An essential nuclear envelope integral membrane protein, Brr6p, required for nuclear transport

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Despite rapid advances in our understanding of the function of the nuclear pore complex in nuclear transport, little is known about the role the nuclear envelope itself may play in this critical process. A small number of integral membrane proteins specific to the envelope have been identified in budding yeast, however, none has been reported to affect transport. We have identified an essential gene, *BRR6*, whose product, Brr6p, behaves like a nuclear envelope integral membrane protein. Notably, the *brr6-1* mutant specifically affects transport of mRNA and a protein reporter containing a nuclear export signal. In addition, Brr6p depletion alters nucleoporin distribution and nuclear envelope morphology, suggesting that the protein is required for the spatial organization of nuclear pores. *BRR6* interacts genetically with a subset of nucleoporins, and Brr6-green fluorescent protein (GFP) localizes in a punctate nuclear rim pattern, suggesting location at or near the nuclear pore. However, Brr6-GFP fails to redistribute in a *Anup133* mutant, distinguishing Brr6p from known proteins of the pore membrane domain. We hypothesize that Brr6p is located adjacent to the nuclear pore and interacts functionally with the pore and transport machinery.

Keywords: integral membrane protein/mRNA export/nuclear envelope/nuclear transport

Introduction

The presence of a nuclear envelope (NE) in eukaryotes divides the cell into nuclear and cytoplasmic compartments, greatly enhancing its capacity to control gene expression. Transport of proteins and ribonucleoprotein particles (RNPs) between the nucleus and cytoplasm through the nuclear pore complex (NPC) is central to this ability (reviewed in Görlich and Kutay, 1999; Nakielnny and Dreyfuss, 1999). The structure of the NPC is conserved from yeast to humans, consisting of a highly symmetrical core structure organized around a central pore channel as well as peripheral cytoplasmic filaments and nucleoplasmic basket structures thought to play key roles in initiation and termination of transport pathways (reviewed in Franke and Scheer, 1974; Davis, 1995; Stoffler *et al.*, 1999; Wentz, 2000). Transport of different types of protein and RNP cargoes through the NPC is mediated by specific protein carriers, including members

of the karyopherin β family and a number of unrelated transport factors such as Ntf2 and Mex67 (reviewed in Pemberton *et al.*, 1998; Wozniak *et al.*, 1998; Görlich and Kutay, 1999).

Interactions between carriers and nucleoporins lining the channel of the pore are thought to be important for transport (reviewed in Fabre and Hurt, 1997; Pemberton *et al.*, 1997; Wozniak *et al.*, 1998; Görlich and Kutay, 1999; Wentz, 2000), although the precise roles of particular nucleoporins are not well understood. Our knowledge of the functional architecture of the yeast nuclear pore has benefited greatly from a detailed study of isolated NPCs carried out recently by Rout *et al.* (2000). The study produced a working model of the NPC, confirming the presence of ~30 yeast nucleoporins at the pore and placing them spatially with respect to known ultrastructural features such as the central channel and nuclear basket. Many of these proteins are likely to function in the targeting of transport substrates to the NPC and their passage to the cytoplasm.

In addition to nucleoporins, the NE around the NPC could also play a key role in transport, possibly providing binding sites for transport factors or anchoring pore structures. Interestingly, an antibody against gp210, a mammalian integral membrane protein located in the pore membrane domain bridging the inner and outer NE membranes (reviewed in Gerace and Burke, 1988; Dingwall and Laskey, 1992; Gant and Wilson, 1997), has been shown to inhibit nuclear import (Greber and Gerace, 1992). Although the mechanism of the inhibition is unknown, the result suggests that integral membrane proteins can affect transport events.

In yeast, three integral membrane proteins specific to the NE have been identified. Two of these, Pom152p and Pom34p (Wozniak *et al.*, 1994; Strambio-de-Castillia *et al.*, 1995; Tcheperegine *et al.*, 1999; Rout *et al.*, 2000), co-purify with yeast nuclear pores. Both are believed to reside in the pore membrane domain and to function in nuclear pore assembly and anchoring. The third, Ndc1p, is an inner envelope protein that interacts with both the NPC and the spindle pole body (Chial *et al.*, 1998). In addition, three yeast proteins, Snl1p, Nem1p and Spo7p, common to the NE and the endoplasmic reticulum, have also been shown to affect the NPC (Ho *et al.*, 1998; Siniosoglou *et al.*, 1998). However, none of the known yeast NE integral membrane proteins has been reported to affect nuclear transport.

Here we report the characterization of an essential yeast protein, Brr6p, which has the properties of an NE integral membrane protein and affects specific nuclear transport pathways. The *BRR6* gene interacts genetically with a subset of nucleoporins, and loss of Brr6p function causes redistribution of Nsp1p and Nup188-green fluorescent protein (GFP) as well as aberrant envelope and pore

Fig. 1. The *brr6-1* mutant accumulates bulk poly(A) RNA in the nucleus and at the nuclear rim. Shown are the mRNA localization patterns in *BRR6* and *brr6-1* cells at 30°C determined by *in situ* hybridization with a digoxigenin-labeled oligo dT50 probe. DAPI staining was used to confirm the locations of the cell nuclei. The inserts in the *brr6-1* panels show dT50 and DAPI staining of a single nucleus enlarged to show the mRNA accumulation at the nuclear rim evident in some mutant cells. Bar, 10 μ m.

morphologies. Strikingly, the *brr6-1* cold-sensitive (cs) allele accumulates mRNA and a nuclear export signal (NES) protein reporter at the nuclear rim. Thus, Brr6p represents the first example of a yeast NE integral membrane protein that impacts nuclear transport.

Results

BRR6 was identified through complementation of the growth defect of *brr6-412*, one of two original isolates of the *brr6-1* cs mutant obtained in an *in situ* hybridization screen for cs mRNA export mutants (see Materials and methods). The 197 aa *BRR6* open reading frame (ORF) [*Saccharomyces cerevisiae* Genome Database (SGD) accession No. YGL247w] is predicted to encode an essential 22.8 kDa protein of unknown function. Disruption of the *BRR6* ORF with the *HIS3* marker confirmed that the gene is essential. The *brr6-1* allele was found to contain a single, conservative arginine to lysine change at amino acid 110. Isogenic *BRR6* and *brr6-1* strains were generated by integrating wild-type and mutant alleles into a $\Delta brr6::HIS3$ deletion strain (see Materials and methods for details). The resulting *brr6-1* mutant showed a moderate growth defect at 30°C, which was exacerbated at 16°C, while the *BRR6* strain was indistinguishable from the wild-type parent (data not shown).

The *brr6-1* mutant accumulates mRNA in the nucleus and at the nuclear periphery

Using a digoxigenin-labeled dT50 probe, we examined the mRNA *in situ* hybridization patterns in *BRR6* and *brr6-1* cells maintained at 30°C (Figure 1) or shifted to 16°C (data not shown). At both temperatures, *BRR6* cells showed the whole cell dT50 staining typical of wild-type cells. In contrast, *brr6-1* cells had clear staining in the cell nucleus at 30°C as well as at 16°C. Thus, *brr6-1* exhibits a constitutive nuclear mRNA export defect. A strain in which the only copy of *BRR6* was under the control of the repressible *GAL1-GAL10* promoter also showed both a growth defect and nuclear mRNA accumulation when grown for 5 h in media containing glucose (data not

Fig. 2. The *brr6-1* mutation causes a defect in NES protein export. Shown are the localization patterns of NLS/NES(GFP)₂ and NLS(GFP)₃ reporters in *BRR6* and *brr6-1* cells as indicated. The NLS/NES construct accumulates at the nuclear rim in *brr6-1* as confirmed by DAPI staining (data not shown). The NLS construct shows nucleoplasmic staining in both mutant and wild-type cells. Bar, 10 μ m.

shown), indicating that these are most likely loss-of-function phenotypes. In some *brr6-1* cells, dT50 signal was clearly concentrated at the nuclear rim (Figure 1, insert), suggesting that *BRR6* may play a role in a step of mRNA export occurring at or near the nuclear pore.

brr6-1 is defective in NES protein transport

The factors known to affect mRNA export in yeast can be divided into two general categories: those that appear to be dedicated to mRNA and those that also affect protein transport pathways (reviewed in Nakielnny and Dreyfuss, 1999). Our *in situ* hybridization results suggested a role for *BRR6* in mRNA export; to assess whether *BRR6* also functions in protein transport, we examined the localization of a number of different GFP-tagged protein transport reporters in living *BRR6* and *brr6-1* cells. Reporters were selected that are known to utilize different protein transport pathways. The set included diffusible and non-diffusible SV40 nuclear localization signal (NLS)-GFP constructs [NLS-GFP, NLS(GFP)₃], an SV40 NLS/NES-GFP reporter [NLS/NES(GFP)₂], a ribosomal protein NLS reporter (L25-GFP) as well as GFP-tagged forms of two known mRNA binding proteins, Npl3p and Nab2p.

Of the reporters tested, only the NLS/NES(GFP)₂ construct showed any change in localization. In *BRR6* cells (Figure 2), the reporter showed the expected wild-type cytoplasmic distribution reported previously (Stade *et al.*, 1997). Interestingly, about half of the *brr6-1* mutant cells with GFP signal showed a pronounced accumulation of the reporter at the nuclear rim, consistent with a defect in NES protein transport. In contrast, the distribution of an NLS(GFP)₃ reporter lacking the NES sequence was unaffected in *brr6-1* (Figure 2). Similarly, no defects were observed using a diffusible NLS-GFP reporter (data not shown) in either steady state experiments or in the kinetic protein import assay developed by Goldfarb and colleagues (Shulga, 1996). The distributions of the L25-GFP reporter, Npl3p and Nab2p were also unchanged in *brr6-1* (data not shown). The effect on NLS/NES(GFP)₂ but not other protein reporters implies a defect specific to the NES protein transport pathway. Interestingly, the NES protein exporter Xpo1p (reviewed in Görlich and Kutay, 1999) continued to show wild-type nucleoplasmic and nuclear rim distribution in *brr6-1* using an Xpo1-GFP fusion protein (data not shown).

Fig. 3. Brr6-GFP localizes to the nuclear rim. (A) The localization patterns of a Brr6-GFP fusion protein expressed from a low copy (CEN/ARS) vector or a high copy 2 μ m plasmid as indicated. At low copy, Brr6-GFP shows predominantly punctate nuclear rim staining. Upon overexpression, Brr6-GFP accumulates at the nuclear and cell peripheries. (B) The effect of *BRR6* (*BRR6*/pRS424) overexpression relative to the empty vector (pRS424) on growth of wild-type (W303), $\Delta nup116::HIS3$, $\Delta nup1::HIS3$ and $\Delta nup188::HIS3$ strains (all W303 strain background) on -TRP medium. *BRR6* overexpression causes dramatic growth defects in $\Delta nup1$ and $\Delta nup188$ cells with only mild effects on wild-type and $\Delta nup116$ cells. (C) Nup188-GFP and Brr6-GFP (*Brr6*-GFP/pRS424) localization patterns in wild-type (RS456) and $\Delta nup133$ cells. In contrast to Nup188-GFP, Brr6-GFP fails to show clustering in the $\Delta nup133$ mutant. Bar, 10 μ m.

Brr6p is located at the nuclear rim

The mRNA export and NES protein transport defects observed in *brr6-1*, along with the apparent absence of effects on other protein reporters, argue that *BRR6* plays a role in mRNA and NES transport pathways. The accumulation of both mRNA and the NES reporter at the nuclear rim suggests that *BRR6* may act at the NE. To determine the location of the *BRR6* gene product, we examined the distribution of a Brr6-GFP fusion protein in living cells by fluorescence microscopy. When Brr6-GFP was expressed from a low copy plasmid, the GFP signal was observed predominantly at the nuclear rim, suggesting that Brr6p associates with the NE (Figure 3A). Notably, the Brr6-GFP rim staining pattern was punctate, similar to that seen with nuclear pore components (Rout *et al.*, 2000). The pattern was distinctly different from the uniform rim distribution of non-pore-associated NE integral membrane proteins, such as Spo7p and Nem1p in yeast (Siniossoglou

et al., 1998), suggesting that Brr6p may be located at or near the pores. Interestingly, overexpression of Brr6-GFP resulted in dramatic localization to both the nuclear and cellular peripheries (Figure 3A). The latter distribution resembles that of the ER markers Sec63-GFP and GFP-HDEL in living yeast cells (Prinz *et al.*, 2000) and most likely represents accumulation of excess Brr6p in the peripheral ER. Similar localization in both the NE and the ER has been shown for Spo7p and Nem1p (Siniossoglou *et al.*, 1998) and for the mammalian nuclear pore membrane protein, Pom121, upon overexpression (Soderqvist *et al.*, 1997).

***BRR6* interacts genetically with nucleoporin genes**

Consistent with the possibility that Brr6p is located at or near the NPC, *BRR6* shows genetic interactions with nuclear pore components. A haploid double mutant carrying a *cs* allele of the nucleoporin, *nup188* (*brr7-1*; A.de Bruyn Kops and C.Guthrie, unpublished results), and the $\Delta brr6::HIS3$ deletion covered by *brr6-1* on a TRP plasmid and wild-type *BRR6* on a URA plasmid are viable, whereas loss of the URA plasmid on 5'-fluoroorotic acid (5-FOA)-containing medium is lethal (data not shown), indicating that *brr6-1* is synthetically lethal with *nup188cs/brr7-1*. Moreover, overexpression of *BRR6* (*BRR6* 2 μ m plasmid) in $\Delta nup188$ and $\Delta nup1$ deletion strains dramatically impaired growth, while overexpression had only a modest effect in wild-type W303 or $\Delta nup116$ cells (Figure 3B). A $\Delta nup2$ strain and a C-terminal *Anic96* deletion mutant [*Anic96*(532–839)] as well as the *xpo1-1* and *gle2-1* (RNA export factor) temperature-sensitive (*ts*) mutants also showed synthetic lethality with *BRR6* using this assay (data not shown). Interestingly, the locations of Nup1p (Rout *et al.*, 2000) and a portion of Nic96p (Fahrenkrog *et al.*, 1998) detected by immuno-electron microscopy coincide with the nuclear basket, pointing to a possible impact of *BRR6* on basket function. Importantly, a number of strains tested containing deletions or mutations in other nucleoporins [including $\Delta nup100$, $\Delta nup133$, $\Delta nup145$, *Anic96*(28–63), and *brr3/gle1cs* and *ts* alleles] showed no effect, indicating that the observed interaction is specific to a subset of nucleoporins and transport components.

***Brr6*-GFP localization is unchanged in a pore-clustering mutant**

Although *BRR6* interacts genetically with nucleoporins, Brr6p was not identified in the recent NPC preparations reported by Rout *et al.* (2000), raising the possibility that Brr6p may interact with the pore without being part of the NPC *per se*. Another criterion that is frequently used to evaluate if a protein is a nucleoporin is whether its distribution is altered in one of several nucleoporin mutants (e.g. $\Delta nup133$; Doye *et al.*, 1994; Pemberton *et al.*, 1995) known to cause pore clustering. To test the behavior of Brr6p in this assay, we compared the distribution of Brr6-GFP and a tagged nucleoporin, Nup188-GFP, in a $\Delta nup133$ strain. As predicted, the pattern of Nup188-GFP signal changed from nuclear rim in the wild-type strain to a characteristic single bright nuclear spot in the $\Delta nup133$ mutant (Figure 3C). Attempts to localize Brr6-GFP in the $\Delta nup133$ and other nucleoporin deletion mutants known to cause pore clustering

using a low copy Brr6-GFP construct were unsuccessful because the GFP signal was very low. Efforts to localize the protein by immunofluorescence using a number of epitope tags have also been unsuccessful, most likely because of the small size, membrane association and apparent low abundance of the protein. Similarly, we have not yet been able to generate a usable antibody against Brr6p itself. However, when the experiment was performed using a high copy Brr6-GFP construct (Figure 3C), wild-type and $\Delta nup133$ cells showed indistinguishable Brr6-GFP distributions. A caveat to these experiments is the possibility that excess Brr6-GFP might remain distributed throughout the NE. Nonetheless, given the dramatic nature of the clustering phenotype, we would still expect to see intensified signal in one location if clustering occurred. The failure of Brr6-GFP to cluster in this assay distinguishes Brr6p from nucleoporins as well as from the pore membrane proteins, Pom152p and Pom34p, which behave like canonical nucleoporins in both this assay and in the NPC preparation (Rout *et al.*, 2000).

The extraction profile of Brr6p from crude lysates typifies an integral membrane protein

The Brr6-GFP localization results indicate that Brr6p is most likely a membrane component concentrated at the NE. As such, Brr6p could be either a peripheral membrane protein, associated with the envelope via interactions with nucleoporins or other envelope-associated proteins, or an integral membrane protein. One of the characteristics that distinguishes integral from peripheral membrane proteins is their relative solubility under different extraction conditions. While peripheral proteins can be readily removed by treatment with high ionic strength or high pH, solubilization of integral membrane proteins requires detergent to disrupt lipid–lipid and lipid–protein interactions. We thus tested the ability of high salt, high pH and detergent to solubilize Brr6p.

Whole-cell extracts were prepared from $\Delta brr6$ cells containing a low copy *BRR6*-GFP construct using four different extraction conditions (see Materials and methods). Extracts were centrifuged at 100 000 g and pellet and supernatant samples were assayed by western blotting with an antibody specific for GFP. Brr6-GFP remained associated with the cell pellets in samples extracted under low salt, high salt or high pH conditions but was efficiently extracted in low salt buffer containing 4% Triton X-100 (Figure 4A). This is the profile expected for an integral membrane protein and is similar to that observed for the known nuclear envelope integral membrane protein Pom152p (Figure 4B). In contrast, HA-tagged Nup188p, a non-integral membrane nuclear pore component, was extracted under both high salt and high pH conditions (Figure 4C). Thus, in crude lysates, Brr6p exhibits properties characteristic of integral membrane proteins.

The C-terminus of Brr6p is sequestered in the NE lumen

Consistent with the solubility behavior of Brr6p in extracts, sequence analysis of the *BRR6* ORF revealed two hydrophobic stretches with the potential to function as transmembrane domains. One is located near the C-terminus (residues 158–174) and a second, somewhat

Fig. 4. The extraction profile of Brr6p from crude lysates typifies an integral membrane protein. Shown are analyses of Brr6-GFP (A), Pom152 (B) and Nup188-HA (C) extracted from whole-cell lysates using four different extraction conditions: (i) 150 mM NaCl; (ii) 1 M NaCl; (iii) 150 mM NaCl, 100 mM sodium carbonate; (iv) 150 mM NaCl, 4% Triton X-100. Supernatant (S) and pellet (P) lanes are indicated. Western blots were probed with antibodies against GFP, Pom152 or the HA tag. Brr6-GFP and Pom152 are present in the supernatant only after detergent treatment, while Nup188-HA is also evident in high salt and high pH supernatants.

weaker candidate, is present near the N-terminus (residues 58–74) (Figure 5A). The use of one or both of these sequences to anchor Brr6 in the membrane would dictate the topology of the protein with respect to the bilayer and hence, the availability of different parts of the protein for functional interactions. We were particularly interested in determining the orientation of the *brr6-1* mutation (aa 110) with respect to the NE lumen. The mutation is located between the two predicted transmembrane sequences and lies within a region of *BRR6* that shows striking homology (31% identical, 49% similar) to a *Saccharomyces cerevisiae* karyopherin gene, *KAP123* (residues 247–312), implicated in ribosomal protein import and mRNA export (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997; Seedorf and Silver, 1997) (Figure 5A). The region of homology comprises ~1/3 of the *BRR6* ORF (residues 52–120) and could reflect the interaction of Brr6p and Kap123p with a

Fig. 5. Trypsin digestion yields a detergent-sensitive C-terminal Brr6 fragment. (A) The *BRR6* ORF, showing the locations of the *brr6-1* mutation (asterisk), the *KAP123* homology and the two predicted transmembrane domains. A sequence alignment of the homologous portions of *BRR6* and *KAP123* is shown. No significant similarity was found between *BRR6* and the karyopherin β family prototype, *KAP95*. (B) The results of trypsin digestion experiments carried out on whole-cell lysates containing Brr6-GFP and polyoma-Brr6 constructs tagged at the C- and N-termini, respectively. Western blot analyses using antibodies against the GFP or polyoma tags demonstrate the protection of a C-terminal Brr6-GFP fragment (asterisk), which is sensitive to detergent. No protected N-terminal fragments are observed. (C) The likely topology of Brr6p in the membrane. The ends of the region of *KAP123* homology (arrows) and the site of the *brr6-1* mutation (asterisk) are indicated.

common transport factor or nucleoporin. For both the region of *KAP123* homology and the *brr6-1* mutation to be accessible to the transport machinery, Brr6p would have to be anchored in the membrane by the C-terminal predicted transmembrane domain, with the C-terminus sequestered in the NE lumen.

To investigate the membrane topology of Brr6p, we used trypsin digestion to determine whether the N- and/or C-termini of the protein were protected from proteolysis in the presence and absence of Triton X-100. We carried out trypsin digests on crude lysates containing Brr6p bearing either a C-terminal GFP tag or an N-terminal polyoma tag. The results of a typical experiment are shown in Figure 5B. In the absence of trypsin, a protein of ~49 kDa corresponding to full-length Brr6-GFP is present in the presence or absence of detergent (lanes 1 and 4). Trypsin digestion in the absence of detergent results in a protected fragment of ~29 kDa apparent mol. wt (lane 2). The fragment is specific to the trypsin digestion because addition of soybean trypsin inhibitor results in primarily the full-

length protein (lane 3). Although it is possible that the trypsin protection results from protein conformation rather than membrane association, the detergent sensitivity (lane 5) argues in favor of protection by the bilayer. The size of the protected Brr6-GFP fragment correlates precisely with the length expected if Brr6p were anchored in the membrane via the C-terminal-most predicted transmembrane domain, with the C-terminus extending into the lumen of the NE.

In extracts containing the N-terminally tagged polyoma-Brr6p there were no protected fragments (Figure 5B). A band of ~26 kDa corresponding to the full-length product was seen in the absence of trypsin or when protease inhibitor was added (lanes 1, 3 and 4). However, no bands were observed when trypsin was included, either in the absence or presence of detergent (lanes 2 and 5). These results are consistent with a topology in which the Brr6 N-terminus, including the site of the *brr6-1* mutation and the region of homology with Kap123p, is located outside the lumen (Figure 5C). Such an orientation would make

interaction of these regions with the transport machinery feasible.

Depletion of Brr6p alters the localization of two nucleoporins

Taken together, the *BRR6* genetic interactions and the Brr6p-GFP localization and extraction data argue that Brr6p is an NE membrane protein located near, and interacting with, the NPC. To further examine the relationship between Brr6p and the NPC, we tested whether the localization of two nucleoporins, Nup188p and Nsp1p, is altered under conditions where *BRR6* expression is inhibited. The localization of a GFP-tagged Nup188 protein was examined in living cells in which the sole copy of Brr6p was expressed from its own promoter (*BRR6-Myc*) or the repressible *GAL1-GAL10* promoter (*galBRR6-Myc*) (see Materials and methods). When these strains were assayed for growth on YEP plates containing galactose or glucose, *BRR6-Myc* grew well on both carbon sources while *galBRR6-Myc* grew on galactose but failed to grow on glucose. When cells cultured in YEP media containing galactose were switched to YEP with glucose, a growth defect was observed in the *galBRR6-Myc* but not in the *BRR6-Myc* strain by 6 h. When Nup188-GFP localization was examined in living cells, the GFP signal became consolidated into a single spot in most of the *galBRR6-Myc* cells by 5 h following a switch to glucose-containing medium (Figure 6A). Co-staining with DAPI confirmed that this spot was located at the nuclear rim (data not shown). No change in Nup188-GFP localization was observed in the *BRR6-Myc* strain. Similar results were obtained when Nsp1p was detected by immunofluorescence microscopy in fixed *galBRR6-Myc* and *BRR6-Myc* cells grown in galactose and switched to glucose medium (Figure 6A). When cells were maintained in galactose, no change in Nup188-GFP or Nsp1p localization was observed in *BRR6-Myc* or the majority of *galBRR6-Myc* cells (data not shown). A small portion (<10%) of *galBRR6-Myc* cells showed concentrations of signal at a few spots along the nuclear rim (data not shown), probably reflecting suboptimal expression of Brr6p from the galactose promoter in some cells. In the *brr6-1* mutant, Nup188-GFP and Nsp1p appeared to be decreased in nuclear rim signal rather than redistributed to a single spot (Figure 6B).

Brr6p depletion causes changes in NPC distribution and envelope morphology

The redistribution of Nup188-GFP and Nsp1p from a punctate rim pattern to a single spot observed upon Brr6p depletion is reminiscent of the pore-clustering phenotype exhibited by *Δnup133* (Doye *et al.*, 1994; Pemberton *et al.*, 1995). Clustering is also seen in a mutant of *GLE2*, an NPC-associated transport factor, as well as in certain nucleoporin mutants (reviewed in Wentle, 2000). In the *gle2-1* mutant, the phenotype is accompanied by major changes in NE morphology (reviewed in Fabre and Hurt, 1997; Wentle, 2000). In other mutants, including *Δnup133*, pores aggregate but little alteration of the envelope is observed (Doye *et al.*, 1994; Aitchison *et al.*, 1995; Heath *et al.*, 1995; Li *et al.*, 1995). To determine whether changes in pore or membrane ultrastructure underlie the Nup188-GFP and Nsp1p redistribution observed following

Fig. 6. Depletion of Brr6p causes redistribution of nucleoporins. (A) Nup188-GFP and Nsp1p localizations under conditions where Brr6p expressed from the *GAL1-GAL10* promoter (*galBRR6-Myc*) was depleted by glucose inhibition. Nup188-GFP was assayed in living cells and Nsp1p was detected by indirect immunofluorescence in fixed cells. Growth in glucose medium for 5 h resulted in a consolidation of the Nup188-GFP and Nsp1p signals (examples indicated with arrows) in the *galBRR6-Myc* strain but not in *BRR6-Myc* in which *BRR6* is under the control of its own promoter. (B) Nup188-GFP and Nsp1p distributions in *BRR6* and *brr6-1* cells. Bar, 10 μ m.

Brr6p depletion, we carried out electron microscopy on *galBRR6-Myc* and *BRR6-Myc* strains grown in galactose and switched to glucose for 5 h.

Aberrant pore and envelope morphology consisting of a bulge in the outer NE overlying a large electron-dense mass was observed in >50% of *galBRR6-Myc* nuclei following a shift to glucose-containing medium (Figure 7A). The abnormal structures appeared both individually and in clusters and strongly resembled the NE ‘herniations’ reported previously for the *gle2-1* and *Δnup116* mutants (Wente and Blobel, 1993; Murphy *et al.*, 1996; Bailer *et al.*, 1998). As with the Nup188-GFP and Nsp1p localization phenotypes, abnormal membrane morphology was seen in a small portion (<5%) of the *galBRR6-Myc* cells maintained in galactose-containing medium. In contrast, the pore and envelope morphologies in the *BRR6-Myc* strain in either medium were indistinguishable from that seen in an untagged wild-type strain, *BRR6*, grown in glucose medium (Figure 7B). It seems probable that the clustered herniations observed following Brr6p depletion are the ultrastructural correlate of the nucleoporin redistribution seen at the light microscopic level. Interestingly, small, isolated herniations were also evident in *brr6-1* cells grown in glucose at 30°C (Figure 7B); however, no clusters were detected in the mutant. These results indicate that Brr6p is required for normal pore distribution and NE morphology.

Fig. 7. Brr6p depletion and the *brr6-1* mutation cause changes in NE morphology. Shown are transmission electron microscope images of thin sections through cells prepared by high-pressure freezing. (A) A single example of *BRR6-Myc* and two examples of *galBRR6-Myc* cells grown in galactose medium and then switched to glucose medium for 5 h. The upper panels show examples of whole nuclei (NU) (bar, 500 nm). Examples of typical pores are marked with an asterisk (*) and are shown at higher magnification in the lower panels (bar, 100 nm). (B) Images of *BRR6* and *brr6-1* cells grown in glucose at 30°C. Upper panels show whole nuclei (bar, 500 nm), lower panels show individual pores (*) at higher magnification (bar, 100 nm).

Discussion

To date only a handful of yeast NE integral membrane proteins have been identified, none of which has been shown to participate in transport processes. We have identified an essential yeast NE component, *BRR6*, whose function affects both mRNA and NES protein transport as well as the spatial organization of nuclear pores.

Brr6p is an integral membrane protein of the NE that interacts functionally with the NPC

Several observations indicate that Brr6p is most likely an integral membrane protein of the nuclear envelope: (i) Brr6p behaves biochemically like an integral membrane protein, being resistant to high pH and requiring detergent for efficient extraction; (ii) a C-terminal Brr6p fragment is protected from trypsin digestion in the absence but not the presence of detergent, consistent with the protein being anchored in the membrane via a predicted transmembrane domain identified in the C-terminus of the *BRR6* ORF; (iii) visualization of GFP-tagged Brr6p expressed at low copy in living cells reveals a predominantly nuclear rim distribution. In addition, two results suggest that Brr6p interacts functionally with the NPC. First, *BRR6* shows specific genetic interactions with the confirmed nucleoporin genes, *NUP1*, *NIC96* and *NUP188* (Rout *et al.*, 2000 and references therein), and with another possible nucleoporin, *NUP2* (Loeb *et al.*, 1993). Secondly, depletion of Brr6p results in the dramatic redistribution of

two nucleoporins, Nup188-GFP and Nsp1p, from a punctate rim pattern to a single spot.

The redistribution of nucleoporins observed upon Brr6p depletion is reminiscent of the pore-clustering phenotype exhibited by a number of nucleoporin mutants, including *Δnup133*, and in a mutant of *GLE2*, an NPC-associated factor believed to function in mRNA export in association with the nucleoporin *NUP116* (reviewed in Wentz, 2000). In both *Δnup133* and *gle2-1*, the phenomenon has been shown to result from lateral movement of the NPCs in the envelope (Belgareh and Doye, 1997; Bucci and Wentz, 1997). Interestingly, the ultrastructural morphology of the envelope and NPC following depletion of Brr6p resembles the distinctive herniations documented previously for both *gle2* and *Δnup116* mutants (Wentz and Blobel, 1993; Murphy *et al.*, 1996; Bailer *et al.*, 1998). As has been noted previously, the herniations differ from the 'grape-like aggregates' of NPCs found in other nucleoporin mutants that show clustering, including *Δnup133*, *Δnup145* and *Δnup120* (reviewed in Pemberton *et al.*, 1995; Murphy *et al.*, 1996). The smaller envelope herniations evident in the *brr6-1* mutant may reflect an earlier stage of the herniation process.

BRR6 functions in nuclear transport

The *brr6-1* mutant accumulates both mRNA and an NLS/NES-GFP fusion protein at the nuclear rim, consistent with a role for *BRR6* in transport events occurring at or near the nuclear pore. Importantly, no defects were detected with a number of other protein transport reporters in *brr6-1*,

arguing that the mutation affects a subset of transport events. In particular, the absence of defects with NLS-GFP constructs lacking an NES implies a specific effect on NES protein transport. Future experiments will be required to determine what aspect of the NES protein transport pathway is affected in *brr6-1*; in principle, the punctate rim pattern could reflect an accumulation of docked NES proteins at either pore face during import or export. However, the genetic interaction between *BRR6* and *NUP1* points to the possibility that *brr6-1* may affect transport events occurring at the nuclear basket.

Interestingly, the presence of both mRNA and NES protein transport defects in *brr6-1* suggests a possible overlap between the two pathways. Such an overlap was proposed earlier based on the extremely rapid nuclear accumulation of both an NES reporter and mRNA in the *xpo1-1* karyopherin mutant (Stade *et al.*, 1997), although a direct role for Xpo1p (Crm1p) in mRNA export remains controversial (Hodge *et al.*, 1999; Neville and Rosbash, 1999). Overlap between mRNA and NES export pathways is further suggested by findings that the vertebrate nucleoporin, Nup98, participates in Crm1p-mediated Rev protein export (Zolotukhin and Felber, 1999) as well as in mRNA export involving the Gle2p homolog, Rae1p (Bailer *et al.*, 1998; Pritchard *et al.*, 1999). The accumulation of both mRNA and the NES reporter at the nuclear rim in *brr6-1* suggests the exciting possibility that Brr6p, as an integral membrane protein, could function at a step in transport where mRNA and NES protein export pathways converge at the NE. Alternatively, the NES accumulation could be a secondary effect of reporter trapped in the envelope herniations present in *brr6-1*. In this case, NES protein export might continue through remaining normal pores, explaining the absence of NES nucleoplasmic signal in the mutant. However, this would not explain the mRNA export defect given that most cells show dramatic nucleoplasmic mRNA accumulation in *brr6-1*.

Brr6p may be located in a novel membrane domain adjacent to the NPC

The punctate character of the Brr6-GFP rim localization pattern, similar to that of nucleoporins, strongly suggests that Brr6p is located at or near the NPC. However, unlike Pom152p and Pom34p, components of the pore membrane domain that bridge the inner and outer NE at the NPC (Rout *et al.*, 2000 and references therein), Brr6-GFP did not relocate in the $\Delta nup133$ clustering assay, nor was Brr6p identified in the recent nuclear pore preparation reported by Rout *et al.* (2000). Thus, based on the available information, Brr6p behaves differently from proteins of the pore membrane domain. These apparent distinctions raise the intriguing possibility that Brr6p is located not in the pore membrane domain but, rather, adjacent to the NPC, perhaps in a novel peri-pore NE domain. To date, our attempts to test this prediction by immuno-electron microscopy using several tagged forms of Brr6p have been unsuccessful owing to low signal. We are generating antibodies against Brr6p itself in continued efforts to localize the protein at the ultra-structural level.

Interestingly, an NPC-associated membrane domain around the pore could explain the phenomenon of minimum pore spacing documented in early ultra-structural studies (reviewed in Franke and Scheer, 1974) and

more recent computer-aided reconstructions from serial sectioned yeast (Winey *et al.*, 1997). In these studies, a minimum pore-free space (120 nm for yeast) was generally observed around each pore such that two NPCs did not abut. The separation between pores, seemingly paradoxical given the high lateral mobility of yeast pores in the envelope (Belgareh and Doye, 1997; Bucci and Went, 1997), could be explained if the area occupied by each NPC were effectively expanded by associated membrane proteins adjacent to the pore.

Peri-pore membrane proteins could also serve to anchor pore-associated structures, such as the nuclear basket, and provide docking sites for transport substrates. It is not yet known where export carriers first encounter their cargoes in the nucleus. Although cargo-carrier complexes are often envisaged to dock at the nuclear basket and evidence for interactions between karyopherins and putative basket components is increasing, transport substrates could also accumulate at the NE. In this regard it is interesting that a recent immuno-electron microscopic study found mRNPs concentrated adjacent to the NPC (Iborra *et al.*, 2000), rather than in the central axis of the nuclear basket, raising the possibility that some mRNPs may accumulate at the membrane before entering the pore. In such a scenario, an NE integral membrane protein such as Brr6p could be important for concentrating mRNP cargoes near the NPC, thus playing a key role in mRNA export from the nucleus.

Materials and methods

Strains and plasmids

The strains used in this work are listed in Table I. Cloning was carried out using published bacterial and yeast vectors (Bluescript, YCp50, pSE358, pRS305 and pRS424; for references see Supplementary data, available at *The EMBO Journal* Online). pJL602 was a gift from Joachim Li. Nup188-GFP (URA) and Npl3-GFP (LEU) constructs were generated by Katrin Stade and Chris Siebel (K.Stade, C.Siebel and C.Guthrie, unpublished results). In addition, the following constructs were obtained from other laboratories: Kap123-GFP (Seedorf and Silver, 1997); NLS-GFP (Shulga, 1996); NLS-(GFP)₃, L25-GFP and Nab2-GFP (gifts from Nicole Miller, Arash Komeili, Arie Kaffman and Erin O'Shea); NLS/NES-(GFP)₂, NLS-(GFP)₂ and Xpo1-GFP (Stade *et al.*, 1997).

Isolation of the *brr6-1* and $\Delta brr6::HIS3$ alleles

The *brr6-1* allele (bad response to refrigeration) was isolated twice (*brr6-275*, *brr6-412*) in an oligo dT50 *in situ* hybridization screen for *cs* mRNA transport mutants of *S.cerevisiae* (A.de Bruyn Kops and C.Guthrie, unpublished results) similar to that used to identify *ts* transport mutants (for references see Supplementary data). The *BRR6* gene (SGD accession No. YGL247w) was cloned by complementation of the *brr6-412 cs* growth defect using a genomic DNA library (Rose *et al.*, 1987). The *BRR6* ORF was disrupted ($\Delta brr6::HIS3$) by replacing the sequence encoding aa 7–196 with the *HIS3* coding sequence in diploid YPH399 and W303 cells. Tetrad analysis of the $\Delta brr6::HIS3$ diploids showed that *BRR6* is essential in both strain backgrounds. Linkage between the *brr6-275* and *brr6-412* mutations and the *BRR6* gene was confirmed in crosses with haploid $\Delta brr6::HIS3$ strains containing *BRR6* on a *URA3* plasmid and subsequent loss of the plasmid on 5-FOA. Recovery of *brr6-275* and *brr6-412* mutations onto plasmids followed by DNA sequence analysis showed a single change at aa 110 (Arg to Lys) for both isolates, which we have designated *brr6-1*. The mutant plasmids conferred both *cs* growth and mRNA transport defects on haploid *brr6::HIS3* deletion strains. Isogenic strains were made by targeted insertion of mutant and wild-type *BRR6* into the *BRR6* downstream sequence in the $\Delta brr6::HIS3$ deletion strains. The resulting strains, *BRR6* and *brr6-1*, contain the wild-type and *brr6-1* alleles and the *LEU2* marker flanked by the $\Delta brr6::HIS3$ gene disruption.

Table I. Yeast strains

Strain	Genotype	Source (this study unless noted)
<i>brr6-275</i> (yDBK22)	<i>MATa trp1-63 leu2-1 ura3-52 his brr6-1cs</i>	outcrossed strain derived from DBY4275 (D.Botstein)
<i>brr6-412</i> (yDBK58)	<i>MATa his brr6-1cs</i>	outcrossed strain derived from DBY4412 (D.Botstein)
<i>Δbrr6::HIS3</i> (yDBK123)	<i>MATa/α trp1-63/trp1-63 leu2-1/leu2-1 ura3-52/ura3-52 his3-200/his3-200 ade2-101/ade2-101 lys2-801/lys2-801 BRR6/brr6::HIS3</i>	disruption of <i>BRR6</i> in YPH399 diploid
<i>Δbrr6::HIS3</i> (yDBK124)	<i>MATa/α ade2-1/ade2-1 ura3-1/ura3-1 his3-11,15/his3-11,15 trp1-1/trp1-1 leu2-3,112/leu2-3,112 BRR6/brr6::HIS3</i>	disruption of <i>BRR6</i> in W303 diploid
<i>Δbrr6::HIS3</i> haploid shuffle strain (yDBK125)	<i>MATα trp1-63 leu2-1 ura3-52 his3-200 ade2-101 lys2-801 brr6::HIS3</i> (YCp50-URA3-BRR6)	segregant of yDBK123
<i>Δbrr6::HIS3</i> haploid shuffle strain (yDBK126)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 brr6::HIS3</i> (YCp50-URA3-BRR6)	segregant of yDBK124
<i>BRR6</i> (yDBK165)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 brr6::HIS3/BRR6(LEU2)</i>	<i>BRR6</i> integrated into <i>Δbrr6::HIS3</i> (W303 background)
<i>brr6-1</i> (yDBK166)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 brr6::HIS3/brr6-1(LEU2)</i>	<i>brr6-1</i> integrated into <i>Δbrr6::HIS3</i> (W303 background)
<i>BRR6-untagged</i> (yDBK155)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 brr6::HIS3</i> (pSE358-TRP1-BRR6)	pDBK55 shuffled into yDBK126
<i>BRR6-GFP</i> (yDBK175)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 brr6::HIS3</i> (pSE358-TRP1-BRR6-GFP)	pDBK75 shuffled into yDBK126
<i>BRR6-Myc</i> (yDBK176)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 brr6::HIS3</i> (pSE358-TRP1-BRR6-myc epitope)	pDBK76 shuffled into yDBK126
<i>polyoma-BRR6</i> (yDBK181)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 brr6::HIS3</i> (pSE358-TRP1-polyoma epitope-BRR6)	pDBK81 shuffled into yDBK126
<i>galBRR6-Myc</i> (yDBK192)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 brr6::HIS3</i> (pJL602-LEU2-BRR6-myc epitope)	pDBK92 shuffled into yDBK126
<i>Δnup188</i> (NP188-2.4)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 nup188-1::HIS3</i>	<i>NUP188</i> disruption (R.Wozniak) ^a
<i>brr7-1</i> (yDBK36)	<i>MATa ade2-101 lys2-801 leu2-1 ura3-52 nup188 cs</i>	isolate from dT50 screen (A.de Bruyn Kops and C.Guthrie, unpublished)
<i>Δnic96</i> (nic96Δ532–839)	<i>MATa ade2 trp1 leu2 ura3 nic96::HIS3</i> (pUN100-LEU2-nic96Δ532–839)	<i>NIC96</i> disruption (E.Hurt) ^a
<i>Δnup1</i> (LDY460)	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can 1-100 nup1::LEU2</i>	<i>NUP1</i> disruption (L.Davis) ^a
<i>Δnup2</i> (JLY48)	<i>MATα ura3-52 leu2-3,112 trp1-289 nup2-4::URA3</i>	<i>NUP2</i> disruption (G.Fink) ^a
<i>Δnup116</i> (SWY27)	<i>MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 nup116-5::HIS3</i>	<i>NUP116</i> disruption (S.Wente) ^a
<i>Δnup133</i> (nup133 ⁻)	<i>MATα ade2 trp1 leu2 ura3 nup133::HIS3</i>	<i>NUP133</i> disruption (E.Hurt) ^a

^aComplete reference provided in Supplementary data.

Epitope tagging

Low copy constructs (CEN/ARS) were made in which *BRR6* was tagged at the C-terminus with the GFP coding sequence or a Myc epitope (EQKLISEEDL) and at the N-terminus with a polyoma epitope (MEYMPME)₂. Tagged and untagged constructs were shuffled into haploid *Δbrr6::HIS3* (W303 strain background) to give strains carrying a single copy of *BRR6*. Comparison of the growth of tagged and untagged strains on YEPD media showed nearly wild-type growth for the tagged strains at 30°C.

Brr6p overexpression and depletion

DNA fragments containing GFP-tagged and untagged *BRR6* were subcloned into 2 μm plasmids (*TRP1*) and transformed into W303 cells. The GFP-tagged construct gave highly elevated GFP fluorescent signal relative to the same fusion on a CEN plasmid, indicating that *BRR6* was overexpressed. Wild-type cells transformed with both constructs showed mild growth defects. To examine the effect of overexpression in selected nucleoporin mutants, strains carrying the untagged *BRR6* 2 μm plasmid were grown on dropout media (-Trp) at room temperature for 3 days.

A *GALI-GALI0* promoter driven, *BRR6-Myc* construct was made in the pJL602 gal vector (*LEU2*). Strains in which the sole copy of *BRR6* was under the control of either the *GALI-GALI0* or the *BRR6* promoter were generated by shuffling into the *Δbrr6::HIS3* haploid deletion strain (W303 background) to give *galBRR6-Myc* and *BRR6-Myc* strains, respectively. Brr6p depletion experiments were carried out in *galBRR6-Myc* and *BRR6-Myc* cells grown at 30°C and harvested at 0 and 5 h following a change from galactose- to glucose-containing media.

Localization of mRNA and proteins

Cells were grown to OD₆₀₀ = 0.15–0.25 in YEPD or appropriate dropout media. Bulk polyA(+) mRNA was localized in fixed, spheroplasted cells by *in situ* hybridization with a digoxigenin-tailed oligo dT50 probe

followed by staining with monoclonal anti-digoxigenin (1:100; Boehringer Mannheim) and FITC-conjugated goat anti-mouse (1:400; Cappel/ICN) antibodies and mounting in glycerol/gelatin containing DAPI (0.5 μg/ml). For localization of GFP-tagged proteins, cells were harvested, resuspended in media and examined immediately by fluorescence microscopy. DAPI (0.5 μg/ml) was added to confirm the location of the cell nucleus. For immunofluorescence, cells were fixed and spheroplasted prior to staining. The following localization studies were carried out: (i) low and high copy Brr6-GFP [in *Δbrr6::HIS3* cells (W303 background) carrying a CEN/ARS Brr6-GFP construct and W303 cells containing a 2 μm Brr6-GFP plasmid at 30°C]; (ii) Nup188-GFP and Brr6-GFP [in *Δnup133* and wild-type cells carrying a Nup188-GFP(URA) or a high copy Brr6-GFP(Trp) plasmid at 25°C]; (iii) Nup188-GFP and Nsp1p localization in *galBRR6-Myc* and *BRR6-Myc* cells under conditions of Brr6p depletion. Nup188-GFP was assayed in living cells. Nsp1p was localized by immunofluorescence using monoclonal anti-Nsp1p (1:1000; 32D6 Aris laboratory) and rhodamine-conjugated goat anti-mouse antibodies (1:1000); (iv) selected GFP-tagged protein import reporters (in *BRR6* and *brr6-1* cells grown in dropout media at 30°C).

Electron microscopy

Electron microscopy was carried out on (i) *BRR6* and *brr6-1* cells grown in YEPD at 30°C and (ii) *BRR6-Myc* and *galBRR6-Myc* cells grown at 30°C in galactose and switched to glucose for 5 h. Cells were harvested (OD₆₀₀ = 0.15–0.25) rapidly by filtration and subjected to high-pressure freezing and freeze substitution before embedding in Epon-Araldite (for reference see Supplementary data). Sample preparation was carried out by Kent McDonald at the Berkeley Electron Microscopy Facility. Embedded cells were sectioned to a thickness of ~65 nm and post-stained with lead citrate (0.2%, 3 min) and aqueous uranyl acetate (1%, 10–30 min).

Biochemical extraction and trypsin digestion of Brr6p

Whole-cell lysate (700 µl) was prepared from 100 ml of *BRR6-GFP* cells ($OD_{600} = 1.0$) by bead-beating in a standard buffer (20 mM HEPES-KOH pH 7.9, 5 mM MgCl₂, 150 mM NaCl) in the presence of protease inhibitors, and incubating for 10 min at 30°C in zymolyase (100T, 1 mg/ml) to disrupt the cell wall. Aliquots (100 µl) of the lysate were frozen then thawed. An equal volume of one of four 2× extraction buffers was added to give the following final concentrations: (i) 150 mM NaCl; (ii) 1 M NaCl; (iii) 150 mM NaCl, 100 mM sodium carbonate pH 11; and (iv) 150 mM NaCl, 4% Triton X-100 (v/v). Lysates were incubated for 30 min on ice, dounced 10 times and pelleted at 100 000 g (4°C) for 30 min in a Beckman TLA-120.1 rotor. Pellet and supernatant samples were analyzed by western blotting. Brr6-GFP, Nup188-HA and Pom152 were detected using the following antibodies: polyclonal anti-GFP (Silver laboratory), monoclonal anti-HA epitope (12CA5; Lerner laboratory) and monoclonal anti-Pom152 (MAB118C3, Rout laboratory).

For trypsin digestion experiments, 400 µl of whole-cell lysate prepared from *BRR6-GFP* or *polyoma-BRR6* cells (see above) were pelleted and resuspended in an equal volume of BTM (10 mM Tris pH 6.5, 0.1 mM MgCl₂), dounced 10 times on ice and pelleted at 100 000 g. Pellets were resuspended in 700 µl of BTM. Aliquots (80 µl) were incubated on ice for 1 h in the presence or absence of trypsin (500 µg/ml), Triton X-100 (1% v/v) and soybean trypsin inhibitor. Samples were collected by trichloroacetic acid precipitation and analyzed by SDS-PAGE and western blotting using polyclonal antisera against GFP (Silver laboratory) or a polyoma epitope (O'Shea laboratory).

Supplementary data

Because of space limitations, only brief descriptions of methods are given here. For greater detail see the Supplementary data, available at *The EMBO Journal* Online.

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