4). Finally, the antibody was dialyzed against PBS.

Immunoblot Analysis

The nuclear envelope polypeptides separated by SDS–PAGE were transferred to nitrocellulose and processed as described by Radu et al. (1993), except that 2% nonfat dry milk replaced gelatin as a blocking agent, and all solutions were buffered with PBS. Blots were incubated with a 1:300 dilution of the affinity-purified antibody, and the detection was done with ^{125}I-labeled protein A.

Immunofluorescence

BRL cells in culture were permeabilized and fixed with cold methanol for 6 min at −200°C and further processed as described by Radu et al. (1993). Nonfat dry milk was used as blocking agent, and the affinity-purified anti-Nup98-2 antibody, incubated at a 1:100 dilution, was visualized by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG.

Immunoelectron Microscopy

Ultrathin cryosections from BRL cells in culture were obtained and processed as described (Radu et al., 1993). The affinity-purified anti-Nup98-2 antibody, incubated at a 1:500 dilution, was detected by goat anti-rabbit IgG bound to 10 nm gold.

Ligand Blotting Assay

E. coli expressing Nup98-1, Nup98-2, and Nup98-3 were lysed in SDS–PAGE sample buffer, subjected to electrophoresis, and transferred to nitrocellulose in 0.2 M Tris–glycine buffer (pH 8.4), 0.01% SDS, 16.6% methanol. The nitrocellulose strips were washed with distilled water and rinsed two times for 10 min each in 40% 2-propanol. The blots were washed extensively with distilled water, blocked for 1 hr at room temperature in blocking buffer (2% Carnation nonfat dry milk in PBS, 0.2% Tween 20, 0.2 mM phenylmethylsulfonyl fluoride), and washed two times for 10 min each in buffer A (20 mM HEPES–KOH [pH 7.3], 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 2 mM DTT) containing 2 mg/ml gelatin (Sigma G-2625). In the meantime, Xenopus fraction A (1 mg/ml in buffer A containing 2 mg/ml gelatin) was incubated with the conjugate NLS–HSA (the nuclear localization signal of the SV-40 T antigen coupled to human serum albumin; 1 μg/ml final protein concentration) for 15 min at room temperature, then Tween 20 was added to a final concentration of 0.2%. The nitrocellulose strips were incubated with the mixture for 1 hr at room temperature, washed in buffer A, and blocked for 5 min in blocking buffer. The bound NLS–HSA conjugate was detected by incubation with rabbit anti-HSA IgG (Sigma, St. Louis, MO) followed by ^{125}I-labeled protein A (New England Nuclear, Boston, MA) and radioautography for 2–5 hr using XAR-5 film (Eastman Kodak, Rochester, NY) at −800°C. Free wild-type and mutant NLSs (Moore and Blobel, 1992) were used in competition experiments.

Depletion of Fraction A Docking Activity

The purified Nup98-2 and Nup98-3 (100 μg each) was immobilized on 100 μl Ni–NTA resin and allowed to refold in a descending gradient of 6–1 M urea, as described in the Qiagen QIAexpress system instructions. The resin batches were equilibrated with buffer A containing 2 mM 2-mercaptoethanol instead of DTT and 5 mM ATP and incubated for 1 hr at room temperature with 600 μl of Xenopus fraction A (4 mg of protein per milliliter) supplemented with 5 mM ATP. The load and the flow-through fractions were tested for the ability to mediate docking of the rhodamine-labeled conjugate NLS–HSA to the nuclear envelope, as described by Adam et al. (1990), with the modifications of Moore and Blobel (1992). BRL cells grown on coverslips were permeabilized for 5 min on ice with 35 μg/ml digitonin, then incubated for 15 min at room temperature with 10 μg/ml NLS–HSA and 1 mg/ml of the load or flow-through fractions (see above). After washing, the cells were fixed with 3% paraformaldehyde and mounted for immunofluorescence.

Acknowledgments

We thank Richard W. Wozniak for providing the peptide sequence information, Jun Sukegawa for the cDNA library, Helen Shio for the preparation of the immunoelectron microscopy specimens, and members of the Rockefeller University Biopolymer Facility for protein sequencing.

Received August 30, 1994; revised February 10, 1995.

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GenBank Accession Number

The accession number for the Nup98 sequence reported in this paper is L39991.