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Identification of an unconventional nuclear localization signal in human ribosomal protein S2

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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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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