A fraction of the yeast nucleoporin Nic96p is localized at the terminal ring of the nuclear basket. When Nic96p was affinity purified from glutaraldehyde-treated spheroplasts, it was found to be associated with Mlp2p. Mlp2p, together with Mlp1p, are the yeast Tpr homologues, which form the nuclear pore-attached intranuclear filaments (Strambio-de-Castillia, C., Blobel, G., and Rout, M. P. (1999) J. Cell Biol. 144, 839–855). Double disruption mutants of MLP1 and MLP2 are viable and apparently not impaired in nucleocytoplasmic transport. However, overproduction of MLP1 causes nuclear accumulation of poly(A)+ RNA in a chromatin-free area of the nucleus.

Nuclear pore complexes are complex structures within the nuclear membrane, which mediate nuclear import and export of transport substrates and shuttling receptors (1). Each nuclear pore complex (NPC) consists of a basic framework (i.e. the ring/spoke complex) which exhibits an 8-fold symmetry and spans the double nuclear membrane (2–4). The NPC is attached to peripheral structures such as the short cytoplasmic filaments, the nuclear basket, the nuclear envelope lattice, pore-attached intranuclear filaments, and the nuclear lamina (5–13). Recently, some of these peripheral elements of the NPC gained increased attention since they were proposed to play an important role in the initial docking step of the transport substrate to the pores and the final release from the pores (8, 14–19). In particular, the large nucleoporin Nup358, located at the tips of the short cytoplasmically attached NPC filaments, is thought to mediate one of the first contacts with transport cargos via the importin/karyopherin αβ complex (15, 19–21). On the other side of the nuclear envelope, release of the import cargo from the nuclear pores appears to occur at the terminal ring of the nuclear basket, and Nup153 might be involved in this final process (16, 18, 22). However, electron microscopy revealed that NPCs do not abruptly end at the nuclear basket, but are connected to a complicated meshwork of intranuclear structures. These structures consist of pore-attached filaments, which deeply penetrate into the nuclear interior or an underlying nuclear envelope lattice (6, 7, 11, 23–26). Therefore, it could be hypothesized that nuclear transport does not end after release of the import cargo from the nuclear basket and followed by intranuclear diffusion, but instead the facilitated transport through the pore could continue on intranuclear “tracks” to distinct intranuclear sites. In a similar way, export cargos may use intranuclear filaments for transport from the nuclear interior to the NPCs. Accordingly, the “gene gating hypothesis” was presented to propose that nuclear pores are connected via intranuclear tracks to distinct locations inside the nucleus (27).

The molecular composition of the intranuclear filamentous system (often referred to as the “nuclear skeleton” or “nuclear matrix”), which has been visualized by a variety of electron microscopy techniques is still poorly characterized (28–36). This is surprising since the nuclear skeleton/nuclear matrix has been assigned many roles in nuclear and chromatin organization, nuclear division, and chromosome segregation, DNA replication and repair, RNA transcription and processing, and intranuclear transport. To date, only the long pore-attached filaments, which deeply penetrate into the nuclear interior and were identified almost 30 years ago by electron microscopy (23), have been characterized on a molecular level. Tpr (for translocated promoter region) was recently shown to be a constituent of these long pore-attached intranuclear fibrils (7, 37, 38). However, it is not completely clear whether Tpr is also located at the short NPC-associated cytoplasmic filaments (5). It was suggested that nuclear pore complex-associated filamentous proteins provide the structural connection between the nuclear interior and the nuclear periphery. However, it is not yet known whether these filaments form channels through which transport occurs, or whether they constitute tracks, along which transport cargos move along (38). Moreover, it was reported that importin/karyopherin β is physically associated with Xenopus Tpr (22) and overproduction of Tpr in mammalian cells inhibits poly(A)+ RNA export, but not protein import (39).

Yeast peripheral NPC elements, such as the nuclear basket and the cytoplasmic filaments can be discerned by electron microscopy (40). In the past, we have focussed on various Nsp1p subcomplexes, one consisting of Nsp1p, Nsp57p, Nup49p, and Nic96p (41), and another subcomplex between Nsp1p and Nup82p (42), in which Nup159p is also present (43, 44). Recently, Nsp1p and its interacting partners were located by immunoelectron microscopy to distinct sites within the NPC fine structure; accordingly, Nsp1p and Nic96p exhibit a dual location on both sides of the central gated channel, and at the terminal ring of the nuclear basket (40). Thus, the fraction of

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A. The abbreviations used are: NPC, nuclear pore complex; PARM, prolamellar body; PFC, postmeiotic filamentous constrictions; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
Nic9p6, which is located at the terminal ring, could directly contact to pore-associated intranuclear filaments.

To identify additional Nic9p6-interacting proteins in yeast, we treated yeast spheroplasts with glaturaldehyde prior to affinity purification. This allowed us to identify Mlp2p, which associates with protein A-tagged Nic9p6 (ProtA-Nic9p6). Mlp2p is highly homologous to Mlp1p, a previously identified myosin-like protein in yeast (45). Both Mlp1p and Mlp2p are the two closest homologues of the higher eukaryotic Tpr protein family.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—The yeast strains used in this study are listed in Table I. Cells were grown in minimal SDC or rich YPD medium. Genetic manipulations of yeast were performed as described (46). The following yeast plasmids were used: pHT4467, ARS/CEN plasmid with the URA3 and ADE3 marker; pUN100, pRS314, pRS316, YCplac33, and pASZ11, ARS/CEN plasmids with the LEU2, URA3, and ADE2 marker, respectively. pRS424 and YEpEplac12, 2 μm plasmids with the TRP1 marker. pRS426, YEp552, and YEp420, 2 μm plasmids with the URA3 marker. pRS425, 2 μm plasmid with the LEU2 marker. Manipulation and analysis of DNA such as restriction analysis, end-filling, ligations, DNA sequencing, and PCR amplifications were performed according to Ref. 47. Gene disruptions were made by the one-step disruption method (48).

**Plasmid Constructions and Gene Disruptions**—Construction of strains with integrated MLP2 constructs was done as follows: by PCR, a NotI site was generated at the stop codon. This yielded plasmid YEpEplac12-MLP2-C (NotI). The genes encoding ProtA and GFP, each available as NotI restriction fragment, were inserted into the corresponding NotI site within YEpEplac12-MLP2-C (NotI). The unique SpeI site within the 3'-UTR of MLP2 was used for the blunt end insertion of the HIS3 gene. For integration of tagged MLP2-ProtA:HIS3 and MLP2-GFP::HIS3, the plasmids were cut with convenient restriction enzymes to release the integration fragments. The RS453 diploid strain was transformed with these linearized DNA fragments and colonies that grew on SDC-His plates were selected. After sorption on YPA plates and tetrad analysis, haploid progeny, which contained the correct integration were selected. Integrations were verified by PCR analysis. Whole cell extracts from these strains were prepared and analyzed for the expression of the GFP- and ProtA-tagged Mlp2p by Western blotting using anti-GFP and anti-ProtA antibodies, respectively. For the disruption of the entire MLP2 ORF, a linear mlp2:HIS3 fragment was generated and used to transform the RS453 diploid strain. Colonies that grew on SDC-His plates and showed the correct integration (as verified by PCR Southern analysis) were sporulated on YPA. Tetrad analysis showed a 4:0 segregation for cell viability on YPD plates, and a 2:2 segregation of the HIS3 marker. To recover the full-length MLP2 gene from the chromosome, the “GAP-repair” method was used. To do so, the joined 5'- and 3'-UTR fragments of MLP2 (derived from pUN100-mlp2) were inserted into high copy number plasmids pRS424 and pRS426. For gap repair, these constructs were digested with NotI/ SpeI, releasing an internal 0.1-kilobase fragment. The gel-purified plasmids were transformed into a haploid RS453 strain carrying integrated MLP2-GFP::HIS3. Colonies were selected on SDC-Trp or SDC-Ura plates, respectively. Transformants were screened for an increased GFP signal in the fluorescence microscope, which allowed identification of colonies which contained pRS424-MLP2-GFP and pRS426-MLP2-GFP, respectively. GST-tagged Mlp2p-C constructs were made by PCR from genomic DNA and cloned into pGEX-4T-3 (Amersham Pharmacia Biotech). For expression of GFP-tagged MLP2-C constructs in yeast, PCR-derived DNA was cloned into pRS425-TEV-ProtA. For tagging of the MLP1 gene, a NotI restriction site was generated by PCR immediately before the stop codon to yield YEp420-MLP1. The generated NotI site of YEp420-MLP1 (NotI) was used to insert ProtA, TEV-Prota, and GFP, as NotI cassettes as described above. To generate GFP-tagged Mlp1p-C (residue 1446–1875), the last 429 amino acids from Mlp1p were amplified by PCR from genomic DNA and cloned into pRS425-SGFP. Affinity Purification of ProtA-Nic9p6 and Seh1p—Affinity purification of ProtA-Nic9p6 and Seh1p-Prota from yeast spheroplasts treated with 0.1% glaturaldehyde was done as follows: a yeast strain expressing ProtA-Nic9p6 (41) was grown in 2 liters of SDC-Leu medium for 14 h at 30 °C to an A600 nm of 1. Cells were collected by centrifugation, suspended in 5 ml of 20T Zymolyase of cells, resuspended in 100 ml of sorbitol buffer (1.2 M sorbitol, 0.02 M KCl, pH 7.4), and split into four equal aliquots. Increasing concentrations of glutaraldehyde were added: 0, 0.01, 0.1, and 1% and it was incubated for 30 min on ice. After two washing steps with sorbitol buffer, ProtA-Nic9p6 affinity purification on IgG-Sepharose beads was done as described previously (49).

**Mass Spectrometry**—Mass spectrometry of bands excised from SDS-polyacrylamide gels was done as described elsewhere (50). Tryptic peptide mixtures obtained as a result of in-gel digestion were analyzed on a matrix-assisted laser desorption time of flight mass spectrometer (REFLEX III, Bruker-Daltonics, Bremen, Germany). Matrix and samples were prepared as described (51). Proteins were identified via non-redundant protein sequence data base search with a list of detected tryptic peptides using PeptideSearch software.

**Purification of Karyopherin α and β and GST-Mlp2p-C from Escherichia coli and Solution Binding Assay—**Expression and purification of recombinant karyopherin α and karyopherin β from E. coli were done as described (52). GST-Mlp2p-C was expressed in bacteria transformed with the plasmid pGEX-4T-3 vector (Amersham Pharmacia Biotech). Induction of GST alone or GST-tagged fusion proteins and their subsequent purification on glutathione-Sepharose beads was done as described (53). After washing with universal buffer, purified karyopherin α (~2 μg) and karyopherin β (~2 μg), either as single subunits or complex, were mixed in 100 μl of buffer with GST beads or GST fusion protein beads. After incubating at 4 °C for 1 h, columns were washed and the bound proteins were eluted by SDS sample buffer. Bound and unbound fractions were analyzed by SDS-PAGE and Coomassie silver staining, or Western blot using the anti-Kap60p and Kap95p antibodies.

**Fluorescence Microscopy**—To detect GFP in vivo, the GFP signal was analyzed in the fluorescence channel of a Zeiss Axioskop fluorescence microscope. Pictures were taken with a Xillix Microimager CCD camera and digital pictures processed by the software program Openlab (Improvision, Coventry, United Kingdom).

**Yeast Survival Analysis after UV Irradiation**—UV light sensitivity of mlp1Δ/Δ (mlp1Δ/+ mlp1+/+), and mlp1Δ/Δ mlp2Δ/Δ mutants was analyzed by diluting freshly growing yeast strains in YPD medium and spotting equivalent amounts of cells (diluted in 10⁻² steps) onto YPD plates. The plates were UV-irradiated (Stratalinker UV cross-linker; 254 nm UV light bulbs; model 1800) with an intensity from 0 to 150,000 μJ/cm² and plates were incubated for 3 days at 30 °C.

**Electron Microscopy**—Glycerol spraying/low-angle rotary metal shadowing of the purified Mlp1p was performed as described (54).
**RESULTS**

**Purification of Nic96p from Glutaraldehyde-fixed Spheroplasts Reveals an Association with Mlp2p Which Is Homologous to the Vertebrate Tpr Proteins**—Affinity purification of ProtA-Nic96p from yeast spheroplasts has shown an interaction with Nsp1p, Nup49p, and Nup57p (41). To find components that associate less strongly with Nic96p, we treated yeast spheroplasts with glutaraldehyde (to stabilize/cross-link protein complexes) prior to affinity purification of ProtA-Nic96p. Interestingly, the ProtA-Nic96p eluate derived from the lysate treated with 0.1%, but not 0.01% glutaraldehyde revealed, besides Nup49p, Nup57p, and Nsp1p, an additional band of about 160 kDa on the silver-stained SDS-polyacrylamide gel (Fig. 1A, lane 8). The 160-kDa band was excised from the gel, in-gel digested with trypsin, and analyzed by matrix-associated laser desorption ionization mass spectrometry, followed by a data base search on the detected peptide masses. In total, 21 peptides from this band were detected which corresponded to a 15% protein sequence coverage. According to our general criteria (51), this sequence coverage and mass accuracy was good enough for the unambiguous identification of this band which corresponds to the yeast ORF YIL149c, which has a predicted molecular mass of 195 kDa. In the course of this work, Blobel and colleagues (56) identified and characterized this ORF and called it Mlp2p, one of the two yeast Tpr homologues. Mlp2p is homologous to yeast Mlp1p (myosin-like protein), which was found earlier as a 218-kDa coiled-coil protein (45). As deduced from the amino acid sequence, Mlp2p (as well as Mlp1 and other Tpr proteins) can be divided into two distinct domains: an amino-terminal domain of roughly 1500 amino acids, which has numerous heptad repeats with the potential to form α-helical coiled-coil structures (56) and a short carboxy-terminal domain of roughly 180 amino acids, which is devoid of heptad repeats, but exhibits one FXFG motif typically found in repeat sequence containing nucleoporins such as Nsp1p and Nup1p, and a putative bipartite basic NLS (see also later). To show that Mlp2p is specifically associated with Nic96p under conditions of glutaraldehyde fixation, another nucleoporin, Seh1p-ProtA, which is organized with five other components in the Nup84p complex (57), was affinity purified under similar conditions from spheroplasts, treated with 0.1% glutaraldehyde. When the two different ProtA-fusion protein preparations were compared by SDS-PAGE and silver staining, the 160-kDa band corresponding to Mlp2p is only seen in the ProtA-Nic96p, but not Seh1p-ProtA eluate (Fig. 1B). This shows that Mlp2p specifically associates with Nic96p under the chosen conditions of glutaraldehyde treatment.

**Mlp2p and Mlp1p Are Nuclear Pore-associated Proteins**—To determine its intracellular location, Mlp2p was tagged with GFP at its carboxy-terminal end and the construct was integrated at the authentic gene locus to replace endogenous MLP2 by MLP2-GFP. Mlp2p-GFP gave a distinct nuclear envelope labeling which was punctate and often excluded from the area in which the nuclear membrane is adjacent to the vacuole (Fig. 2A). This staining is typical for a NPC distribution in yeast and suggests that Mlp2p is restricted to the part of nuclear envelope which also contains nuclear pores. This conclusion is further supported by the finding that Mlp2p-GFP co-clusters with nuclear pores in *nup133* cells (Fig. 2A; see also Ref. 58). However, a residual intranuclear staining also becomes visible under these conditions. Accordingly, Mlp2p is nuclear pore-associated, but it is also found inside the nucleus. Since Mlp1p is highly homologous to Mlp2p, Mlp1p was also tagged with GFP and its subcellular location was determined by fluorescence microscopy. Similar to Mlp2p, Mlp1p shows a nuclear pore distribution in yeast (Fig. 2B). This result is in contrast to a previous finding, in which Mlp1p was located to dot-like structures adjacent to the nucleus by indirect immunofluorescence microscopy (45). However, in this case Mlp1p was overproduced, whereas here the GFP-tagged MLP1 was expressed under its authentic promoter and inserted into a low copy ARS/CEN plasmid. To test whether overproduced Mlp1p-GFP and Mlp2p-GFP form aggregates, both fusion genes were inserted into high copy number plasmids and expressed in yeast. Overproduction of Mlp2p-GFP or Mlp1p-GFP caused the appearance of an extremely bright fluorescent spot, often close to the nuclear envelope (data not shown). This suggests that overproduction causes aggregate formation of both Mlp2p and Mlp1p (see also Ref. 56). Since it is possible that Mlp2p and Mlp1p form a coiled-coil heterodimer, we overexpressed both proteins in yeast. However, the same GFP aggregates formed under the condition of co-overproduction (data not shown).

To determine the localization of Mlp1p and Mlp2p on the ultrastructural level, strains expressing Mlp1p-ProtA and Mlp2p-ProtA, respectively, were prepared by pre-embedding
immunoelectron microscopy using a colloidal gold-conjugated anti-ProtA antibody (40). As shown in Fig. 2C, the anti-ProtA antibody labeled the nuclear periphery of the nuclear pores of both ProtA-tagged Mlp1p and Mlp2p. For Mlp2-ProtA, gold particles were located at 51 ± 10 nm (mean ± S.D., n = 15) from the central plane of the pore. Gold particles were also found in the nucleus. However, the number of nuclear gold particles significantly varied from cell to cell (going from 0 to 30% of the total gold particles), and their distribution seemed to be random with an unclear association with closest pores. In contrast to Mlp2p, most of the gold particles for Mlp1p-ProtA were found in the nucleus (60% nuclear, 38% at the pores, and 2% cytoplasmic). For the gold particles associated with pores, two distinct locations were found, one at 90 ± 20 nm (mean ± S.D., n = 15; see Fig. 2C, arrowheads) and the other at 180 ± 38 nm (mean ± S.D., n = 15; see Fig. 2C, arrows) from the central plane of the pore. In both cases, the gold particles were often aligned on tracks and seemed to be associated with pore-attached filaments (see Fig. 2C).

Fig. 2. Mlp1p and Mlp2p are NPC-associated proteins. Nuclear envelope and nuclear pore location of Mlp2p-GFP (A) and Mlp1p-GFP (B). A haploid double mutant MLP2-GFP::HIS3/nup133::HIS3 was generated. Mlp1p-GFP was expressed from a single copy plasmid (ARS/CEN) transformed into the mlp1Δ single or mlp1Δ/nup133Δ double disrupted strain. Shown are fluorescence and Nomarski pictures. In the case of MLP2-GFP::HIS3 cells (upper panel in A), the fluorescence and Nomarski pictures were merged. C, immunoelectron microscopy localization of Mlp1p and Mlp2p. a, overview of nuclei cross-sections of Mlp1p-ProtA and Mlp2p-ProtA pre-embedding labeled with anti-ProtA antibody directly conjugated to 8-nm colloidal gold particles. b-d, a gallery of selected examples of gold-labeled nuclear pore cross-sections from Mlp1p-ProtA and Mlp2p-ProtA strains. Mlp2p was located at the nuclear periphery of the pore (at 51 ± 10 nm from the central plane of the pore), whereas Mlp1p was found associated with pore-attached filaments at two distinct locations: at 90 ± 20 nm (arrowheads) and 180 ± 38 nm (arrows) from the central plane of the pore. c, cytoplasm; n, nucleus. Scale bars correspond to 200 (a) and 100 (b-d) nm.

Fig. 3. Purification and structure of Mlp1p. A, affinity purification of Mlp1p-TEV-ProtA by IgG-Sepharose chromatography and cleavage by the TEV protease, or acetic acid elution, was performed as described under “Experimental Procedures.” A cell homogenate (1), soluble supernatant (2), insoluble pellet (3), flow-through (4), and a 200-fold equivalent of the TEV-cleaved and eluted Mlp1p (5) were analyzed by SDS–8% PAGE, followed by Coomassie staining and Western blotting, using anti-ProtA antibodies. A protein standard, i.e. 10-kDa ladder with a stronger 50-kDa band, is also shown (6). The position of Mlp1p is indicated. In the case of Western blot analysis, Mlp1p-ProtA was not cleaved by TEV, but eluted from the IgG-Sepharose column by acetic acid. B, electron microscopic appearance of purified yeast Mlp1p after glycerol spraying/low-angle rotary metal shadowing. A gallery of long, mostly curved filamentous molecules with a tendency to anneal head to tail (see last panel down) is displayed. The scale bar represents 100 nm.

We analyzed the structure of purified Mlp1p by electron microscopy after glycerol spraying/low-angle rotary metal shadowing (54). Although the isolated Mlp1p was more than 95% pure, as judged from the SDS-polyacrylamide gel, the sample yielded a rather heterogeneous morphology in the electron microscope. Nevertheless, about 10% of the particles appeared as long, thin and mostly curved filamentous molecules (Fig. 3B). In a control, another yeast ProtA-tagged nucleoporin (ProtA-Nup85p) was affinity purified and prepared for glycerol
spraying/low-angle rotary metal shadowing under identical conditions, but no thin and filamentous structures were seen in the electron microscope.2

A Short Sequence within C-domain of Mlp2p Binds in Vitro to the Karyopherin a/β complex—Since both GFP-tagged Mlp2p and Mlp1p exhibit, besides a distinct nuclear envelope location, an intranuclear staining (see also Fig. 2), it is possible that they are imported into the nucleus with the help of an NLS. It was found recently that the C-domain, but not the coiled-coil N-domain of mammalian Tpr, contains a nuclear localization signal (39, 59). According to these findings, we tested whether the C-domains of Mlp2p (see also Fig. 4A for sequence) and Mlp1p exhibit a nuclear localization sequence which can target an attached reporter protein into the nucleus. Therefore, the C-domains of Mlp2p and Mlp1p were fused to GFP and the in vivo location of the corresponding fusion proteins was determined by fluorescence microscopy (Fig. 4B). This revealed that both Mlp2p-C and Mlp1p-C domains can mediate efficient transport of GFP into the nucleus. GFP alone does not accumulate inside the nucleus (60). Although both GFP fusion proteins are strongly concentrated inside the nucleus, GFP-Mlp2p-C, but not GFP-Mlp1p-C, was also detected in the cytoplasm. Since GFP-Mlp2p-C has a calculated molecular mass of ~40 kDa, it may not be efficiently retained inside the nucleus due to diffusion back into the cytoplasm. In contrast, GFP-Mlp1p-C is larger (~60 kDa) and therefore may be better retained inside the nucleus. We therefore made a 2 x GFP-Mlp2p-C construct and expressed it in yeast. The extent of nuclear accumulation of 2 x GFP-Mlp2p-C increased and the cytoplasmic signal decreased. However, a small cytoplasmic signal was still visible (Fig. 4B). This could suggest that the NLS of Mlp2p is less strong as compared with the Mlp1p-NLS. In conclusion, the C-domains of Mlp2p and Mlp1p contain NLSs which most likely target the corresponding full-length proteins into the nucleus.

The COOH-terminal domain of Mlp2p contains a sequence which resembles a bipartite NLS; in addition, this domain also exhibits a FSFG motif typically found in repeat sequences containing nucleoporins (Fig. 4A). We therefore tested whether the C-domain of Mlp2p

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2 U. Aebi, unpublished results.
can interact with karyopherin α, karyopherin β, or both. The last 180 amino acids from Mlp2p were fused to the GST protein and GST-Mlp2p-C was expressed in E. coli. After purification by glutathione affinity chromatography, recombinant and purified karyopherin α (Kap60p) and karyopherin β (Kap95p) were added separately or as a reconstituted complex to the immobilized GST-Mlp2p-C (Fig. 4C). Kap60p or Kap95p monomers did not significantly bind to GST-Mlp2p-C; in contrast, a strong and cooperative binding of the Kap60p-Kap95p complex to GST-Mlp2p-C beads was observed (Fig. 4C). The immobilized GST alone served as a negative control, to which no binding of the karyopherin α/β complex could be detected (Fig. 4C).

It was recently shown that the karyopherin α/β complex cooperatively binds to FXFG, but not GLFG repeat sequences-containing nucleoporins (52). This could suggest that the C-domain of Mlp2p binds via its FXFG containing sequence or via a NLS to karyopherin α/β. We therefore separated the sequence containing the FSFG motif (residue 1500–1649) from the putative bipartite NLS (residue 1640–1679) and fused both domains to GST. Whereas GST-Mlp2p-C-FSFG no longer bound to karyopherin α/β, the GST-Mlp2p-C-NLS fusion construct retained karyopherin α/β binding activity (Fig. 4D). This shows that the putative bipartite NLS within Mlp2p-C can interact with karyopherin α/β. Finally, this bipartite-type NLS was fused to GFP and expressed in yeast. This revealed that the NLS-like sequence, but not the FSFG-containing part, exhibits nuclear targeting activity (Fig. 4E).

The mlp1Δ/mlp2Δ Double Mutant Is Viable, but Shows an Increased Sensitivity to UV Light—To study the in vivo role of Mlp2p and to find out whether it functionally overlaps with Mlp1p, the complete MLP2 ORF was replaced by the HIS3 gene in a diploid yeast strain (see “Experimental Procedures”). After sporulation of the heterozygous mlp2Δ:HIS3/MLP2 diploid strain and tetrad analysis, four viable spores were recovered, of which the two mlp2Δ:HIS3 containing progeny (further designated as mlp2Δ+) grew similar as compared with the MLP2Δ progeny (data not shown). Since Mlp1p may compensate for the loss of the Mlp2p function, a haploid double disruptant was generated by mating and tetrad analysis. This mlp1Δ/ + mlp2Δ− strain was still viable and no clear growth defect was noticed at the various tested temperatures as compared with MLP1Δ/MLP2Δ+ progeny. However, a dot-spot growth analysis revealed that the double disrupted strain forms heterogeneous colonies (Fig. 5). In addition to colonies of normal size, smaller colonies also became visible in mlp1Δ/ + mlp2Δ− mutants. This shows that the two yeast Tpr-like proteins are required for optimal cell growth. Since mlp1Δ mutants were shown to have an increased sensitivity to UV light (45), and the observed heterogeneous colony size in the double mutant would be consistent with a defect in DNA repair, we analyzed whether the mlp1Δ/ + mlp2Δ− null mutants are hypersensitive to UV light causing DNA damage (61). Indeed, the mlp1Δ/ + mlp2Δ− double disrupted strain displayed a significantly increased sensitivity to ultraviolet radiation (Fig. 5), whereas the single disrupted mlp1Δ− and mlp2Δ− strains were less sensitive to this treatment (Fig. 5). Thus, the mlp1Δ/ + mlp2Δ− double mutant is vulnerable to DNA damaging methods such as UV light irradiation (see also “Discussion”).

Poly(A)+ RNA Export Is Inhibited in Mlp1p-overproducing Cells—To find out whether nucleocytoplasmic transport is affected in the mlp2Δ or mlp1Δ disruption mutants, nuclear protein import of Npl3p-GFP and NLS-GFP-lacZ, and mRNA export were analyzed. No apparent defect in nuclear import and export was observed in neither the single nor the double disrupted strains (data not shown). However, overproduction of Mlp1p (achieved by transforming a haploid RS453 wild-type strain with a high-copy number plasmid containing MLP1) caused nuclear accumulation of mRNA (Fig. 6A). In contrast, overproduction of MLP2-GFP did not reveal such impairment, whereas overexpression of both MLP1 and MLP2-GFP enhanced the inhibition of poly(A)+ RNA export. We noticed that the poly(A)+ RNA signal which accumulated in Mlp1p-overproducing cells did not coincide with the DNA staining. To find out in which part of the nucleus the mRNA accumulated, indirect immunofluorescence was performed using anti-Nsp1p (Fig. 6B) and anti-Nop1p antibodies (Fig. 6C). Clearly, the bright poly(A)+ RNA spot seen in Mlp1p-overproducing cells is confined within the nucleus, in an area which is devoid of chromatin, but is not the nucleolus (Fig. 6, B and C, see also “Discussion”).

DISCUSSION

Nucleocytoplasmic trafficking through the nuclear pore complex is a bidirectional process. However, it is not known whether guided transport continues after translocation through the pore channel on NPC-attached intranuclear and cytoplasmic filaments. During nuclear protein import, termination of transport was suggested to occur at the nuclear basket (i.e. the terminal ring) in Xenopus oocyte nuclei (16), but in vivo facilitated transport of certain import cargos may continue from the nuclear pores to distinct intranuclear sites (for discussion see Ref. 60). Conversely, intranuclear transport of export cargoes (e.g. mRNPs) may also be facilitated by guided transport on track-like structures.

Infranuclear filaments, which are attached to the inner side of nuclear pore complexes could play a role in facilitated intranuclear transport steps, either by serving as tracks or forming intranuclear channels. It is now clear that Tpr proteins are constituents of the NPC-attached intranuclear filaments. Our work shows that the yeast Tpr protein, Mlp2p, physically associates with Nic96p (or a member of the Nic96p complex). Although this interaction was found when spheroplasts were treated with glutaraldehyde prior to affinity purification of ProtA-Nic96p, it seems that Mlp2p was not covalently cross-linked to Nic96p; the apparent molecular mass of the putative cross-link product on SDS-PAGE (~160 kDa) does not match with the calculated molecular mass (~300 kDa). How glutaraldehyde fixation allows a better co-enrichment of Mlp2p during ProtA-Nic96p purification is not clear. However, Mlp2p does not unspecifically co-purify with any nucleoporin during purification from glutaraldehyde-treated spheroplasts as a
yeast? Although both proteins exhibit a strong homology, they appear distinct in appearance. In the main, thus giving the molecules their long filamentous structure. Heptad-repeat containing NH2-terminal domain, they may adapt a different shape in specimen preparation. As both Mlp2p and Mlp1p harbor a long cation and/or spraying, and bend, kink, or fold back during the process. Mlp1p molecules may have a tendency to break during purification, but the long and thin structure of Mlp2p is more flexible and can adapt to different environments during purification.

The reason for this behavior is not known, but the long and thin structure of Mlp2p is more dynamic at the NPC, as recently suggested for vertebrates. Nic96p is an NPC-attached intranuclear filament protein. Mlp1p interacts with Nic96p, and Mlp2p provides an anchoring site for Nic96p on the nuclear basket of the NPC. Whether Mlp1p also interacts with Nic96p to form a complex and mediates nuclear accumulation of a GFP reporter protein, as shown in Figure 6.

Since no heterodimer formation between Mlp1p and Mlp2p was observed, these coiled-coil proteins may be monomers or homodimers. However, more work is required to address this. In an attempt to obtain a first glimpse of the structure of the yeast Tpr proteins, Mlp1p was purified and analyzed by electron microscopy after glycerol spraying/low-angle rotary metal shadowing. This revealed that Mlp1p is a thin, mostly curved filamentous molecule with a length in excess of 100 nm. Although biochemically pure, morphologically the sample appeared rather heterogeneous with only about 10% of the particles exhibiting an elongated structure. The reason for this behavior is not known, but the long and thin Mlp1p molecules may have a tendency to break during purification and/or spraying, and bend, kink, or fold back during specimen preparation. As both Mlp2p and Mlp1p harbor a long heptad-repeat containing NH2-terminal domain, they may dimerize to form a parallel 2-stranded α-helical coiled-coil domain, thus giving the molecules their long filamentous appearance.

Do Mlp1p and Mlp2p perform an overlapping function in yeast? Although both proteins exhibit a strong homology, they do not overlap in vivo in a synthetically lethal relationship. One possibility is that both have a redundant role in optimizing nucleocytoplasmic transport reactions, e.g., in nuclear protein import (56) and mRNA export from intranuclear sites to the nuclear pores. Mlp1p and Mlp2p may have additional roles, e.g., in intranuclear organization or linking intranuclear structures such as peripheral chromatin or chromatin-associated proteins to the nuclear envelope and/or nuclear pores. It has been shown by immunoelectron microscopy that chromatin directly contacts the NPC-attached intranuclear filaments (63). Interesting in this context is the increased sensitivity of the mlp1/mlp2 double disruption mutant to UV light. In Saccharomyces cerevisiae, telomeric chromatin is associated with the nuclear envelope; SIR proteins and Rap1p play an important role in the segregation of telomeric heterochromatin to the nuclear envelope (64). Recently, it was found that cells lacking the chromatin assembly factor-1 exhibit an increased ultraviolet radiation sensitivity and reduction of telomeric silencing (65). Thus, chromatin assembly and organization with respect to telomeric silencing and DNA repair are processes in yeast that appear to require the nuclear periphery.

The C-domains of both Mlp2p and Mlp1p contain, like their higher eukaryotic counterparts (39, 59), nuclear targeting signals. In the case of Mlp2p, we could identify a short sequence at the COOH-terminal end that not only resembles a basic bipartite NLS, but binds cooperatively in vitro to the karyopherin α/β complex and mediates nuclear accumulation of a GFP reporter protein. Thus, the observed interaction between Mlp2p and karyopherin α/β may be used for nuclear uptake of Mlp2p. Since the C-domain of Mlp2p contains one FXFG motif and an extended sequence with moderate resemblance to degenerate repeat domains of classical nucleoporins, it is possible that the C-domain of Mlp2p directly interacts with karyopherin β and other β-like transport factors. However, the significance of this FSFG motif within Mlp2p remains to be shown. In any case, the C-domains of Mlp2p and Mlp1p appear to extend from the predicted filament-forming N-domain and therefore should be accessible to bind to transport factors.

Although nuclear mRNA export is not inhibited in mlp1− or mlp2− cells, overproduction of Mlp1p causes poly(A)+ RNA accumulation inside the nucleus. Interestingly, mRNA accumulates in a distinct area of the nucleus which is devoid of chromatin and is neither the nucleolar compartment. Since overproduction of Mlp1p causes formation of intranuclear aggregates, it is possible that mRNPs get trapped in these Mlp1p-containing intranuclear structures. It has been shown recently...
that overproduction of Mlp1p causes an extensive electron-dense fibrillargranular network, which extends from the nuclear envelope toward the nuclear interior and is highly permeable, even for large macromolecules (56). It is thus possible that mRNPs together with transport factors become trapped within these Mlp2p aggregates. Interestingly in this context we found an association of Mlp2p with the Mex67p/Mtr2p mRNA export complex under conditions of dominant-negative RanGTP expression. It is possible that this interaction represents a translational intermediate trapped on intranuclear tracks, allowing co-isolation of Mlp2p with Mex67p/Mtr2p.

In summary, we characterized two NPC-associated proteins, Mlp2p and Mlp1p, which are the closest yeast homologues of higher eukaryotic Tpr proteins. We found that purified Nic96p is associated with a pool of Mlp2p. This implies that Nic96p (or a Nic96p complex at the terminal ring) links the yeast Tpr protein Mlp2p to the nuclear pores. Since Mlp2p can interact with protein import and nuclear mRNA export factors, and overproduction of Mlp1p inhibits mRNA export, we suggest that yeast Tpr proteins form filaments, which participate in intranuclear transport toward and away from the nuclear pores.

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