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Two functionally distinct domains generated by *in vivo* cleavage of Nup145p: a novel biogenesis pathway for nucleoporins

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Nup145p is an essential yeast nucleoporin involved in nuclear export of polyadenylated RNAs. We demonstrate here that Nup145p is cleaved *in vivo* to yield two functionally distinct domains: a carboxy-terminal domain (C-Nup145p) which is located at the nuclear pore complex (NPC) and assembles into the Nup84p complex, and a GLFG-containing amino-terminal domain (N-Nup145p) which is not part of this complex. Whereas the essential C-Nup145p accomplishes the functions required for efficient mRNA export and normal NPC distribution, N-Nup145p, which is homologous to the GLFG-containing nucleoporins Nup100p and Nup116p, is not necessary for cell growth. However, the N-Nup145p becomes essential in a *nup188* mutant background. Strikingly, generation of a free N-domain is a prerequisite for complementation of this peculiar synthetic lethal mutant. These data suggest that N- and C-domains of Nup145p perform independent functions, and that the *in vivo* cleavage observed is of functional importance.

Keywords: mRNA export/nuclear pore complex distribution/nucleoporin/protein cleavage/yeast

Introduction

Nuclear pore complexes (NPCs) are evolutionarily conserved structures that allow passage of soluble material through the nuclear envelope. While the aqueous channels of the NPCs allow passive diffusion of small molecules (<40 kDa), most proteins and RNAs are translocated through the pore by a process involving steps of GTP hydrolysis (for review see Gorlich and Mattaj, 1996). Active nucleocytoplasmic transport through NPCs is bidirectional, signal-mediated and involves different soluble shuttling factors (for review see Gerace, 1995; Koepp and Silver, 1996).

Yeast NPCs are of estimated molecular weight 66 MDa and could be composed of about 100 distinct proteins (called Nups or nucleoporins; Rout and Blobel, 1993). The role of NPC proteins in transport reactions is not yet elucidated even if there are numerous yeast nucleoporin mutants that accumulate mRNA inside the nucleus or are impaired in nuclear protein import under restrictive conditions. Nup1p, Nsp1p, Nic96p, Nup49p and Nup57p are yeast NPC proteins implicated in protein uptake into the nucleus (Nehrbass *et al.*, 1990; Grandi *et al.*, 1993, 1995b; Bogerd *et al.*, 1994). For some of them, a physical interaction with the cytoplasmic receptors that recognize nuclear localization sequences (NLSs) has been demonstrated, suggesting that these nucleoporins may serve as docking sites for the import complexes (Belanger *et al.*, 1994; Dingwall *et al.*, 1995; Iovine *et al.*, 1995; Rexach and Blobel, 1995). Nuclear pore proteins may also act as a receptor for factors involved in export reactions, since a cellular protein (Rab/hRip), which contains several FG repeat sequences typically found in nucleoporins, binds to a nuclear export sequence (NES) of the HIV Rev protein which, itself, exports unspliced and partially spliced viral mRNA from the nucleus to the cytoplasm (Stutz and Rosbash, 1994; Bogerd *et al.*, 1995; Fritz *et al.*, 1995).

Efficient mRNA export from the nucleus to the cytoplasm similarly requires a large number of yeast nucleoporins. Indeed, mutations in Nup1p, Nup82p/Nle4p, Nup116p, Rat7/Nup159p, Rat3/Nup133p, Nup49p and some components of the Nup84p sub-complex (Nup84p, Nup85p/Rat9p, Nup120p/Rat2p) lead to the accumulation of mRNA in the nucleus (Wente and Blobel, 1993; Bogerd *et al.*, 1994; Doye *et al.*, 1994; Aitchison *et al.*, 1995a; Gorsch *et al.*, 1995; Grandi *et al.*, 1995a; Hurwitz and Blobel, 1995; Kraemer *et al.*, 1995; Li *et al.*, 1995; Goldstein *et al.*, 1996; Siniossoglou *et al.*, 1996). In some cases, like in Nup116p deletion mutants, it has been suggested that a defect in export of polyadenylated RNAs might be indirectly caused by structurally sealed NPCs (Wente and Blobel, 1993). However, many other of these mutants do not exhibit structural NPC abnormalities, suggesting that these nucleoporins might directly participate in RNA export reactions. That an NES-containing protein essentially involved in mRNA export, Gle1p, is found in association with NPCs suggests that at least some export factors, like import factors, have transient interactions with nucleoporins (Murphy and Wente, 1996). It is likely that some of these NPC components would interact with mediators of mRNA export, like the hnRNP proteins, which are tightly bound to mRNA, accompany mRNA during passage from the site of synthesis to

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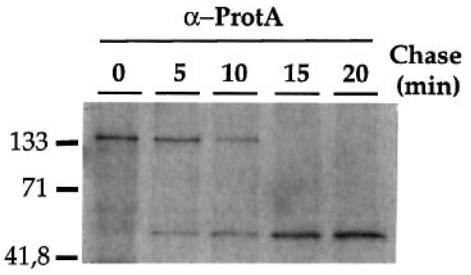

Fig. 1. Nup145p is cleaved *in vivo*. Cells were pulse-labelled for 5 min and chased for the indicated time points. Proteins were extracted and immunoprecipitated with IgGs recognizing ProtA–Nup145p (Fabre *et al.*, 1994). Samples were analysed by SDS–PAGE, followed by fluorography. Molecular weights of a protein standard (kDa) are indicated on the left.

the cytoplasm, and shuttle between the nucleus and the cytoplasm (Dreyfuss *et al.*, 1993).

Nup145p is one of the NPC proteins whose depletion from yeast cells causes a nuclear retention of poly(A)$^+$ RNAs, but does not directly affect nuclear protein import (Fabre *et al.*, 1994). Upon biochemical purification, Nup145p is cleaved into two distinct moieties: a carboxy-terminal domain (C-Nup145p) which does not share any sequence homology to known proteins, and an amino-terminal domain (N-Nup145p) that has homology to two other yeast nucleoporins, Nup100p and Nup116p (Fabre *et al.*, 1994; Wente and Blobel, 1994). This homology includes the presence of GLFG repeat sequences and a nucleoporin RNA-binding motif (NRM) which binds to homopolymeric RNA *in vitro* (Fabre *et al.*, 1994). Here we report that cleavage of Nup145p occurs *in vivo* and describe that the two different domains perform separate functions. The C-Nup145p assembles into the Nup84p complex and is required for normal NPC distribution and mRNA export. The N-Nup145p is not essential for cell growth, but becomes crucial if other nucleoporins are mutated.

Results

Nup145p is cleaved in vivo

Although the *NUP145* gene potentially codes for a 145 kDa nucleoporin, it is detected as two proteolytically cleaved products of 65 and 80 kDa (Fabre *et al.*, 1994; Wente and Blobel, 1994). To determine whether the event that cleaves Nup145p occurs *in vivo* and thus may have a functional relevance, maturation of Nup145p was followed in living cells by pulse–chase analysis. For this purpose, ProtA–Nup145p, an amino-terminally tagged form of the protein that can functionally replace the essential Nup145p (Fabre *et al.*, 1994), was used. Exponentially growing yeast cells were pulse-labelled for 5 min with [^{35}S]methionine/cysteine before chase with excess of unlabelled amino acids. ProtA–Nup145p was then immunoprecipitated from cell aliquots taken after various time points of chase (see Materials and methods). After pulse-labelling of yeast cells, a precursor polypeptide of expected molecular weight (~136 kDa) was detected (Figure 1). During the chase, this precursor band was still visible after 5 min, but started to vanish after 10 min. Concomitant to the disappearance of the precursor, a new band of 57 kDa which corresponds to the tagged N-Nup145p is detected. Similar results were obtained when wild-type Nup145p was immunoprecipitated with an antibody specific for the amino-terminal domain of the protein (data not shown). When a mutant form of Nup145p (ProtA–Nup145NC1p, see below), which is not cleaved, was used in a similar pulse–chase experiment, solely the full-length ProtA–Nup145NC1p was detected (not shown). These experiments indicate that Nup145p is initially synthesized as a precursor, before it is cleaved *in vivo* into an amino- and carboxy-terminal domain. These data also explain why under steady-state conditions Nup145p is mainly found in its cleaved two forms, N-Nup145p and C-Nup145p (Fabre *et al.*, 1994; Wente and Blobel, 1994).

Cleavage site of Nup145p is conserved during evolution

To determine the cleavage site within Nup145p and the residues crucial for *in vivo* processing, intragenic mutants impaired in Nup145p maturation were sought. The generation of a 65 kDa amino-terminal and 80 kDa carboxy-terminal fragment and sequence data derived from an amino-terminal peptide of C-Nup145p (Wente and Blobel, 1994) suggested that cleavage should occur between F_{605} and S_{606} (Figure 2). We therefore performed a random PCR mutagenesis of the DNA surrounding the S_{606} encoding region and obtained a collection of non-cleavable (NC) *NUP145* mutant alleles (see Materials and methods). DNA sequencing of the mutant alleles revealed that non-cleavable forms of Nup145p carry mutations in $F_{606}\rightarrow$S (NC1) and $H_{604}\rightarrow$P (called NC2), respectively. An additional $W_{608}\rightarrow$R or L mutation is found in both mutants. The W_{608} mutation alone (NC3), however, did not inhibit the cleavage reaction (Figure 2A).

Databases were searched for proteins sharing homology with Nup145p. Nuclear pore proteins Nup116p and Nup100p from yeast, and Nup98p from human and rat, were found similar to N-Nup145p (Fabre *et al.*, 1994; Wente and Blobel, 1994; Radu *et al.*, 1995). In addition, a fragment of an EST sequence of *Caenorhabditis elegans* was found to match the NRM of Nup145p (Fabre *et al.*, 1994). Release of the entire genomic sequence corresponding to this cDNA in AceDB (ZK328.5B) reveals that homology with Nup145p is extended (Figure 2B). In particular, amino acids that were defined as important for Nup145p maturation (see above) are conserved between hNup98, rNup98, ZK328.5B and Nup145p. This cDNA might encode a putative *C.elegans* nuclear pore protein since the most amino-terminal domain contains several GLFG motifs (not shown).

The non-cleavable mutant proteins Nup145NC1p and Nup145NC2p were functional, since they could fully complement the growth arrest of a Δ*nup145* null strain (not shown; see Materials and methods). These data confirm our earlier conclusion that cleavage of Nup145p into an N- and C-terminal domain is not required for its essential *in vivo* function, at least not in a genetic background in which other nucleoporins are intact (see also later).

Post-translational maturation has a role in intracellular targeting of Nup145p

To determine whether the cleavage of Nup145p has a role in the localization of the two generated domains,

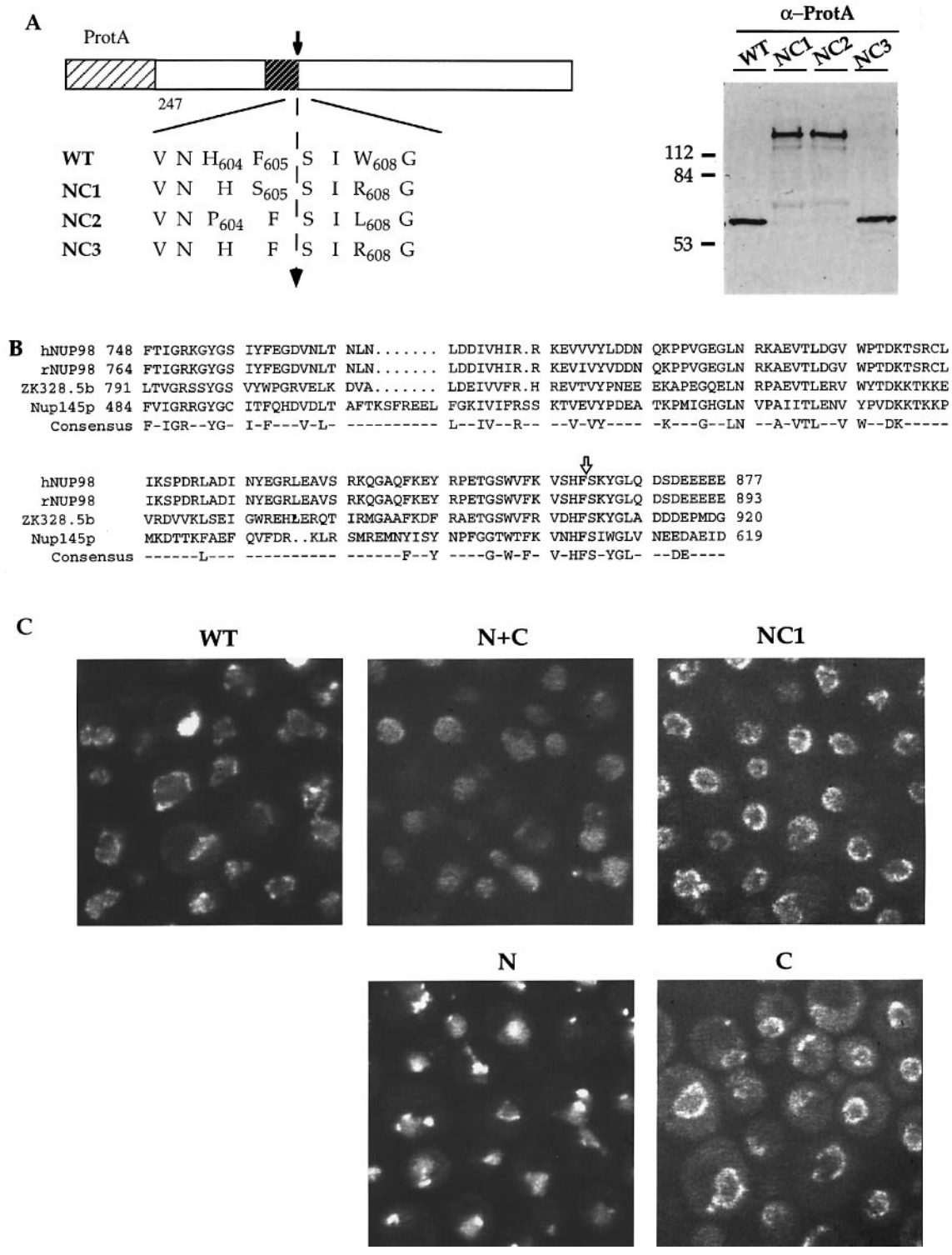

Fig. 2. Analysis of mutants defective in cleavage of Nup145p. (**A**) A schematic representation of the ProtA–Nup145p fusion protein is shown in the left part of the figure. In the construct, GLFG repeat sequences were replaced at amino acid position 247 by the protein A tag. The sequence around S_{606} is depicted for wild-type Nup145p and the three mutants NC1, NC2 and NC3. In the right part of the panel, whole-cell extracts derived from cells expressing ProtA–Nup145p (WT) and mutants ProtA–Nup145NCp (NC1, NC2 and NC3) were analysed by SDS–PAGE and Western blotting using anti-protein A antibodies (α-ProtA). Molecular weights (kDa) are indicated on the left. A band corresponding to the cleaved ProtA–Nup145Np (57 kDa) is observed when extracts were derived from strains expressing WT or NC3 constructs. A band corresponding to uncleaved ProtA–Nup145p (136 kDa) is seen when extracts were derived from strains expressing NC1 or NC2 constructs. (**B**) Sequence comparison between Nup145p, human and rat Nup98 and a cDNA clone from *C.elegans*. The consensus sequence is shown and the position of the putative cleavage site is indicated by an arrow. (**C**) Subcellular localization of cleaved and non-cleaved Nup145p fusion proteins as revealed by direct confocal fluorescence microscopy. Strains expressing GFP–Nup145p (WT) and GFP–Nup145NC1p (NC1) show a punctuate staining of the nuclear periphery, while strains expressing GFP–Nup145Np together with a free Nup145Cp (N+C) show both a ring-like and a nucleoplasmic staining. When expressed independently of each other, GFP–Nup145Np (N) is found both at the NPCs (clustered because of the lack of C–Nup145p) and in the nucleoplasm, while GFP–Nup145Cp (C) is only located at the pores.

subcellular localization of N-Nup145p when part of the wild-type precursor was compared with that of N-Nup145p when it is expressed separately from C-Nup145p and when it is not releasable from the latter (Figure 2C). Nup145p, N-Nup145p and Nup145NC1p were thus tagged at their amino-terminal ends with the green fluorescent protein (GFP, see Materials and methods). GFP–Nup145p, like GFP–Nup145NC1p, extends from M_1 to Y_{1318}, GFP–Nup145Np extends from M_1 to F_{605}, and GFP–Nup145Cp (or ProtA–Nup145Cp used in this *trans*-complementation experiment) extends from S_{606} to Y_{1318}. Subcellular localization was examined in either the Δ*nup145* null strain, or in nup133$^-$ cells, in which the nuclear pores cluster (Doye *et al.*, 1994). Confocal microscopic analysis of living cells is shown in Figure 2C. As expected, GFP–Nup145p shows a punctuate nuclear envelope staining (see also Fabre *et al.*, 1994). This staining co-localizes with clustered NPCs in the nup133$^-$ strain (not shown). A similar localization is observed with GFP–Nup145NC1p, indicating that cleavage is not required for proper localization of the amino-terminal end of the protein at the NPC. However, when GFP–Nup145Np is localized in the presence of C-Nup145p provided in *trans*, the N-terminal domain shows both an NPC and a nucleoplasmic staining.

To determine whether N- and C-moieties of Nup145p depend on each other to be localized at the NPC, GFP–Nup145Np or GFP–Nup145Cp were independently expressed. When tested for functionality, GFP–Nup145Cp alone could fully complement the Δ*nup145* null mutant. In contrast, expression of GFP–Nup145Np could only partly complement the lethal phenotype of the Δ*nup145* null mutant. This is seen by a reduced growth rate of the mutant at 30°C ($t_{1/2}$ = 3.5 h as compared with $t_{1/2}$ = 1.2 h for wild-type cells) and growth arrest of cells at 37°C. This result shows that C-Nup145p performs the most crucial function for cell viability, whereas N-Nup145p is dispensable for cell growth. Confocal analysis showed that GFP–Nup154Np is localized both at the NPCs (clustered because of the lack of C-Nup145p; see below) and in the nucleoplasm, while GFP–Nup145Cp is located only at the nuclear pores.

Taken together, these data indicate that both N- and C-domains of Nup145p contain signals for NPC targeting, but association of N-Nup145p with nuclear pores is efficient only when it is derived from *in vivo* cleavage of the full-length Nup145p.

The carboxy-terminal domain of Nup145p assembles into the Nup84p nucleoporin complex

In order to unravel the function of N-Nup145p and C-Nup145p, interacting components for both domains were searched. For this purpose, the amino-terminal ends of each domain were tagged with protein A which allows affinity purification under non-denaturing conditions by IgG–Sepharose chromatography (Figure 3A). ProtA–Nup145Np extends from S_{247} to F_{605}, and ProtA–Nup145Cp from S_{606} to Y_{1318} and complement Δ*nup145* null mutant in a manner identical to the GFP fusion proteins.

When interacting proteins were analysed by SDS–polyacrylamide gel electrophoresis followed by Coomassie blue staining (Figure 3B) and Western blotting (Figure 3C), it was found that ProtA–Nup145Cp (estimated molecular

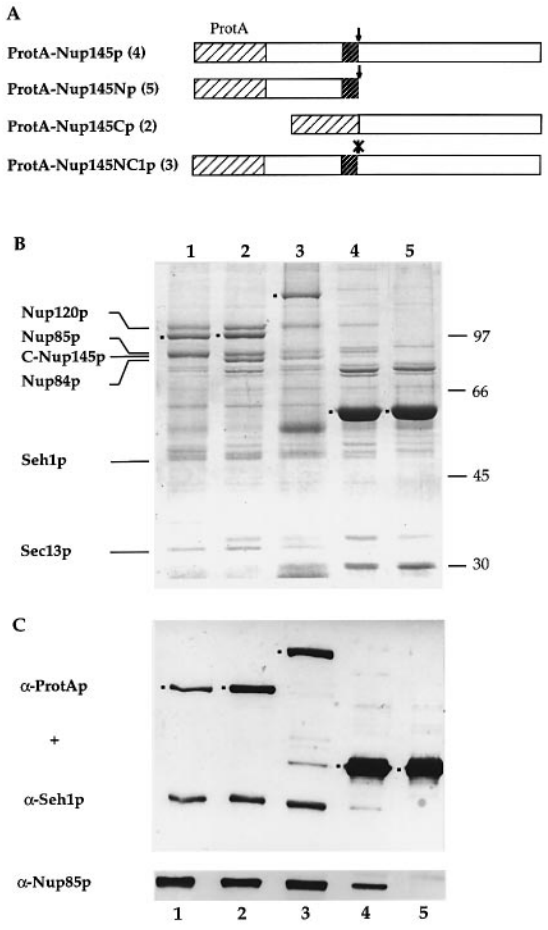

Fig. 3. The carboxy-terminal domain of Nup145p assembles into the Nup84p nucleoporin complex. (**A**) A schematic representation of the ProtA–Nup145p fusion proteins is shown; ProtA–Nup145Cp (2), ProtA–Nup145NC1p (3), ProtA–Nup145p (4) and ProtA–Nup145Np (5). Affinity purification of ProtA fusion proteins by IgG–Sepharose chromatography was performed as described in Materials and methods. Eluates from affinity chromatography with ProtA–Nup84p (1), and ProtA–Nup145p fusion proteins were analysed by SDS–PAGE and Coomassie blue staining (**B**), or Western blotting (**C**) using IgG coupled to HRP to detect the protein A moiety of the fusion proteins (α-ProtAp) or antibodies specific for Seh1p (α-Seh1p) and Nup85p (α-Nup85p). The positions of the proteins of the Nup84p complex are indicated. The positions of ProtA fusion proteins are marked by a filled square. Note that C-Nup145p co-migrates with Nup85p in the complex when purified via ProtA–Nup84p.

weight of 96 kDa, Figure 3B, lane 2; and corresponding Western blot, Figure 3C, lane 2) is in physical association with several prominent bands. The pattern of these major co-purifying bands strikingly resembles the pattern of a previously described complex which includes Nup120p, Nup85p, band IV, Nup84p, Seh1p and Sec13p (Siniossoglou *et al.*, 1996; Figure 3B, lane 1). This suggested that C-Nup145p could be part of this nucleoporin complex. Band IV was isolated from an SDS–polyacrylamide gel in an essentially pure form; this was possible when the complex was affinity purified via ProtA–Nup84p from a nup84$^-$ strain in which Nup85p was not full-length, but amino-terminally truncated (Siniossoglou *et al.*, 1996). Mass spectroscopic analysis unequivocally showed that band IV is the carboxy-terminal domain of Nup145p. In addition, band IV is recognized by antibodies raised against C-Nup145p (data not shown). By Western blotting

it was further verified that Nup85p, Seh1p (Figure 3C), Nup84p and Sec13p (data not shown) are major co-purifying bands of the ProtA–Nup145Cp eluate. The 120 kDa band is likely to be Nup120p, because it co-migrates with the Nup120p band in the Nup84p complex (Figure 3B, compare lanes 1 and 2). Peptide sequence analysis of a 70 kDa band (Figure 3B, lane 2) showed that it corresponds to Hsp70p. However, Hsp70p is often detected in protein purification, arguing that it may be an unspecific contaminant (Craig *et al.*, 1993). These data demonstrate that C-Nup145p is part of Nup84p sub-complex.

When full-length ProtA–Nup145p was affinity purified, a 57 kDa band corresponding to ProtA–Nup145Np bound efficiently to the IgG–Sepharose column, but the bands corresponding to the other members of the Nup84p complex (Nup120p, Nup85p, C-Nup145p, Nup84p, Seh1p and Sec13p) were also associated (Figure 3B and C, lane 4). However, these bands were clearly sub-stoichiometric as compared with results obtained with ProtA–Nup145Cp (Figure 3B, lane 2). These data indicate that N-Nup145p may not be part of the complex after cleavage. To test for this, ProtA–Nup145NC1p was isolated by IgG–Sepharose chromatography. It was observed that Nup120p, Nup85p, Nup84p, Seh1p and Sec13p could be recovered in approximately stoichiometric ratios (Figure 3B, lane 3). Taking these results together, we suggest that Nup145p precursor is targeted to the NPC Nup84p sub-complex, and after cleavage, the carboxy-terminal domain remains in interaction with it, while the amino-terminal part is (largely) released from the complex (see also Discussion).

To determine further the possible interacting components of free N-Nup145p, affinity purification was performed using a lysate derived from strain FYEF95 (ΔC) which expresses solely ProtA–Nup145Np. Although ProtA–Nup145Np could be efficiently affinity purified, none of the components of the Nup84p complex was associated in significant amounts (Figure 3B, lane 5). This result was also confirmed by Western analysis using antibodies against Nup85p and Seh1p (Figure 3C, lane 5). An identical pattern was obtained when a ProtA–Nup145Np fusion protein containing the GLFG repeats was used (not shown). The stronger band which migrates in the 70 kDa region, also detected in ProtA–Nup145p eluate, was analysed and shown to correspond to Hsp70p. These data indicate that under these conditions N-Nup145p does not have stable interacting partners.

Yeast cells lacking the carboxy-terminal domain of Nup145p are impaired in mRNA export and NPC distribution

The data described above indicated that C-Nup145p assembles into the Nup84p complex. As members of this complex (e.g. Nup84p, Nup85p and Nup120p) participate in mRNA export and normal NPC distribution (Siniossoglou *et al.*, 1996), we suspected that C-Nup145p performs a similar function. Nuclear export of polyadenylated RNA was examined in the FYEF95 (ΔC) strain (Figure 4A). At 30°C, a temperature at which this strain grows, albeit very slowly (see also above), ~30% of cells accumulate mRNA inside the nucleus. Following transfer to 37°C, progressively more and more cells show nuclear poly(A)$^+$ accumulation, with 100% of cells positive after 7 h (Figure 4A). In contrast, no apparent defect in nuclear protein import could be detected as judged by the nuclear accumulation of a Matα-2–lacZ reporter protein (not shown). Interestingly, >80% of the FYEF95 (ΔC) cells were able to recover growth if shifted back from 37°C to 30°C, indicating that the mutant is thermoreversible.

In this mutant, a nucleolar disintegration was also observed. The nucleolus was fragmented into several foci in ~10% of the cells at 30°C and in 30% of the cells when shifted for 7 h to 37°C. Furthermore, we show that lack of C-Nup145p induces nuclear pore clustering. This was demonstrated by the use of a nucleoporin tagged with GFP (GFP–Nup49p; Belgareh and Doye, 1997). Whereas in wild-type cells GFP–Nup49p gives a typical ring-like staining of the nuclear envelope, the same NPC reporter construct clusters in 100% of FYEF95 (ΔC) cells, even when grown at 30°C (Figure 4A). This is in agreement with the observation in Figure 2C that GFP–Nup145Np, when expressed alone, is partly localized in clustered NPCs. NPC clustering was further analysed by electron microscopy (Figure 4B). In a wild-type strain, a normal nuclear and NPC morphology was observed. In the strain lacking C-Nup145p, nuclear envelope morphology appears unaffected, but NPCs heavily cluster at one site of the nuclear membrane and no double nuclear membrane could be seen between the clustered NPCs (Figure 4B). On the other hand, in strain FYEF94 (ΔN) which expresses solely C-Nup145p, NPCs show no tendency for clustering (Figure 4C). This is consistent with the observation that GFP–Nup145Cp is targeted to apparently normal NPCs (see Figure 2C).

We conclude that, like other members of the Nup84p NPC sub-complex, the carboxy-terminal domain of Nup145p, but not its amino-terminal part, is required for a normal distribution of NPCs within the nuclear membrane, as well as for normal nuclear export of polyadenylated RNAs.

Isolation of a synthetically lethal mutants in which the amino-terminal domain of Nup145p is required for cell growth

Since N-Nup145p alone does not stably interact with other NPC components and is not required for cell growth, a colony sectoring assay was designed to identify mutated genes that show synthetic lethality when N-Nup145p is missing (see Materials and methods). A synthetically lethal mutant (called nsl7) could be obtained from such a screen. nsl7 was complemented by the cleavable ProtA–Nup145p and ProtA–Nup145Np, but not by ProtA–Nup145Cp (Figure 5). This shows that the presence of the amino-terminal domain of Nup145p in nsl7 is essential for cell growth. We next tested whether cleavage of Nup145p is a prerequisite to complement the synthetic lethal phenotype of this mutant. Strikingly, ProtA–Nup145NC1p, which is no longer cleaved *in vivo* (see also Figure 2), was strongly impaired in restoring growth of the nsl7 strain on 5-FOA-containing plates (Figure 5A). We therefore conclude that in a certain mutant background a separate Nup145p amino-terminal domain is essential for cell growth. If the release of this domain from the precursor protein is inhibited from full-length Nup145p, it cannot perform its function.

The mutation which caused synthetic lethality in nsl7

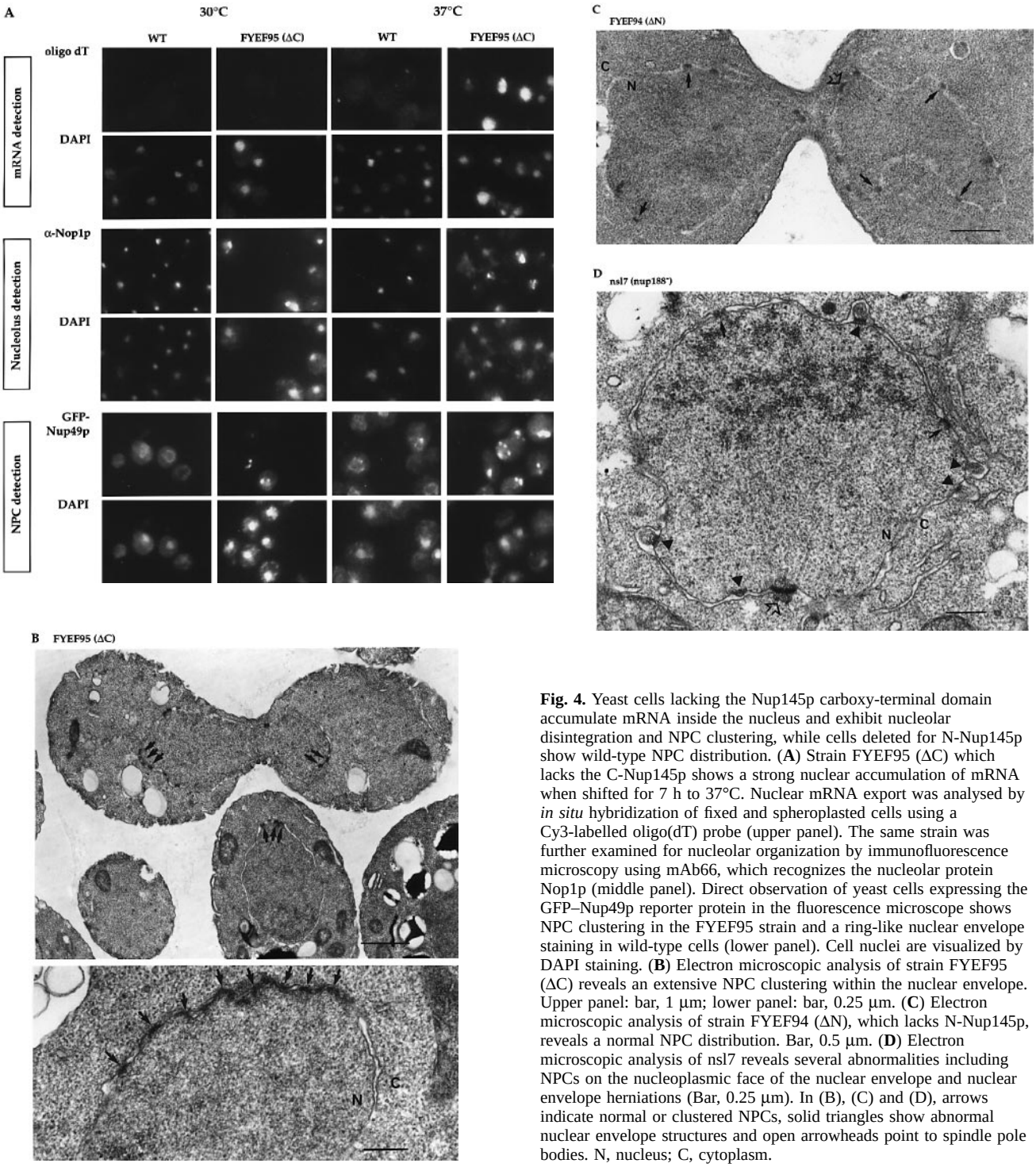

Fig. 4. Yeast cells lacking the Nup145p carboxy-terminal domain accumulate mRNA inside the nucleus and exhibit nucleolar disintegration and NPC clustering, while cells deleted for N-Nup145p show wild-type NPC distribution. (**A**) Strain FYEF95 (ΔC) which lacks the C-Nup145p shows a strong nuclear accumulation of mRNA when shifted for 7 h to 37°C. Nuclear mRNA export was analysed by *in situ* hybridization of fixed and spheroplasted cells using a Cy3-labelled oligo(dT) probe (upper panel). The same strain was further examined for nucleolar organization by immunofluorescence microscopy using mAb66, which recognizes the nucleolar protein Nop1p (middle panel). Direct observation of yeast cells expressing the GFP–Nup49p reporter protein in the fluorescence microscope shows NPC clustering in the FYEF95 strain and a ring-like nuclear envelope staining in wild-type cells (lower panel). Cell nuclei are visualized by DAPI staining. (**B**) Electron microscopic analysis of strain FYEF95 (ΔC) reveals an extensive NPC clustering within the nuclear envelope. Upper panel: bar, 1 μm; lower panel: bar, 0.25 μm. (**C**) Electron microscopic analysis of strain FYEF94 (ΔN), which lacks N-Nup145p, reveals a normal NPC distribution. Bar, 0.5 μm. (**D**) Electron microscopic analysis of nsl7 reveals several abnormalities including NPCs on the nucleoplasmic face of the nuclear envelope and nuclear envelope herniations (Bar, 0.25 μm). In (B), (C) and (D), arrows indicate normal or clustered NPCs, solid triangles show abnormal nuclear envelope structures and open arrowheads point to spindle pole bodies. N, nucleus; C, cytoplasm.

was complemented with a yeast genomic library insert containing *NUP188* which encodes one of the major NPC constituents in yeast (Nehrbass *et al.*, 1996; Zabel *et al.*, 1996; see Materials and methods). This suggested that a recessive mutation of *NUP188* in nsl7 is responsible for synthetic lethality when N-Nup145p is missing. This idea was confirmed in two ways. First, transformation of nsl7 cells with a wild-type *NUP188* could complement the nsl7 mutant, while a mutated form of *NUP188* (*nup188ΔSpeI*) could not (Figure 5A). Second, by combining the disruption allele of *NUP145* with a knockout allele of *NUP188*, it was found that the requirement for a free N-Nup145p was identical to the one observed in nsl7 (Figure 5B). To determine whether the mutation in nsl7 corresponds to a *nup188* null or truncation allele, we recovered the genomic mutated form of *NUP188* and tagged it. Full-length Nup188p was seen by Western blot analysis (not shown), arguing that synthetic lethality may be due to a more subtle mutation within Nup188p.

In order to test if the genetic interaction between N-Nup145p and Nup188p reflects a physical association, IgG affinity purification was performed in a strain which co-expresses a functional Myc–Nup188p and ProtA–Nup145p (see Materials and methods). Under conditions

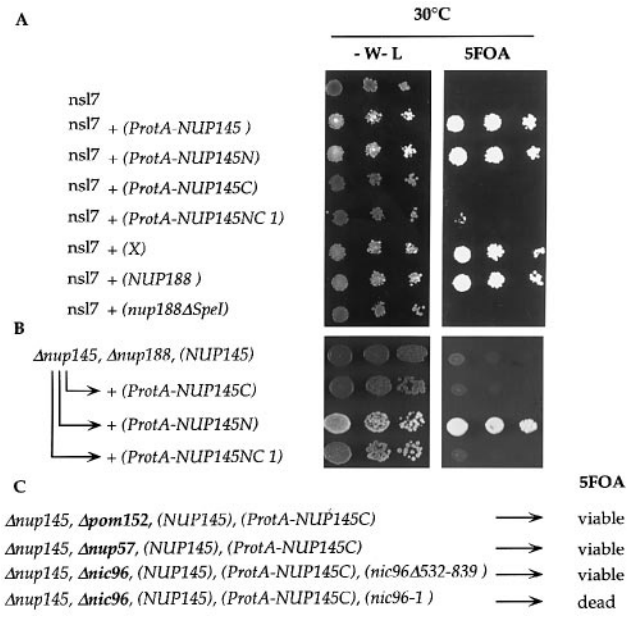

Fig. 5. A mutant allele of *NUP145* lacking the amino-terminal domain is synthetic lethal with *NUP188* and *NIC96* but not with *POM152* or *NUP157*. (**A**) The synthetic lethal strain nsl7, which carries a mutation in *NUP188*, a *nup145::HIS3* disruption and harbours plasmids *(ARS/CEN–URA3–ADE3–NUP145*) and *(ARS/CEN–TRP1–ProtA–NUP145C)*, was transformed with the indicated plasmids (between brackets). Growth on 5-FOA plates is possible when cells have lost the *URA3*-carrying plasmid. It therefore indicates no synthetic lethal relationship between the mutant *nup188* allele and *NUP145* genes. No growth on 5-FOA plates reveals synthetic lethality. X corresponds to an *ARS–CEN–LEU2* plasmid containing a complementing genomic insert. This insert was shown to include *NUP188*. nsl7 is complemented by *NUP188*, *ProtA–NUP145* and *ProtA–NUP145N*, but not by *ProtA–NUP145C*, *ProtA–NUP145NC1* and truncated *nup188ΔSpeI*. The same number of cells and diluted 10 to 10 were dot-spotted onto glucose plates lacking tryptophan and leucine (-W-L) or glucose plates containing 5-FOA and incubated for 3 days at 30°C before pictures were taken. (**B**) The strain which carries a knockout in both *NUP188* and *NUP145* and harbours plasmids *(ARS/CEN–URA3–ADE3–NUP145*) and *(ARS/CEN–TRP1–ProtA–NUP145C)* was transformed with the indicated plasmids. The pairwise deletion mutant is complemented by *ProtA–NUP145N*, but not by *ProtA–NUP145C* and *ProtA–NUP145NC1*. (**C**) Analysis of synthetic lethality relationships among the various interacting Nup188p components and the mutant allele of *NUP145* lacking the amino-terminal domain was carried out as in (B). To test whether pairwise combination of mutant alleles gives synthetic lethality, cells were plated on 5-FOA. Dead, no growth on 5-FOA; viable, growth on 5-FOA.

which allowed complete solubilization of Myc–Nup188p and detection of the Nup84p NPC sub-complex, we could not find Myc–Nup188p in the eluate fraction of ProtA–Nup145p (data not shown). However, this result does not exclude that Nup188p physically interacts with N-Nup145p *in vivo*, but this association is not stable for our biochemical purification.

We also analysed whether phenotypic abnormalities are associated with the mutation of *NUP188* in the nsl7 strain. Only morphological defects in NPC organization revealed by electron microscopy could be detected (Figure 4D). These abnormalities include NPCs which are only found on the nucleoplasmic face of the nuclear envelope and nuclear envelope herniations and are similar to those observed in strains mutated in *NUP188* (Nehrbass *et al.*, 1996). To determine further the cause of the synthetic lethality between the lack of N-Nup145p and mutation in *NUP188*, we analysed whether in the nsl7 mutant, depletion of N-Nup145p might have an effect on nuclear transport processes. To test for this, *NUP145* was expressed under the inducible *GAL10* promoter (see Fabre *et al.*, 1994) in the constitutive presence of C-Nup145p. We found that depletion of Nup145p in such a context had no effect on mRNA accumulation, nor on NLS-dependent nuclear protein import (data not shown).

Nup188p interacts physically or genetically with several other nucleoporins including Pom152p (Nehrbass *et al.*, 1996), Nic96p (Zabel *et al.*, 1996) and Nup157p (Aitchison *et al.*, 1995b). We, therefore, tested whether N-Nup145p is required for cell growth when these nucleoporins are mutated. No synthetic lethality was observed in yeast strains in which N-Nup145p was absent and *POM152* or *NUP157* were disrupted. In the case of *NIC96*, which encodes an essential nucleoporin (Grandi *et al.*, 1993), two mutant alleles were tested. While a thermosensitive mutant which maps in the central domain of Nic96p (*nic96-1*; Grandi *et al.*, 1995b) requires N-Nup145p in order to grow, a mutant allele which lacks the non-essential carboxy-terminal domain of Nic96p (*nic96Δ532–839*; Grandi *et al.*, 1995b) does not (Figure 5C). These results demonstrate that N-Nup145p becomes essential if other components of the nuclear pores, i.e. Nup188p and Nic96p, are mutated (see also Discussion).

Discussion

One important finding of this paper is that a proteolytic cleavage of nucleoporin Nup145p creates, *in vivo*, two domains that function separately. Nup145p processing is the first example of a proteolytic maturation of a nuclear pore protein. While this maturation is dispensable for the activity of C-Nup145p, it is essential for the function of N-Nup145p.

Our data have shown that the cleaved C-domain of Nup145p is incorporated into a large nucleoporin complex consisting of Nup120p, Nup85p, Nup84p, Seh1p and Sec13p. This gives a clue as to how C-Nup145p gets targeted to the nuclear membrane and stably associates with nuclear pores. The Nup84p complex performs an essential role in nuclear pore biogenesis, and deletion of any Nup-member from the complex (e.g. Nup120p, Nup85p and Nup84p) has drastic consequences for nuclear envelope and NPC organization (Siniossoglou *et al.*, 1996). Lack of C-Nup145p also leads to an extensive NPC clustering, reminiscent of the phenotype observed in nup84$^-$ cells, but different from the grape-like NPC structures and nuclear envelope distortions observed in nup85$^-$ and nup120$^-$ cells (Aitchison *et al.*, 1995a; Goldstein *et al.*, 1996; Siniossoglou *et al.*, 1996). Thus, individual members of the Nup84p complex including C-Nup145p perform similar, but not identical functions in NPC and nuclear membrane biogenesis. Altogether these results confirm and extend recent results obtained through analysis of C-terminal truncations of Nup145p (Dockendorff *et al.*, 1997; Emtage *et al.*, 1997). On the other hand, lack of N-Nup145p has no effect on NPC distribution; the grape-like NPC structures previously observed in a mutant strain in which the amino-terminal domain of Nup145p was deleted may have been caused by a concomitant reduction in the expression of the

carboxy-terminal domain of Nup145p (Wente and Blobel, 1994). Beside their overlapping roles in NPC biogenesis, components of the Nup84p complex are required for efficient mRNA export. In this respect, mutants lacking C-Nup145p are not distinguishable from other Nup mutants of the Nup84p complex.

N-Nup145p, when provided as a free entity, is targeted to the NPCs and becomes nucleoplasmic. A similar dual intracellular location was found for the GLFG nucleoporin Nup98p from *Xenopus*, which is homologous to N-Nup145p, Nup116p and Nup100p (Powers *et al.*, 1995). Interestingly, Nup98p was recently shown to be involved in nuclear export of mRNA, rRNA and snRNA (Powers *et al.*, 1997). It is, therefore, attractive to speculate that, upon travelling between the intranuclear compartment and the nuclear pores, Nup98p is involved in RNA export mechanisms. We do not have direct evidence for participation of N-Nup145p in RNA transport reactions; however, the NRM domain (nucleoporin RNA-binding motif) found in N-Nup145p was shown to be genetically linked to a corresponding domain in Nup116p and Nup100p (Fabre *et al.*, 1994). This domain binds homopolymeric RNA *in vitro* and deletion of *NUP100* in combination with deletion of both NRMs of Nup116p and Nup145p leads to defective RNA export reactions (Fabre *et al.*, 1995). First analysis of synthetic lethality between the lack of N-Nup145p and the *NUP188* mutant allele shows that defects in mRNA export and NLS-dependent nuclear protein import are not the primary cause of cell death. It will be interesting to determine whether under these conditions other nuclear export or import pathways are affected.

Post-translational proteolytic maturation of proteins is a widely used mechanism to control and regulate many different cellular processes, including protein targeting and translocation, assembly of protein complexes, enzymatic activation/inactivation, signal transduction and viral biogenesis (for reviews, see Palmenberg, 1990; Rehemtulla and Kaufman, 1992; Resnick and Zasloff, 1992; Little, 1993). Post-translational modifications of NPC proteins, like the attachment of *N*-acetylglucosamine residues to the protein backbone of FXFG-repeat sequence-containing nucleoporins, have been reported (Davis and Blobel, 1987; Holt *et al.*, 1987; Starr and Hanover, 1990). Although the role of this modification is not clear, it may occur concomitantly with distinct assembly steps of nucleoporins into NPC structures (Davis and Blobel, 1987; Starr and Hanover, 1990). In the case of Nup145p, the half-time of precursor maturation is ~10 min, suggesting that maturation does not occur immediately after completion of Nup145p precursor synthesis. This delay in processing, together with our biochemical and localization results, suggests that assembly of Nup145p into the NPC precedes the cleavage reaction. After processing, Nup145p N- and C-domains perform separate functions and even physically dissociate. We note, however, that simultaneous deletion of the two domains leads to an intragenic synthetic lethality, since the individual deletions can complement the lethal phenotype of a Δ*nup145* null mutant, at least at 30°C. Thus, the two domains may be involved in similar pathways.

The sequence around the site of proteolytic cleavage consists of the tetrapeptide 603-N-H-F-S-606 which does not resemble the consensus cleavage site of any known protease. The cleavage site does, however, show some similarities to consensus sites for protein splicing in inteins (reviewed by Shub and Goodrich-Blair, 1992; Cooper and Stevens, 1995). In such cases, excision of an internal segment (called intein) of a polypeptide and religation of the flanking regions (called exteins) is catalysed by the intein itself. In intein–extein junctions, serines or cysteines are found and histidine and asparagine amino acids are conserved in the carboxy-terminal end of the intein. These residues are essential for the protein splicing activity. Thus, Nup145p may catalyse self-cleavage by a mechanism similar to protein splicing, except that no religation of peptides is needed. Alternatively, a specific protease may exist in yeast to cleave Nup145p. Accordingly, mutation of the conserved residues would cause inhibition of self-cleavage or recognition by a specific protease. There may be an exposed hinge region between the amino- and carboxy-terminal domain of Nup145p which is easily attacked by (a) cellular protease(s). Upon mutation of this hinge region, a change of conformation would render the site less accessible for proteolytic attack. Interestingly, secondary structure predictions suggest the existence of a β-sheet structure around the cleavage site which is disrupted in mutants of processing of Nup145p. However, this putative conformational change does not affect the functions of C-Nup145p and N-Nup145p. Indeed, non-cleavable mutants of Nup145p can complement the *NUP145* deletion and restore the temperature-sensitive growth phenotype of a strain deleted for the NRM domains of *NUP100* and *NUP116*, while the carboxy-terminal domain alone cannot (Fabre *et al.*, 1994 and unpublished results).

What could be the reason for the proteolytic cleavage of a nuclear pore protein in living cells? One clue as to why this may occur is the observation that Nup145p precursor cleavage becomes essential in a mutant background in which another nuclear pore protein, Nup188p, is mutated. Lethality could be explained either because: (i) cleavage is necessary; (ii) N-Nup145p is not provided as a free entity; or (iii) C-Nup145p remains as a part of the precursor. Yet, the cleavage is not a prerequisite for the function of C-Nup145p because the non-cleavable form of Nup145p is functional in the *nup188*-mutated background (on condition that free N-Nup145p is provided). In addition, the fact that N-Nup145p, when expressed separately, can complement the synthetic lethal phenotype of the nsl7 mutant argues that cleavage *per se* is not required for functionality of the amino-terminal domain and that this domain has an important role after cleavage. This raises the question of why these two Nup145p domains are covalently linked to each other in the initial phase of Nup145p biogenesis. One possibility, suggested by our results, is to ensure correct and/or efficient targeting of N-Nup145p to the NPCs. Another explanation is that, by this mechanism, stoichiometric expression of both domains is guaranteed. Finally, it could be that the Nup145p precursor has a temporary function on its own during NPC assembly, before the two halves are cleaved and perform separate functions. It will be now of great interest to find out how nucleoporin cleavage is mechanistically linked to NPC biogenesis and/or function.

Table I. Yeast strains used in the study

Strain	Genotype
FYBL2-5D	*Matα ura3–Δ851 trp1Δ63 leu2Δ1*
FYEF88	*Matα ura3–Δ851 trp1Δ63 leu2Δ1 Δnup145 (ARS–CEN–URA3–NUP145)*
FYEF94	*Matα ura3–Δ851 trp1Δ63 leu2Δ1 Δnup145 (ARS–CEN–TRP1–ProtA–NUP145C)*
FYEF95	*Matα ura3–Δ851 trp1Δ63 leu2Δ1 Δnup145 (ARS–CEN–LEU2–ProtA–NUP145N)*
FYEF121	*Matα ade2 ade3 trp1 leu2 his3 ura3 nup145::HIS3 (ARS–CEN–TRP1–ProtA–NUP145C, ARS–CEN–URA3–ADE3–NUP145)*
FYEF172	*Matα ade2 ade3 trp1 leu2 his3 ura3 pom152::KAN nup145::HIS3 (ARS–CEN–TRP1–ProtA–NUP145C, ARS–CEN–URA3–ADE3–NUP145)*
FYEF177	*Mat***a** *ade2 trp1 leu2 his3 ura3 nup188::HIS3 nup145::HIS3 (ARS–CEN–TRP1–ProtA–NUP145C, ARS–CEN–URA3–ADE3–NUP145)*
FYEF178	*Matα trp1 leu2 his3 ura3 nup157::URA3 nup145::HIS3 (ARS–CEN–TRP1–ProtA–NUP145C, ARS–CEN–LEU2–NUP145)*
FYEF179	*Matα ade2 trp1 leu2 his3 ura3 nic96:: HIS3 nup145::HIS3 (ARS–CEN–TRP1–ProtA–NUP145C, ARS–CEN–URA3–ADE3–NUP145 ARS–CEN–LEU2–nic96Δ532–839)*
FYEF181	*Matα ade2 trp1 leu2 his3 ura3 nic96:: HIS3 nup145::HIS3 (ARS–CEN–TRP1–ProtA–NUP145C, ARS–CEN–URA3–ADE3–NUP145 ARS–CEN–LEU2–nic96–1)*

Materials and methods

Yeast strains, media and microbiological techniques

Standard yeast complex and minimal media were used (Sherman *et al.*, 1986). Yeast transformation was performed with the one-step procedure (Chen *et al.*, 1992) or by electroporation (modified from Meilhoc *et al.*, 1990). Yeast strains used in this study are listed in Table I (plasmids are indicated between brackets).

FYEF88 was constructed according to Fairhead *et al.* (1996). In order to avoid putative chromosomal rearrangements between *NUP145*-containing plasmids and the genomic copy, the ORF of *NUP145* was completely deleted from its ATG start codon to the TAA stop codon. This was performed by transforming yeast FYBL2-5D (see Table I and Fairhead *et al.*, 1996) with the complementary regions of 5′ and 3′ intergenes of *NUP145* cloned in recombinant plasmids containing overlapping fragments of the selection marker *URA3* (named pUR and pRA). Since *NUP145* was known to be essential, FYBL2-5D was previously transformed with a centromeric plasmid carrying *NUP145* linked to the *TRP1* marker. Transformants were then selected on glucose-containing plates lacking uracil and tryptophan.

In order to recover the *URA3* marker, chromosomal deletants containing two *I-Sce*I sites flanking *URA3* (provided by pUR and pRA) were transformed with a plasmid expressing the *I-Sce*I gene under the control of a galactose-inducible promoter and the *LEU2d* selection marker. Clones growing in galactose medium were then checked for the absence of *URA3* and verified for correct genomic organization by Southern blotting. This strain was further transformed with a centromeric plasmid containing *NUP145–URA3* and loss of the *TRP1*-carrying plasmid was performed by successive replica plating. The strain FYEF88 or Δ*nup145*, was used for all plasmid transformations described below.

Pulse–chase labelling and immunoprecipitation

Cells were grown at 30°C in minimal medium (2% glucose, 0.67% nitrogen base) supplemented with the required nutrients. After collecting the cells by centrifugation, the pellet was resuspended in the same medium to yield a final cell density of 4.5×10^8 cells/ml. After a 5 min pre-incubation, cells were labelled for 5 min with 250 μCi/ml, 1000 Ci/mmol L-[^{35}S]methionine, L-[^{35}S]cysteine (ICN) and chased with a 300-fold excess of non-radiolabelled amino acids. Aliquots of 0.3 ml (1.55×10^8 cells) were withdrawn at the indicated time points of the chase and mixed with 1 ml of 1% SDS, 25 mM methionine, 25 mM cysteine and 1 mM Pefabloc (Boehringer). Whole-cell extracts were prepared by the glass bead lysis method. Samples were then centrifuged at 4°C and supernatants were precleared by three incubations each of 1 h at 4°C with 100 μl protein A–Sepharose (1 g/50 ml) in 9 ml of TNET buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 0.1% Triton X-100, 0.02% SDS). Finally, 2 μg of antibodies against protein A (Sigma) were added to the pre-absorbed lysate and it was incubated for 1 h at 4°C, before 150 μl of protein A–Sepharose CL-4B (20 mg/ml) were used to precipitate the antigen–antibody complexes (overnight incubation at 4°C on a turning wheel). ProtA–Sepharose beads were finally washed three times with TNET buffer. Bound antigen was eluted with 50 μl of 2× Laemmli's sample buffer and 20 μl were subjected to SDS–8% polyacrylamide gel electrophoresis. Gels were fixed, dried and autoradiographed.

Mutagenesis of the cleavage site within Nup145p

A PCR-based protocol was used (Morrison and Desrosiers, 1993) for random mutagenesis of DNA encoding amino acids 600–608 of Nup145p. Briefly, two degenerate oligonucleotide primers (DG1: 5′-TNCAAAGNCAANCATNNTAGCANTNGGGG-3′; DG2: 5′-AAATGCNAAAANGATNGACTNTGAAAGTCC-3′) were synthesized to generate random mutations in the putative cleavage site. These two oligonucleotides, used in combination with two 'outside' primers, generated two PCR-derived fragments which overlapped within 24 nucleotides of the randomly mutagenized region. These two PCR products were mixed, annealed and used in a third PCR reaction with the two 'outside' oligonucleotides, allowing mutagenized DNA to be subcloned into the *Xba*I–*Aat*II sites of *ProtA–NUP145* (Fabre *et al.*, 1994), cloned into pUN100 (Elledge and Davis, 1988). This library of *NUP145* mutagenized within the putative cleavage site was used to transform the shuffle strain FYEF88 and transformants expressing a non-cleavable Nup145p were identified by Western blot analysis using an antibody against protein A, coupled to horseradish peroxidase (Dakopatts, Denmark). DNA fragments derived from PCR amplification were sequenced and mutations responsible for lack of cleavage were identified. Position W_{608} was found to be mutated in all our mutants and in most clones of the library. However, this mutation either alone or in combination with a mutation in F_{600} affects the cleavage reaction only slightly.

Construction of ProtA–NUP145 and GFP–NUP145 gene fusions and detection of the fusion proteins in yeast

Plasmid expressing the fusion *ProtA–NUP145N* was obtained by substituting the *ProtA–NUP145* cassette described above by in-frame fusion of PCR products encoding the N-terminal region of *NUP145* and *NUP145* terminator. Cloned PCR fragments (636 bp) were verified by sequencing. Translation of *NUP145N* ends with the sequence VNHL(605) which corresponds to the predicted carboxy-terminal end of N-Nup145p, except for replacement of $F_{605}\rightarrow$L (numbers correspond to the positions in the wild-type sequence of Nup145p). Similarly, plasmid expressing the fusion *ProtA–NUP145C* was obtained by substituting the *ProtA–NUP145* cassette by in-frame fusion of PCR products corresponding to the *NOP1* promoter–protein A cassette (Fabre *et al.*, 1994) and the region encoding for the carboxy-terminal domain of Nup145p. Cloned PCR fragments (2047 bp) were verified by sequencing. Translation of the junction *ProtA–NUP145C* is **LIN**$\mathbf{I}_{607}$WGL (bold letters indicate residues added between protein A and C-Nup145p sequence). Expression of each construct under the authentic promoter was obtained by replacing the *NOP1* promoter by a PCR product corresponding to the *NUP145* promoter. In-frame GFP fusions were obtained by replacement of protein A tag from previous constructs by GFP sequence which was isolated as a restriction fragment from pVD241GFP (containing the S65T/V163A GFP variant, a generous gift from V.Doye).

Each ProtA or GFP fusion protein was verified to be expressed to similar levels by Western blot analysis. Whole-cell extracts of haploid strains expressing the different ProtA or GFP fusion constructs were prepared from cells grown in YPD medium or in selective media if required and according to Fabre *et al.* (1994). An equivalent of 0.05 OD_{600} of cells was analysed on SDS–8% polyacrylamide gels, followed by immunoblotting using IgG coupled to horseradish peroxidase (1:5000; Dakopatts, Denmark) to detect ProtA fusion proteins or using anti-GFP

polyclonal antibodies (1:1000; Clontech) followed by incubation with anti-rabbit-HRP (1:5000; Sigma).

Affinity purification of ProtA fusion proteins

Affinity purification of ProtA fusion proteins by IgG–Sepharose chromatography from whole-cell extracts under non-denaturing conditions was done according to Grandi *et al.* (1993) and Siniossoglou *et al.* (1996). Strains expressing the fusion proteins were spheroplasted and lysed in 1% Triton X-100, 150 mM KCl, 20 mM Tris–HCl, pH 8, 5 mM $MgCl_2$, supplemented by a cocktail of protease inhibitors. After centrifugation (27 000 *g*, 10 min), the supernatant was recovered and loaded on an IgG–Sepharose column (Pharmacia). After several washes, elution of the attached proteins from the ProtA-tagged fusions was done by acidic pH.

For detection of a physical interaction between ProtA–Nup145p and Myc–Nup188p, lysis and ProtA purification were performed as above, but an anti-c-Myc monoclonal antibody (9E10, Boehringer-Mannheim) at 10 μg/ml and a HRP-sheep anti-mouse antibody (secondary antibody, dilution 1:5000) were used for Western blot analysis.

Immunofluorescence microscopy

To immunolocalize the nucleolar marker Nop1p, exponentially growing cells were fixed in 3.7% formaldehyde for 30 min. Cells were converted to spheroplasts using 0.5 mg/ml zymolyase 100.000T as described by Wimmer *et al.* (1992), immobilized on poly-lysine-coated coverslips and then incubated for 15 min in PBS/0.2 M glycine and 30 min in PBS/0.2% BSA. Nop1p was detected with monoclonal antibody A66 in a dilution of 1:100. Secondary antibodies, donkey anti-rabbit IgG coupled to Cy3 or donkey anti-mouse coupled to FITC (Jackson Immunoresearch Laboratories, Inc.) were diluted to 1:100. GFP–Nup49p was observed directly on fixed cells. Hoechst 33258 was used to stain DNA. Cells were examined in a Leitz DMRB fluorescence microscope.

Confocal microscopy

To localize GFP fusion proteins in the cell, growing cells were transferred in Sykes Moore Chambers (Bellco, Vineland, NJ) filled with 1.2 ml selective medium and directly observed with a Leica inverted microscope equipped with a ×100/1.4 objective lens. Scanning was performed with a True Confocal Scanner LEICA TCS 4D and GFP fluorescence signal was detected with the fluorescein channel. Acquisitions of four focal planes, 0.8 to 1.2 μm apart, were made.

Mass spectrometry analysis

Bands of interest were excised from the gel and subjected to proteolytic digestion with an excess of trypsin as described (Wilm *et al.*, 1996). Then 0.3 μl of digestion supernatant were deposited directly into an acidified water droplet placed on top of a fast evaporation matrix surface. Peptide mass maps were recorded on a Bruker Reflex Matrix Assisted Laser Desorption Ionization Time of Flight mass spectrometer equipped with a 337 nm N2 laser (Bruker-Franzen, Bremen, Germany).

Electron microscopy

20 ml of cells growing in exponential phase were washed once with Buffer A (40 mM Kpi, pH 6.5, 0.5 mM $MgCl_2$) and fixed overnight at 4°C with 2 ml of buffer A containing 2% fresh paraformaldehyde (Merck), 2% glutaraldehyde (Sigma). After treatment with a reducing buffer (0.2 M Tris–HCl, pH 9.4, 20 mM EDTA, 0.1 M β-mercaptoethanol) cells were spheroplasted for 3 h in phosphate–citrate buffer containing 0.2 mg/ml of zymolyase 20T and 100 μl of glusulase. After washing three times with 0.1 M Na-acetate, pH 6.1, cells were post-fixed in 2% osmium tetroxide and 1% uranyl acetate and further dehydrated in acetone and propylenoxide. Embedding was performed in propylenoxide/epon. Forty nm sections collected on nickel grids were contrasted by staining with uranyl acetate and Reynold's lead. Specimens were visualized with a Zeiss EM109 electron microscope operating at 80 kV.

Analysis of poly(A)$^+$ RNA export

Export of polyadenylated RNA from the nucleus was examined according to Fabre *et al.* (1994) using the FYEF95 (ΔC) mutant strain shifted to 37°C for 0.5, 1, 3, 5, 7 and 10 h, or grown at 30°C. The *in situ* hybridization steps were conducted exactly as described by Amberg *et al.* (1992). The oligo(dT) probe was directly coupled to Cy3. Photographs were taken with the 100× objective on a Leitz DMRB fluorescence microscope.

Isolation of synthetic lethal mutants

The synthetic lethal screen was performed on the basis of the *ade2/ade3*-dependent red/white sectoring colony assay (Wimmer *et al.*, 1992) using a strain deleted for the *NUP145* gene (FYEF121, see Table I) and complemented by both an *ARS–CEN–URA3–ADE3–NUP145* plasmid and an *ARS–CEN–TRP1–ProtA–NUP145C*. This strain performs a distinct red/white colony sectoring on SDC-trp plates at 30°C. UV-induced mutagenesis was performed as described by Wimmer *et al.* (1992) allowing a survival rate of 10%. From 60 000 screened colonies, one non-sectoring mutant fulfilled the requirement to be synthetically lethal with the lack of N-Nup145p but not with the lack of C-Nup145p. Accordingly, this sl mutant (nsl7) was complemented by *ARS–CEN–LEU2–ProtA–NUP145N*. nsl7 was further transformed with a yeast genomic library inserted into pUN100 (*ARS–CEN–LEU2*-based plasmid), as described by Wimmer *et al.* (1992). Three transformants displaying a red/white sectoring phenotype were obtained. Restriction analysis from plasmid DNA recovered from these transformants showed that two plasmids contained a genomic insert corresponding to *NUP145*, while one contained an unrelated insert of 12 kb. Sequencing of the ends of this latter insert revealed that it contained *NUP188*. Complementing activity was finally restricted solely to *NUP188* (Zabel *et al.*, 1996) by transforming nsl7 with pUN100 plasmids carrying either the *NUP188* wild-type gene or a *nup188ΔSpeI* mutant gene, which contains an internal out-of-frame deletion. Only the full-length *NUP188* was able to complement synthetic lethality in strain nsl7.

Pairwise combinations between disruption of *NUP145* and *NUP188*, *NUP157* or *NIC96* were obtained by mating FYEF121 (Table I) with respectively NP188-4-1-2, NP157-2.1 (Aitchison *et al.*, 1995b), nic96-1 or nic96(Δ532–839) (Grandi *et al.*, 1995). Tetratypes were selected after sporulation and double disruption was verified by Southern blot analysis. Haploids with the correct pattern were further transformed by *ProtA–NUP145N* and *ProtA–NUP145NC1* (carried on *ARS–CEN–LEU2* plasmids; see Table I). Synthetic lethality was tested by checking either growth on 5-FOA or, in the case of FYEF178 (Table I), the capacity to segregate *ProtA–NUP145C*-carrying plasmid. Since *NIC96* disruption is lethal to cells, their viability is dependent on the *LEU2* plasmid which carries the different alleles of *NIC96*. Thus, only the requirement for N-Nup145p could be tested.

In the case of *POM152*, pairwise disruption could not be obtained through mating. Therefore, *POM152* was directly disrupted in FYEF121 according to Fairhead *et al.* (1996).

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