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Biochemical and Biophysical Research Communications 335 (2005) 146–153
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Identification of an unconventional nuclear localization signal in human ribosomal protein S2

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Received 12 July 2005
Available online 25 July 2005

Abstract

Ribosomal proteins must be imported into the nucleus after being synthesized in the cytoplasm. Since the rpS2 amino acid sequence does not contain a typical nuclear localization signal, we used deletion mutant analysis and rpS2–β-galactosidase chimeric proteins to identify the nuclear targeting domains in rpS2. Nuclear rpS2 is strictly localized in the nucleoplasm and is not targeted to the nucleoli. Subcellular localization analysis of deletion mutants of rpS2–β-galactosidase chimeras identified a central domain comprising 72 amino acids which is necessary and sufficient to target the chimeric β-galactosidase to the nucleus. The nuclear targeting domain shares no significant similarity to already characterized nuclear localization signals in ribosomal proteins or other nuclear proteins. Although a Nup153 fragment containing the importinβ binding site fused to VP22 blocks nuclear import of rpS2–β-galactosidase fusion proteins, nuclear uptake of rpS2 could be mediated by several import receptors since it binds to importinα/β and transportin.

Keywords: Ribosomal protein S2; Subcellular localization; Nuclear localization sequence; VP22; Nup153

In eukaryotes, the nucleolar compartment of the nucleus is the main site for assembly of the 40S and 60S subunits of the ribosomes, but late maturation events are localized in the nucleoplasm and the cytoplasm. During ribosome synthesis, a pre-ribosomal RNA transcript is processed to the mature 18S rRNA part of the 40S subunit and to the 5.8S and 28S rRNA components of the large subunit. As soon as the 18S rRNA is completely transcribed, it acquires a structure already close to the mature form and assembles with ribosomal proteins [1]. Since ribosomal proteins are synthesized in the cytoplasm, around 80 ribosomal proteins have to be coordinately transported into the nucleus. To ensure a rapid and timely nucleocytoplasmic transport, ribosomal proteins are actively imported by binding to nuclear transport receptors, the karyopherins or importins. Most of the nuclear import of cargo proteins is mediated by the importinβ-type transport receptors. They circulate between the cytoplasm and nucleus, and transport proteins through the nuclear pore complex by binding to specific nuclear localization signals (NLS) encoded in the sequence of the cargo proteins. In the nucleus, importins bind to RanGTP, which triggers the release of the cargo protein and facilitates cargo loading onto exportins, also members of the importinβ-protein superfamily. After the release of the cargo the importin-RanGTP cycles back to the cytoplasm where the bound GTP is hydrolyzed and RanGDP dissociates from the importins. Additional co-factors which bind to nucleotide-bound and -free Ran add more regulatory complexity to the basic model. In addition, some of the importinβ family members are able to function as nuclear export and import receptors [2,3]. Nuclear

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doi:10.1016/j.bbrc.2005.07.069

import is mediated by recognition of specific, in general basic, import signals. Studies of ribosomal protein import in yeast *Saccharomyces cerevisiae* and in mammalian cells suggest that a single ribosomal protein could be translocated by interacting with multiple import receptors independently. A prototype of a highly basic import domain (termed BIB, β-like import receptor binding domain) was characterized in ribosomal protein L23a which probably applied to the majority of ribosomal proteins. The BIB domain can bind at least four different transport receptors including importinβ, importin5, importin7, and transportin. Interestingly, transportin can bind simultaneously to the M9 peptide and to the BIB domain, and may be able to co-import protein cargos containing different types of NLS [4,5]. Recently, it was demonstrated that binding of importins to ribosomal proteins is shielding ribosomal proteins and other highly basic proteins against aggregation with cytoplasmic polyanions such as tRNA [6]. The nuclear export of the pre-60S particles requires Nmd3p which carries a nuclear export signal (NES) and couples as an adapter protein the large subunit protein Rp110p to the nuclear export machinery. It is most likely that the nuclear export of the pre-40S particles also relays on a NES containing adapter protein [7,8].

The gene that encodes the ribosomal protein S2 is essential and its knock-out is lethal in *S. cerevisiae* ([9], unpublished data). The gene *string of perls* (sop) encodes the *Drosophila* rpS2 and a mutant allele of sop blocks development at a mid-stage of oogenesis, suggesting a specific developmental role during oogenesis for rpS2 in addition to its function as part of the small ribosomal subunit [10,11]. In addition, human ribosomal protein (rp) is one of the few rp genes upregulated in certain human cancers and in the presence of mutant p53 [12–14]. In this report, we provide evidence that nuclear import of the small ribosomal protein S2 (rpS2) differs from the majority of ribosomal proteins in that it is not targeted to the nucleoli and nuclear import of rpS2 is mediated by an unusual non-basic nuclear localization signal. Nevertheless, we show that rpS2 interacts with multiple import receptors.

Materials and methods

Cell culture. COS-1 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). For transient DNA transfections, 20 μg of purified plasmid DNA was introduced into 5×10^5 COS-1 cells by electroporation (450 V/250 μF) using a Bio-Rad Gene-Pulser. Between 48 and 72 h after transfection, the cells were harvested for immunoblot analysis or processed for immunofluorescence.

Immunofluorescence. COS-1 cells grown on glass coverslips were transfected with the appropriate plasmids, and 48 h later the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The cells were then permeabilized with 0.2% Triton X-100 in PBS for 4 min and treated with 3% bovine serum albumin in PBS to block non-specific binding of the antibodies. The coverslips were exposed to primary antibodies, and fluorescently tagged secondary antibodies were diluted in 3% bovine serum albumin, PBS. After washing in PBS, the stained cells were mounted in 90% glycerol containing *p*-phenylenediamine and viewed with a 100× oil immersion lens on a Zeiss microscope equipped with the appropriate barrier filters for Texas red optics or a Leica Laser Scanning Spectral Confocal Microscope. Mouse FGF3 C-terminal antipeptide rabbit polyclonal serum was diluted 1 in 200 in PBS. β-Galactosidase was detected using a monoclonal antibody, kindly supplied by H. Durban, Imperial Cancer Research Fund, and rpS2 was detected using a mouse monoclonal antibody against the RGS(His)$_6$ tag (Qiagen).

Plasmid constructions. pS2-1.1 was constructed by inserting the anti-RGS(His)$_6$ epitope upstream and in-frame of the coding region of human ribosomal protein S2 (rpS2) cDNA. The modified human rpS2 cDNA was then inserted into the expression vector pKC4 under control of the early simian virus 40 (SV40) promoter. To obtain the plasmids pS2-1.2, pS2-1.3, and pS2-1.4, PCR was used to delete the N-terminal 62, 113, and 139 amino acids of rpS2, respectively. The vector pKC4.16, which expresses a mutant FGF3 lacking the signal peptide, has been described previously [15]. pS2-1.5 was produced by deleting the 19 C-terminal amino acids in construct pS2-1.2 by PCR. pS2-1.6 and pS2-1.7 were generated by deleting the internal amino acid sequence from residue 51 to 151 and between amino acid 51 and 226 by deleting the corresponding *Sac*I–*Sac*I fragments of the pS2-1.1. The β-galactosidase-rpS2 fusion proteins were based on the expression plasmids pGAL1.0 and pGAL1.1, which have been previously described. Using PCR, partial sequences of rpS2 were amplified with 3′ primers that introduced an *Xho*I site and 5′ primers that introduced an *Xba*I site and a Kozak optimized translation start site. The resulting PCR fragments were inserted into the single *Xho*I site and the *Xba*I site of pGAL1.1, replacing the *fgf3* sequences. To generate the Nup-VP22 mutants, PCR was employed to generate the N-terminal and C-terminal Nup153 fragments corresponding to Nup153 N′ and Nup153 FG [16] which were TOPO cloned into the pV22/myc-His2 vector (Invitrogen).

Immunoblot analysis. For preparing yeast cell lysates, the yeast cells were first disrupted using glass beads. Samples from equivalent numbers of yeast cells were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 12.5% or 15% polyacrylamide gels, transferred to nitrocellulose membranes (Schleicher and Schuell), and then probed with a mouse monoclonal antibody against the RGS(His)$_6$ tag (Qiagen). The immunoreactive proteins were detected by enhanced chemiluminescence using horseradish peroxidase-coupled anti-rabbit immunoglobulin antibodies as described by the manufacturer (Amersham International).

Results

RpS2 is localized in the nucleoplasm and cytoplasm, but is excluded from the nucleoli

The rpS2 proteins are highly conserved during evolution with no residue exchange between the mouse and the human protein, and there is 59% identity between the human and the *S. cerevisiae* amino acid sequence (Fig. 1).

To determine the subcellular localization of human rpS2, we constructed a mutant rpS2 protein containing a RGS(His)$_6$ tag at the N-terminus. The intracellular distribution of rpS2 was investigated by conventional and confocal immunofluorescence microscopy. COS-1

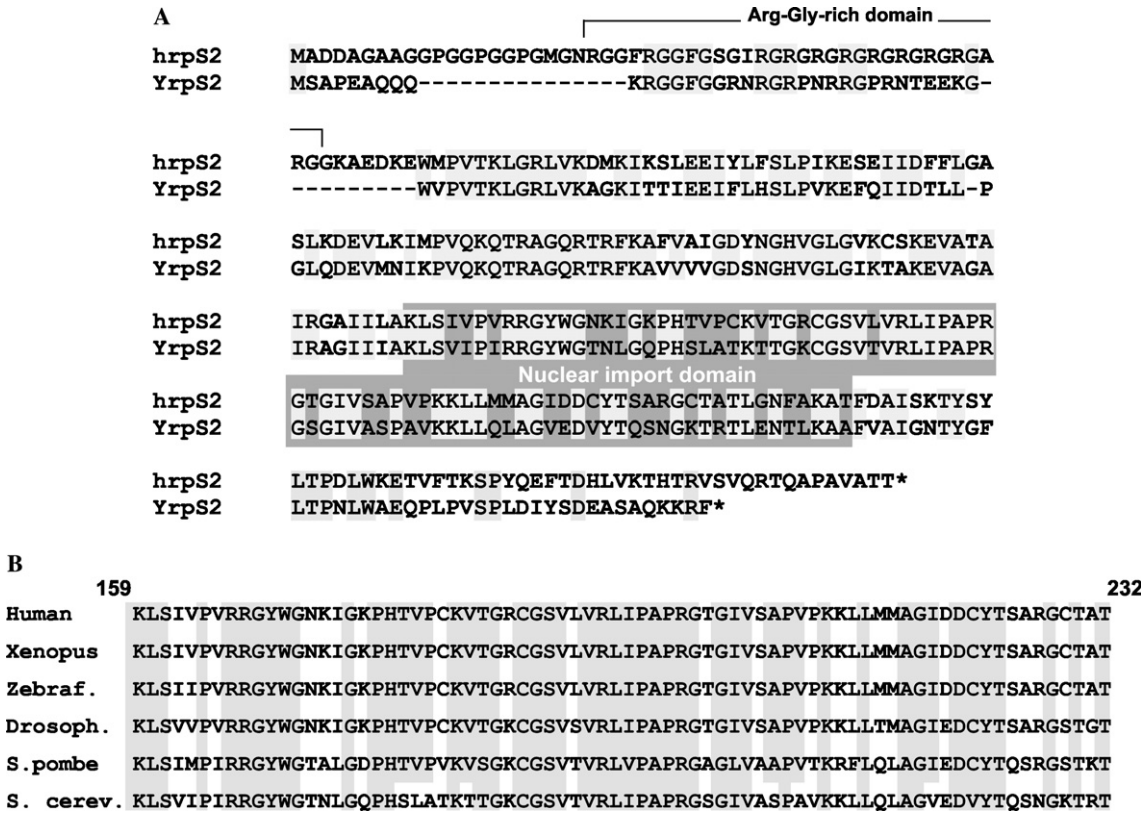

Fig. 1. (A) Amino acid sequence of human rpS2 (hrpS2) and comparison to *S. cerevisiae* rpS2 (YrpS2). Identical amino acids are indicated by gray boxes. The central domain implicated in nuclear targeting is boxed. The N-terminal Arg–Gly-rich region is also boxed. (B) Comparison of the amino acid sequence containing the identified nuclear targeting domain from different species.

cells were transfected with the pKCrpS2-1.1 encoding the mutant His-rpS2 protein. The His-tagged proteins were detected with a monoclonal anti-RGS(His)$_6$ antibody and the immunocomplexes were visualized using a species-specific secondary FITC-conjugated antibody. Surprisingly, in the cell nucleus, His-rpS2 is strictly localized in the nucleoplasm with nucleolar exclusion. An additional cytoplasmic staining was observed in the cells. Analyzing more than 200 transfected cells, 67% demonstrated a more nuclear than cytoplasmic staining and 33% vice versa. Only in few cells we could detect exclusive cytoplasmic staining (Fig. 2A). Using more than three independent transfections with different DNA preparations, we could not detect a nucleolar localization of the His-tagged rpS2. Applying laser scanning microscopy, we dissected several transfected cells and did not find His-rpS2 immunoreactivity in the nucleoli (Fig. 2B, lower panel). In order to exclude the possibility that introducing the N-terminal tag might disturb the intracellular localization of rpS2 and consequently its function, we generated yeast clones in which the endogenous rpS2 gene (SUP44) had been knocked out and the N-terminally histidine-tagged human or its yeast homologue rpS2 expressed under an inducible GAL1 promoter. We took advantage of the fact that the SUP44 gene is only present as a single copy in the haploid genome and its knock-out is lethal ([9] and own unpublished results). The expression of the human His-rpS2 was analyzed by Western blot using anti-RGS(His)$_6$ monoclonal antibody in yeast cells cultured with or without galactose (Fig. 3). Yeast clones expressing human His-rpS2 or yeast His-rpS2 were analyzed for their proliferation under conditions where gene expression was suppressed or induced. In dextrose-containing medium, the yeast clones did not show any cell growth, while in medium containing 2% galactose the yeast clones demonstrated an exponential growth independent of the expressed rpS2 homologue, suggesting that the N-terminally histidine-tagged mutants are functionally active and second that the human rpS2 homologue can functionally replace the yeast homologue (Fig. 3).

RpS2 uses an unusual nuclear import signal

A highly basic motif similar to the BIB domain characterized in some ribosomal proteins which mediates the binding to different nuclear import receptors and the targeting of the ribosomal proteins to the nucleus and nucleoli is not present in the amino acid sequence of rpS2 [4,5]. The sequence of rpS2 also does not contain

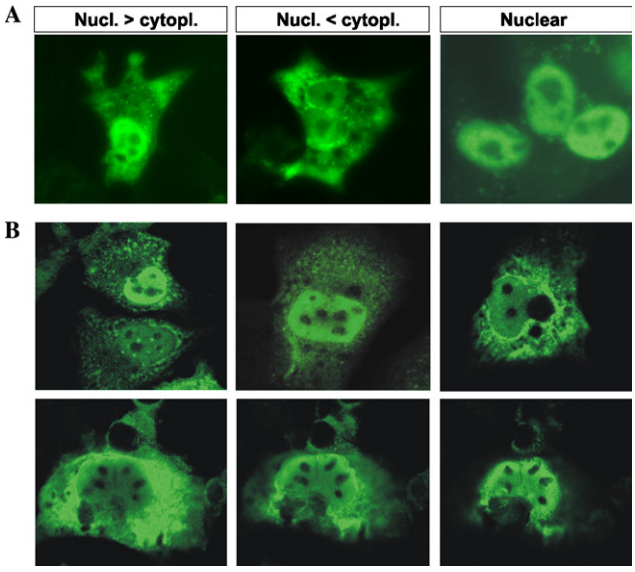

Fig. 2. Nuclear rpS2 is excluded from the nucleoli. (A) COS-1 cells transiently transfected with RGS(His)$_6$-tagged human rpS2 (pKC-rpS2-1.1) were grown on coverslips for 48 h, fixed in 4% paraformaldehyde, and permeabilized with Triton X-100 and then stained with an anti-RGS(His)$_6$ monoclonal antibody (Qiagen) against the His epitope. The rpS2 His-tagged protein complexes were visualized by conventional indirect immunofluorescence using a secondary goat anti-mouse immunoglobulin tagged with fluorescein. Three representative immunostainings are shown. (B) Subcellular localization of His-tagged human rpS2 protein analyzed by laser scanning microscopy demonstrating that rpS2 is excluded from the nucleoli. Lower panel shows a dissection of one pKC-rpS2-1.1 transfected cell. His-tagged rpS2 is found in the cytoplasm and nucleoplasm.

a classical or a bipartite NLS. However, the N-terminal 63 amino acids of the rpS2 protein are rich in Arg-Gly residues, including two consecutive copies of the RGGF motif and eight RG tandem repeats. RG-rich sequences are considered to be involved in nucleolar localization and binding to RNA.

To investigate the possible targeting motifs for nuclear import of rpS2, we generated a set of rpS2 deletion mutants and expressed the mutants in COS-1 cells. The subcellular distribution of the mutant proteins was monitored by indirect immunofluorescence as described previously [17]. Deletion of the N-terminal Arg-Gly-rich region still resulted in nuclear uptake of the truncated protein and a similar subcellular distribution but more pronounced nuclear staining as detected with the wild type rpS2. Further deletions until the N-terminal half of the protein had been truncated did not change the nuclear localization. In addition, deletion of C-terminal amino acids did not affect the nuclear localization of the mutant protein. Deletion mutants lacking large parts of the internal sequences still enter the nucleus, possibly due to their reduced size by passive diffusion (Fig. 4). Therefore, we tested fragments of rpS2 for their ability to confer nuclear import upon a heterologous cytoplasmic protein. Segments of rpS2 were fused to the bacterial lacZ gene and the subcellular localization of the chimeric proteins was examined by indirect immunofluorescence (Fig. 5). The parental plasmid (pGAL1.0) encoding the β-galactosidase showed the

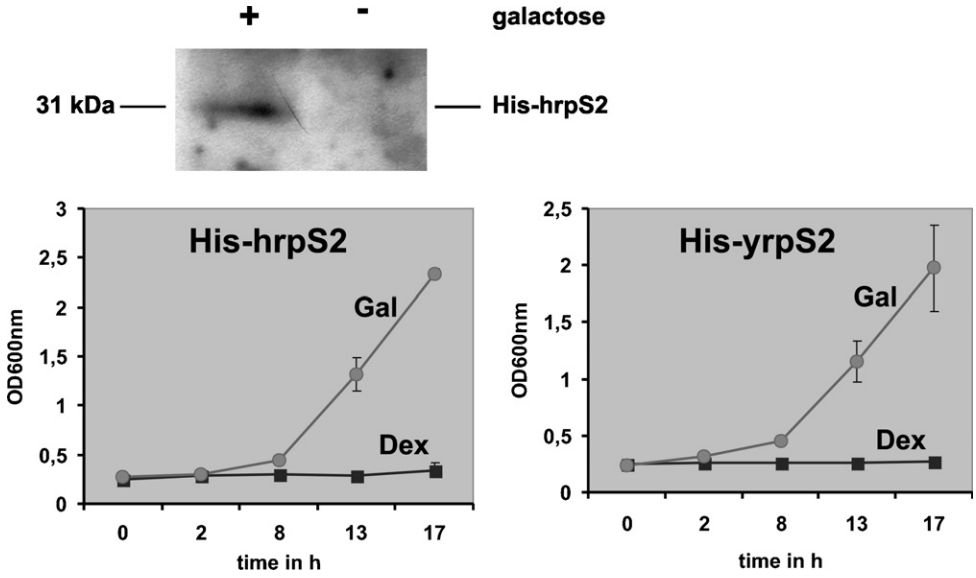

Fig. 3. Human His-tagged rpS2 can functionally replace the rpS2 yeast homologue. Expression of His-tagged human rpS2 protein under control of the GAL1 promoter in INVSc1 cells in which the Yeast rpS2 encoding gene SUP44 had been deleted. INVSc1 cells induced with medium supplemented with 2% galactose expressed a 31 kDa protein detectable with the anti-RGS(His)$_6$ monoclonal antibody. Growth of SUP 44-deleted INVSc1 cells transformed with either the pYes-hS2 or pYes-YS2 expression plasmid is clearly dependent on the expression of rpS2 no matter if the human or the yeast homologue is expressed.

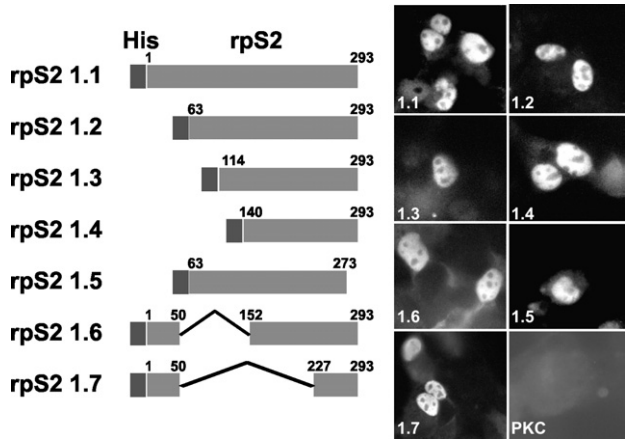

Fig. 4. Subcellular distribution of rpS2 deletion mutant proteins. A series of mutant cDNAs with deletion of various parts of the rpS2 sequence is depicted schematically. The subcellular distribution of rpS2-related proteins in COS-1 cells transiently transfected with these cDNAs is shown. The location of the rpS2-related proteins was determined by staining with a monoclonal antibody against the His epitope, followed by the addition of a secondary antibody conjugated to fluorescein.

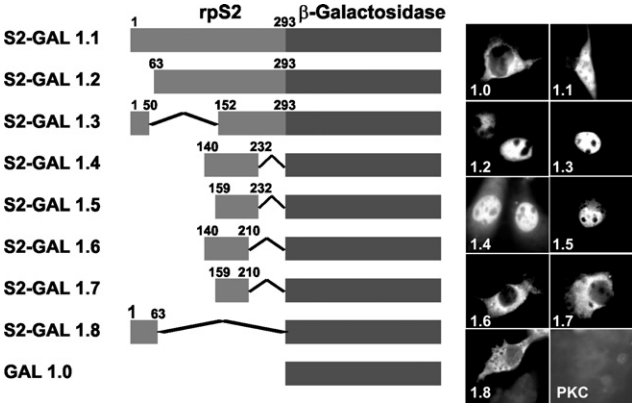

Fig. 5. Properties of rpS2 sequences appended to β-galactosidase as nuclear targeting signals. Schematic depiction of S2aa-Gal showing the position of the diverse rpS2 fragments N-terminally fused to β-galactosidase. Following transfection of the expression constructs into COS-1 cells, the localization of the rpS2-galactosidase proteins was determined by immunofluorescence using a monoclonal antibody against β-galactosidase.

expected cytoplasmic staining. In contrast, the construct with the entire rpS2 sequence fused to the N-terminus of the β-galactosidase is equally distributed between the nucleus and the cytoplasm, and is similar to the subcellular distribution of His-rpS2 excluded from the nucleoli. The chimeric protein containing rpS2 sequences between amino acids 63 and 293 also accumulates in the nucleus but with exclusion of the nucleoli. Deletion of the sequence between the amino acids 51 and 151 did not diminish the nuclear uptake of the fusion protein. Although rpS2 deletion analysis might have suggested that the first 63 amino acids could contain a NLS, when fused to β-galactosidase the N-terminal domain was not sufficient to confer nuclear import upon β-galactosidase. The sequences between amino acids 140 and 232 or between 159 and 232, however, are sufficient to allow the nuclear import of β-galactosidase. Further N-terminal or C-terminal shortening of the sequence between residues 210 and 232 resulted in two mutants which are exclusively located in the cell cytoplasm, suggesting that there is a nuclear targeting motif contained in the segment of amino acids 159–232 which is sufficient to direct a heterologous cytoplasmic protein into the cell nucleus (Fig. 5). We tried to trim further down the sequences necessary and sufficient to target β-galactosidase into the nucleus. However, N-terminal and C-terminal trimming of the rpS2 segment at amino acids 159 and 232 resulted in a fusion protein which lost its capacity for nuclear localization (Fig. 6).

RpS2 binds to two nuclear import receptors representing separate import pathways

The import of nuclear proteins is generally mediated by binding to nuclear transport receptors. Transportin and importinβ import pathways appear to intersect on the nucleoporin Nup153 [18]. The primary structure of Nup153 can be divided into three main parts, the non-repetitive N-terminal region which interacts with the import receptor transportin, a central five zinc finger containing region and a C-terminal domain with spaced FXFG repeats [19]. The C-terminal region is important for binding to several import receptors and adding the C-terminal region of Nup153 to in vitro import assays inhibited the importinα/importinβ-mediated nuclear uptake of cargoes but leaves the transportin-mediated nuclear import intact [16]. We fused fragments of Nup153 which had shown dominant-negative mutant activity in in vitro nuclear import assays to the N-terminus of VP22 protein. VP22 is a herpes simplex virus-1 protein that has the ability to translocate between mammalian cells. VP22 or VP22-fusion proteins are exported from the expressing cells via a non-Golgi secretion pathway and localize into the nucleus of non-expressing adjacent cells [20–22]. COS-1 cells were transfected with cDNAs encoding VP22 N-terminal and C-terminal Nup153 fusion proteins, Nup-N′-VP and Nup-FG-VP, respectively. Subcellular localization of the chimeric proteins was detected by indirect immunofluorescence using a monoclonal anti-myc antibody recognizing the C-terminal myc tag of the VP22 protein (Fig. 7A, upper panel). Nup-N′-VP and Nup-FG-VP accumulate in nearly all cells at the nuclear membrane. In COS-1 cells, co-transfected with the chimeric VP22 protein and with the rpS2-(S2-GAL1.2) fusion protein with the deletion of the first 62 N-terminal amino acids, the rpS2-GAL could still be detected by histochemistry in the nucleus of COS-1 cells co-transfected with the Nup-N′-VP

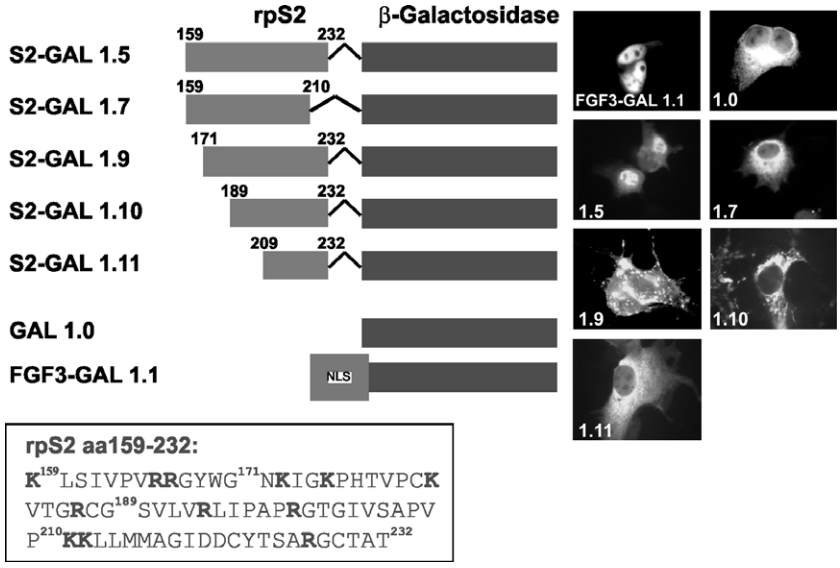

Fig. 6. Subfragments of the identified nuclear targeting domain are not sufficient to confer nuclear localization to β-galactosidase. N-terminal and C-terminal deletion mutants derived from the $S2_{159–232}$-Gal construct are depicted schematically. The different expression plasmids were introduced into COS-1 cells and the subcellular distribution of rpS2–β-galactosidase fusion proteins was monitored by immunofluorescence using a monoclonal antibody against β-galactosidase. FGF3-Gal1.1 containing the N-terminal bipartite NLS of FGF3 served as positive control. The sequence of the identified domain active in nuclear targeting is shown and the basic residues highlighted.

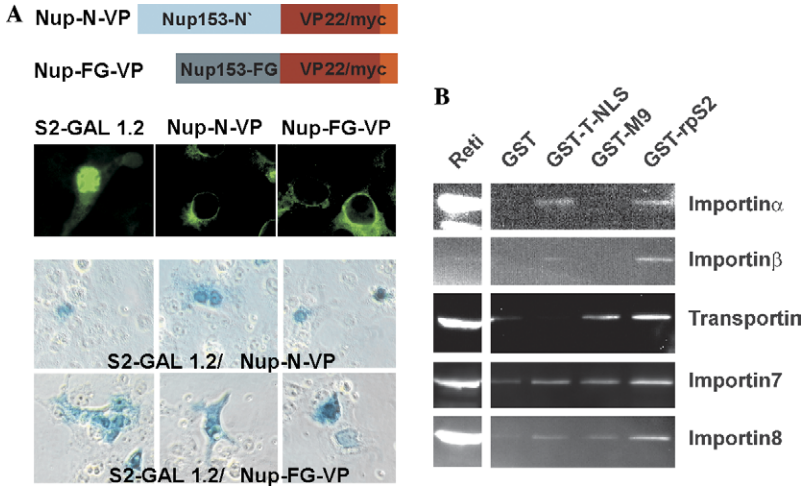

Fig. 7. Nuclear uptake of rpS2–β-galactosidase fusion proteins is selectively inhibited by Nup153N′-VP22, but rpS2 binds to distinct import receptors. (A) Fragments of Nup153 which block selectively the importinα/β or transportin-mediated pathway were fused to VP22. COS-1 cells were co-transfected with cDNAs encoding the Nup-VP22 fusion proteins and a cDNA encoding the $S2_{63–293}$–β-galactosidase fusion protein. The location of the fusion proteins was determined by staining with a monoclonal antibody against myc-tagged VP22, followed by the addition of a secondary antibody conjugated to fluorescein. Localization of the rpS2–β-galactosidase fusion protein was monitored by the galactosidase activity. (B) The binding of nuclear import receptors to rpS2 was assessed by affinity precipitation. GST, M9-GST, T_{NLS}-GST, and rpS2-GST fusion proteins were expressed in *E. coli*, and extracts were bound to GSH–Sepharose. The washed beads were incubated with reticulolysates and the retained proteins were analyzed with specific antibodies as indicated.

mutant but co-transfection with the C-terminal region of Nup153 abolished the nuclear uptake of the rpS2-1.2-GAL fusion protein (Fig. 7A). The result suggests that the nuclear import of 63–293 aa rpS2-GAL protein into the nucleus is mainly dependent on the importinα/β-mediated transport. To further investigate the nuclear uptake of rpS2 and since analysis of the nuclear import of ribosomal proteins revealed that their nuclear import is mediated by transport receptors which bind to distinct cargo proteins via unrelated nuclear targeting signals, we performed a column retention assay to identify possible rpS2 interacting nuclear transport receptors. Recombinant GST fusion proteins were generated by N-terminal GST fusion to the full-length rpS2 sequence

or to control peptides containing the classical large T NLS and the M9 NLS, respectively. The recombinant proteins were expressed in *Escherichia coli* with appropriate cDNAs and bound to GSH–Sepharose. The proteins were then tested for their ability to bind to the receptor protein in their native form present in reticulocyte lysates or nuclear extracts from HeLa cells. After extensive washing, the bound proteins were eluted in SDS–PAGE buffer, separated by SDS–PAGE, and analyzed by Western blotting using transport receptor-specific antibodies. The result showed that the GST-largeT-NLS protein retained the importinα/importinβ complex but not transportin, while the GST-M9 construct clearly interacts with transportin, but not with the importinα/importinβ complex. In contrast, GST-rpS2 interacts with the importinα/β complex and with the nuclear transport receptor transportin. Importin7 and 8 weakly associated with both GST-NLS test constructs and demonstrated a slightly higher binding affinity for the rpS2-GST fusion protein. Therefore, specific interaction is clearly demonstrated for transportin and the importinα/β nuclear import receptors. Surprisingly, despite no obvious sequence homology of the NLS identified in rpS2 to already characterized nuclear targeting motifs, rpS2 can bind to nuclear import receptors of two separate nuclear import pathways (Fig. 7B).

Discussion

In the present study, we demonstrated that the ribosomal protein S2 is one of the few ribosomal proteins not associated with the nucleoli. We showed that rpS2 is excluded from the nucleoli by confocal laser scanning microscopy and conventional immunofluorescence microscopy using a His-tagged form of rpS2. Since the gene encoding rpS2 is highly conserved during the evolution and essential for viability in *S. cerevisiae* [9], we used these features of rpS2 to exclude the possibility that the N-terminal histidine tag might interfere with the distribution of the protein. The yeast and human-tagged rpS2 both were tested for their ability to replace the knocked out endogenous yeast rpS2 which is present as only one copy in the haploid yeast genome. Both proteins are able to functionally replace the wild type protein, suggesting that the function and therefore most likely the localization of the His-tagged forms is the same as the wild type ribosomal protein. In addition, we detected cytoplasmic staining with the His-tagged rpS2 which is a good indication that the tagged protein is incorporated into ribosomal subunits. Recently, Bulygin et al. demonstrated that rpS2 is one of the five ribosomal proteins which comes into contact with the first position of a codon when placed in the A site of the ribosome. This finding is in good agreement with its proposed function to ensure the fidelity of protein synthesis [23]. In addition, this function of rpS2 is consistent with a location near the surface of the small ribosomal subunit and with its subcellular localization, suggesting that it may join the subunit at a later maturation step in the nucleoplasm.

The sequence of the majority of ribosomal proteins contains basic motifs which may function as classical NLS or contain highly basic domains similar to the BIB sequence (β-like import receptor binding domain) identified in rpL23a, which is essential and sufficient for nuclear and nucleolar targeting [4]. In contrast, the identified nuclear targeting motif in rpS2 is much less basic (p*I* 10.62) and has a similar composition of basic residues as the total protein (p*I* 10.66). Nevertheless, this domain is able to translocate a heterologous cytoplasmic protein-like β-GAL into the nucleus. Both, the flanking N-terminal or C-terminal sequences did not have nuclear targeting activity when fused to β-GAL. Analysis of ribosomal protein import in mammalian and yeast revealed that some ribosomal proteins can interact with and be imported by multiple nuclear import receptors. Transportin can bind via two separate binding sites to classical basic NLS and to the unrelated glycine-rich M9 transport signal [4,6]. The two import pathways seem to intersect at the nucleoporin, Nup153; and a Nup153 fragment containing the import β-binding site can act as a dominant-negative mutant to block the importinβ-mediated nuclear uptake but leaves the transportin pathway intact. A second mutant containing the transportin binding site has the opposite effect [16]. We generated fusion mutants with the herpes simplex virus-1 VP22 protein and the two Nup53 fragments to translocate the chimeric proteins into the untransfected cells. Nearly all cells exhibit a nuclear rim staining, suggesting that the chimeric proteins are locked on the nuclear pore complex. Interestingly, nuclear uptake of rpS2-1.2-βGAL, missing the N-terminal domain, via the importinβ-pathway is inhibited while blocking the transportin-mediated pathway did not affect the nuclear uptake of the rpS2-1.2-βGAL construct, suggesting that the identified internal nuclear import signal interacts mainly with the import α/β import receptor system. Since Arg-Gly-rich domains are often implicated in protein–protein interaction, transportin or other transport receptors might interact with the N-terminal domain of rpS2 in addition to the internal NLS. This would explain the discrepancy between the pull-down assay and the functional import assay. However, the N-terminal sequences on its own were not sufficient to confer nuclear import onto β-galactosidase. The post-translational methylation of Gly-Arg-rich N-terminus of high molecular weight forms of FGF2 has been implicated in the nuclear retention of FGF2. Recently, the N-terminal Arg-Gly repeat of rpS2 has been identified as a natural substrate for mammalian protein arginine methyltransferase 3, which might have a function

in nuclear accumulation of the ribosomal protein [11,24,25]. The ribosomal protein S2 has been reported to be upregulated in tumor cells and in proliferating hepatocytes after partial hepatectomy correlating elevated rpS2 with cell growth control. In summary, we provided evidence that the ribosomal protein rpS2, which is encoded by an essential gene in *S. cerevisiae*, uses an unconventional NLS for nuclear import, but similar to other ribosomal proteins interacts with different nuclear transport receptors.

Acknowledgments

The authors are indebted to Dirk Görlich for providing the antibodies against importin7, importin8, and importinβ. The authors thank Douglass J. Forbes for kindly providing cDNAs of the Nup153 dominant-negative mutants. This work was partially supported by grants from the German Research Foundation.

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