Ribosomal Protein S9 Is a Novel B23/NPM-binding Protein Required for Normal Cell Proliferation

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B23 (NPM/nucleophosmin) is a multifunctional nucleolar protein and a member of the nucleoplasm superfamily of acidic histone chaperones. B23 is essential for normal embryonic development and plays an important role in genomic stability, ribosome biogenesis, and anti-apoptotic signaling. Altered protein expression or genomic mutation of B23 is encountered in many different forms of cancer. Although described as multifunctional, a genuine molecular function of B23 is not fully understood. Here we show that B23 is associated with a protein complex consisting of ribosomal proteins and ribosome-associated RNA helicases. A novel, RNA-independent interaction between ribosomal protein S9 (RPS9) and B23 was further investigated. We found that S9 binding requires an intact B23 oligomerization domain. Depletion of S9 by small interfering RNA resulted in decreased protein synthesis and G1 cell cycle arrest, in association with induction of p53 target genes. We determined that S9 is a short-lived protein in the absence of ribosome biogenesis, and proteasomal inhibition significantly increased S9 protein level. Overexpression of B23 facilitated nucleolar storage of S9, whereas knockdown of B23 led to diminished levels of nucleolar S9. Our results suggest that B23 selectively stores, and protects ribosomal protein S9 in nucleoli and therefore could facilitate ribosome biogenesis.

Ribosome biogenesis is a complex process involving many different proteins acting at various stages from early ribosomal RNA (rRNA) synthesis, followed by processing, subunit assembly, and nuclear export of the ribosome (1). Synthesis of rRNA and assembly of the ribosomal subunits occurs in the nucleolus. B23 (also known as NPM, nucleophosmin or NO38) is an essential protein that associates with the ribosomal subunits and accumulates predominantly in the nucleolus (2). B23 is essential for normal embryonic development, maintenance of genomic stability, and normal ribosome biogenesis as was shown by knocking out the B23 (Npm1) gene in mice (3). Indeed, B23 is a multifunctional protein containing an N-terminal oligomerization domain harboring most of the in vitro chaperone activity, a central acidic domain that is required for its ribonuclease activity, and a C-terminal domain mediating binding to nucleic acids (4–6). B23 forms oligomers and the crystal structure of the Xenopus B23 (NO38) core has been solved (7). The oligomers are cyclic pentamers, similar to other members of the nucleoplasm family (7, 8). The oligomer is the predominant form in cells and is thermostable as well as resistant to disruption by low concentrations of SDS (9–11). B23 interacts with other nucleolar proteins, for example, the alternative reading frame (ARF), tumor suppressor protein (12, 13), and nucleolin/C23 (14). B23 can also bind viral proteins, such as Rev and Rex, to promote their nucleolar localization (15, 16). More recently, B23 has been implicated in pre-mRNA splicing (17) and in nuclear export of the L5/5S ribosomal RNA complex (18).

B23 plays an important role in tumor development through several distinct mechanisms. First, translocations in which the B23 oligomerization domain is fused to transcription factors or kinases are common in lymphomas and leukemias (19). Second, C-terminal mutations have been reported to frequently occur in acute myelogenic leukemia with a normal karyotype. These mutations create a novel nuclear export signal, and disrupt a nucleolar localization motif (20). Third, increased levels of B23 protein have been noted in transformed cells, but whether this is a contributing factor to, or merely a consequence of increased cancer cell growth remains unclear (2). Fourth, loss of chromosome 5q that encodes for B23 occurs in myelodysplastic syndromes (2). This loss of B23 causes genomic instability perhaps due to unrestricted centrosome duplication (3, 21), and reduces stability of the ARF tumor suppressor (22, 23). An interesting novel B23 function is its ability to mediate the anti-apoptotic effects of nerve growth factor by acting as a receptor for phosphatidylinositol (3,4,5)-trisphosphate in the nucleus (24). In line with these findings, it was demonstrated that B23 overexpression increases cell survival after ultraviolet irradiation, possibly in collaboration with proliferating cell nuclear antigen (25, 26). Paradoxically, in some cell types, loss of B23 can result in apoptosis (3, 13), which is probably related to the important role of...
B23 in normal cell function. B23 can bind to histones H3, H4, and H2B, and assemble nucleosomes in vitro, like other members of the nucleoplasm protein superfamily (7, 27). These findings have led to the interesting idea that B23 could be a histone chaperone in the nucleolus (7). Indeed, B23 can associate with chromatin to stimulate acetylation-dependent transcription (28).

To further investigate the physiological function of B23, we set out to identify novel B23-associated proteins using an unbiased approach. We identified ribosomal protein S9 as being one of the most abundant soluble B23-binding proteins, supporting a role for B23 in ribosome biogenesis. Furthermore, we could show an important role of S9 in cell growth and proliferation and that B23 promotes the stability and nucleolar localization of S9.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—U2OS osteosarcoma, H1299 lung carcinoma, and normal human fibroblasts WI38 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin in a humidified incubator. To generate stable S9-FLAG or S9-GFP cell lines, parental U2OS cells were transfected with each plasmid separately and selected in 400 μg/ml of G418 (Sigma). Stable clones were expanded and analyzed for S9 expression.

**Immunoprecipitation**—For mass spectrometry analysis, confluent U2OS cells were cultured in p100 plates and infected with adenovirus expressing Myc-tagged B23. Cells were lysed in 0.1% Nonidet P-40 lysis buffer followed by immunoprecipitation using a rabbit Myc polyclonal antibody as indicated. In some experiments, RNAse A (100 μg/ml) (Sigma) was included during the preparation of the lysate and during co-IP. Proteins were resolved on SDS-PAGE and the gels were stained with either silver or Coomassie Blue (Bio-Rad) according to standard protocols. Visible bands were cut out and analyzed using mass spectrometry at the University of North Carolina Chapel Hill core facility. Details for Western blotting and IP have been published (29).

**Plasmids and Transfections**—Full-length cDNA encoding human ribosomal protein S9 (RPS9) cDNA (clone MGC, 5482; IMAGE, 3452221; accession number, BC000802) was subcloned using standard PCR methods from pCMV-SPORT6 (Invitrogen) and into the pCDNA3 FLAG-3C, pCDNA3, pEGFP-N1, and pGEX-3X plasmids. Primer sequences and PCR conditions are available upon request. Cells were transfected using FuGENE 6 reagent according to the manufacturer’s instructions. Western blotting and cell imaging. From this screen, Ambion RPS9 siRNA number 9201 (sense 5’-GGAUUUCUCAGAGAGACGT-3’) was chosen as the main siRNA with the most efficient knockdown. A similar phenotype as described was observed with three other oligos. The siScr and siB23 sequences have been described (13). Transfection of siRNA oligos was performed using Oligofectamine according to the manufacturer’s instructions (Invitrogen).

**Antibodies**—Two S9-derived peptides synthesized by the UNC Chapel Hill peptide core facility were conjugated to keyhole limpet hemocyanin and used for rabbit immunization to generate novel S9 antisera. The S9 antisera were purified using the Sulfolink column purification kit (Pierce). The specificity and affinity were tested on recombinant GST-S9 using whole cell extracts. Anti-senser S9-009 (CRKTYTPRRPFKESR) was used for straight Western blotting and immunofluorescence. Anti-senser S9-162 (RSPYGGRPGRVRRKNC) was used in Western blotting, co-immunoprecipitation, and immunofluorescence applications. Purified antisera toward L5, L11, and L23 have been described (30, 31). Rabbit anti-Myc and rabbit anti-FLAG antibodies were generously provided by Yue Xiong (UNC Chapel Hill). Monoclonal antibodies toward Myc (clone 9E10), anti-FLAG clone M2 (Sigma), β-actin (Sigma), α,β-tubulin (Santa Cruz), ribosomal protein S6 (Cell Signaling Technology), Mdm2 (clone 2A10), p53 (clone DO1, Lab Vision-Neomarkers), p21 (H252, LabVision-Neomarkers), B23/NPM (Zymed Laboratories Inc.), and C23/nucleolin (Santa Cruz) were purchased commercially.

In *vitro* Translation and Glutathione S-Transferase (GST) Pull-down Assay—Promega TnT-coupled kit was used for in vitro transcription and translation (IVT) according to the manufacturer’s instructions. Purification, expression, and elution of GST fusion proteins from beads were carried out according to standard protocols (Amersham Biosciences). For the in vitro binding assay, GST fusion proteins bound to glutathione-Sepharose 4B beads were incubated with 2–4 μl of IVT product in a final volume of 200 μl of binding buffer (29) and rotated at +4°C overnight followed by extensive washing in binding buffer. Bound material was eluted from the beads in sample buffer and resolved using SDS-PAGE followed by Coomassie Blue staining, gel drying, and autoradiography.

**Immunofluorescence**—Procedures for immunofluorescence have been described (29). In brief, cells growing in 6-well plates were washed in phosphate-buffered saline, fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.2% Triton X-100 (Sigma) for 3 min, and kept 30 min in blocking buffer (0.5% bovine serum albumin in 1× phosphate-buffered saline). Following primary and secondary antibody incubations in blocking buffer with extensive washings in between, the stained cells were analyzed using an Olympus IX-81 microscope with SPOT-camera and software as described.

**Infection**—To generate Ad-S9, Ad-S9-EGFP-N1, and Ad-S9-FLAG3C the corresponding DNA was amplified using PCR and inserted into the pCMV-Shuttle vector. Adenoviruses were produced in 293QBT cells according to the manufacturer’s instructions (Strategene). Cells were infected in Dulbecco’s modified Eagle’s medium with 0.1% fetal bovine serum for 2 h at which time fresh complete medium was added. Ad-MycB23 and Ad-GFP have been described (13).

**RNA and Protein Synthesis Measurements**—For estimation of *de novo* protein synthesis cells were starved in methionine-free Dulbecco’s modified Eagle’s medium supplemented with...
dialyzed fetal bovine serum for 30 min followed by a 2-h pulse with \( ^{35} \text{S} \)Met (Amersham Biosciences). Protein extracts were prepared in 0.5% Nonidet-P40 lysis buffer and total \( ^{35} \text{S} \)Met incorporation was measured using liquid scintillation counting and expressed as counts per minute/\( \text{mg} \) of protein. Protein concentrations were determined using the Bradford reagent and readings at 595 nm. To determine the half-life of S9, U2OS cells were harvested at the indicated time points, followed by Western blotting and autoradiography. Actinomycin D (Sigma) at a final concentration of 5 nM or dimethyl sulfoxide (DMSO) as vehicle control were added and maintained throughout the assay. For estimation of the ribosomal RNA synthesis rate, pulse-chase experiments using \( ^{3} \text{H} \)methionine were carried out. Cells on 60-mm plates were starved of methionine for 30 min with methionine-free medium and then labeled with L-\( ^{3} \text{H} \)methionine (Amersham Biosciences) for 30 min. After washing with phosphate-buffered saline, total RNA was purified using TRIzol reagent (Invitrogen) and the incorporated radioactivity was measured by a liquid scintillation counter.

**RESULTS**

\( ^{35} \text{S} \)Methionine Incorporation into S9—To identify novel B23 interacting proteins we performed a co-IP experiment using lysates from U2OS cells infected with Ad-MycB23 or a mock GFP virus. We found that soluble Myc-B23 protein was associated with a protein complex composed of ribosomal proteins (r-proteins) and RNA helicases, most likely representing pre-ribosomal particles at various stages of their maturation (Fig. 1A). Among the most prominent non-ribosomal proteins associated with B23 were RNA helicase A (DHX9), RNA helicase Gu/II (DDX21), and DEAD box RNA helicase 1 (DDX1). In addition, we were able to identify over 15 r-proteins of both the large and small ribosome subunit (Table 1). Not all bands were chosen for analysis as they indeed were assumed r-proteins. Given the suspected RNA-dependent interactions within this complex we included RNase A (100 \( \mu \text{g/ml} \)) before and during immunoprecipitation. We could then observe Myc-B23 in a complex with much fewer proteins and the most prominent of those were endogenous B23, which can oligomerize with Myc-B23 (5, 29), and ribosomal protein S9 (Fig. 1B). In total, over 42 peptides matching S9 was identified by mass spectrometry (Table 2).

**Cell Proliferation and Flow Cytometry**—For measurements of proliferation, cells were seeded in 6-well plates and transfected followed by counting each day using trypan blue in triplicate. For flow cytometric analysis, cells were collected after the indicated treatments by trypsinization, fixed with 70% ethanol, stained with propidium iodide, and analyzed as described previously (32).

**S9, a Novel B23 Interacting Protein**

**TABLE 1**

| Protein Accession | Mol. mass (Da) | Peptides\(^a\) | MS and MS/MS score \(^b\) |
|-------------------|---------------|----------------|--------------------------|
| RNA helicase A    | AAB48855      | 141,979.7      | 9                        | 172                      |
| DDX21             | AAH08071      | 872,90.4       | 24                       | 387                      |
| DDX1              | Q6JR1         | 78,815.5       | 4                        | 98                       |
| RPS3A             | CAE98774      | 29,286.4       | 5                        | 117                      |
| RPS6              | AAH09427      | 28,140.8       | 9                        | 308                      |
| RPL8              | RSRIL8        | 20,007.3       | 7                        | 188                      |
| RPL7a             | RSHU7A        | 29,877         | 3                        | 100                      |
| RPS9              | BAC34330      | 25,177.7       | 3                        | 123                      |
| RPL18             | S3852         | 21,162.1       | 7                        | 123                      |
| RPL21             | SS9513        | 18,513.4       | 4                        | 159                      |
| RPL11             | AA18970       | 20,111.6       | 3                        | 70                       |
| RPL26             | AAA60279      | 17,277.5       | 6                        | 123                      |
| RPL12             | Q9NGO2        | 17,831.4       | 3                        | 98                       |
| RPS11             | R3HU41       | 18,419         | 5                        | 84                       |
| RPS13             | R3R13         | 17,211.7       | 10                       | 233                      |
| RPL38             | RL28_HUMAN    | 15,606.6       | 5                        | 91                       |

\(^a\) Number of peptides that match the theoretical digest of the primary protein identified.

\(^b\) Score of the quality of the peptide-mass fingerprint match and the quality of the MS/MS peptide fragment ion matches (if MS/MS data was generated). Scores of 95 or greater are considered significant.
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—to define the protein domain in B23 that is required for binding S9. We carried out co-IP and GST pull-down experiments using a series of B23 deletion mutants. Whereas WT B23, and B23(10–294) bound to S9-FLAG in cells (Fig. 2A) and on S9-GST beads (Fig. 3B), further sequential deletions of the N-terminal region of B23 abolished most, if not all, B23-S9 binding (Fig. 3, A and B). These results may suggest that the binding region for S9 resides within amino acid residues 10–25 in B23. However, since WT and B23(10–294) mutant form oligomers whereas the B23(25–294) mutant and others are severely impaired in oligomerization activity (5, 7, 29), the B23-S9 interaction can also depend on B23 oligomerization. In fact, the binding of S9 to B23 was completely eliminated when removing the 100–117 highly conserved region in the B23 core region (Fig. 3C), further sequential deletions of the N-terminal region of B23 abolished most, if not all, B23-S9 binding (Fig. 3, A and B). These results may suggest that the binding region for S9 resides within amino acid residues 10–25 in B23. However, since WT and B23(10–294) mutant form oligomers whereas the B23(25–294) mutant and others are severely impaired in oligomerization activity (5, 7, 29), the B23-S9 interaction can also depend on B23 oligomerization. In fact, the binding of S9 to B23 was completely eliminated when removing the 100–117 highly conserved region in the B23 core region (Fig. 3B, lane 6), or when introducing point mutations in the B23 oligomerization domain (data not shown), similar to that which was observed in the case of the ARF tumor suppressor (29). Strong binding was seen between S9 and the B23(1–192) mutant suggesting that the B23 oligomerization domain cooperates with the central acidic domains of B23 in binding S9 (Fig. 3A, lane 9). Binding data and status of B23 oligomerization is summarized in Fig. 3, C and D. We conclude that B23 binding to S9 is dependent on an intact B23 oligomerization domain.

S9 Is Required for Normal Cell Proliferation—To investigate the physiological role of S9 in cell growth we used siRNA to deplete S9 in primary human fibroblasts (W138) and the WT p53 containing tumor cell line U2OS. From an initial screening of six siRNA oligonucleotides one S9 siRNA oligo was chosen with 90% knockdown of free (non-ribosome bound) endogenous S9 and without adverse toxicity. 72 h after transfection the cell proliferation rate was reduced by more than 50% compared
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**FIGURE 3.** An intact B23 oligomerization domain is required for S9 binding. A, mapping of the S9 binding domain in B23 using co-immunoprecipitation. Extracts from U2OS cells transfected with the indicated plasmids encoding WT and deletions of Myc-B23 were used in immunoprecipitations with a Myc rabbit polyclonal antibody and the precipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with an anti-FLAG antibody (M2) or a Myc monoclonal antibody (9E10). B, mapping of the B23 domain for S9 binding using in vitro GST binding assay. transfected with a nitrocellulose membrane, and blotted with an anti-FLAG antibody (M2) or a Myc monoclonal antibody (9E10). B, mapping of the B23 domain for S9 binding using in vitro GST binding assay. S9-bound B23 was detected by autoradiography. Shown also is input of GST, GST-S9, and the IVT product. Lower migrating IVT bands of B23 represent initiation of translation from the first and downstream AUGs in B23, given that the Myc tag is at the N terminus. Also, note that the first 10 amino acids in B23 is a methionine-rich region leading to a lower radioactive signal for the other constructs lacking this part of B23. C, a schematic of B23 depicts its N-terminal oligomerization domain (OD; residues 1 to 117), nuclear localization signal (NLS) and nuclear export signal (NES), a C-terminal heterodimerization domain (HeD; 187 to 259), and acidic region. D, schematic representation of Myc-B23 deletion mutants and their binding to S9-FLAG. Oligomerization status of B23 is also indicated as described in more detail elsewhere.

with control cells (siScr) (Fig. 4B), and S9 knockdown cells exhibited a flat morphology (Fig. 4A). This apparent growth arrest indeed corresponded to an increase in the G1 phase cell population and a decrease of nucleolar or ribosomal stress and mediates a G1 cell cycle arrest (31, 34). As is shown in Fig. 4D, G1 cell cycle arrest in U2OS was associated with a pronounced increase in p21 and a minor increase in MDM2 (Fig. 4D, left panel). The basal level of the WT p53 protein is elevated in the U2OS tumor cell line compared with primary WI38 cells and did not further increase upon S9 depletion or siRNA transfection per se. Primary WI38 fibroblasts showed both an increase in p53 protein level and a modest p21 increase but markedly elevated levels of MDM2 protein (Fig. 4D, right panel).

To investigate whether ribosome function could be altered as a consequence of S9 loss, we measured protein synthesis in cells. Interestingly, the rate of protein synthesis was reproducibly and significantly decreased following depletion of S9 with ~30% (Fig. 4E), whereas in contrast, the ribosomal RNA synthesis was not affected (data not shown). We conclude from these experiments that S9 is important for sustained cell growth (protein synthesis) and as a consequence cell proliferation.

**B23 Increases S9 Steady-state Level—**Ribosomal proteins are generally considered metabolically stable. However, r-proteins can be short-lived in the absence of ribosome biogenesis (35, 36). It has been shown that B23 interaction with tumor suppressor ARF, an unstable nucleolar protein, serves to sequester ARF in the nucleolus and stabilize the protein (23, 37). To elucidate the functional significance of the B23-S9 interaction, we first examined whether B23 is important in controlling the S9 protein level. First, we performed [35S]Met labeling while at the same time exposing cells to a low concentration of actinomycin D (5 nM) to selectively block rRNA synthesis and ribosome biogenesis. Labeling and chase was followed by immunoprecipitation to investigate the half-life of newly synthesized and free S9 protein. The results showed that newly synthesized free S9 is highly unstable in the presence of actinomycin D with a half-life of less than 2 h, but not under normal growth conditions when a significant pool of S9 remains stable (Fig. 5A). Second, to examine whether the degradation of S9 at least in part through the proteasome-mediated pathway, we treated U2OS cells with MG132 proteasome inhibitor and analyzed the level of S9. We found that endogenous S9 accumulated after MG132 treatment (Fig. 5B), indicating that S9 is rapidly degraded through proteolysis. This result was in agreement with a proteomic study of nucleolar protein dynamics in response to MG132 showing accumulation of r-proteins in the nucleolus (38). Next, we found that co-infection of U2OS cells with viruses expressing Myc-B23 or S9-FLAG resulted in higher levels of S9 (Fig. 5C). Moreover, enforced expression of B23 could also stabilize endogenous S9 in H1299 tumor cells.
Transfection with WT B23 plasmid, but not empty vector, could also increase S9 (Fig. 5E). In contrast, reducing levels of B23 by siRNA in WI38 or U2OS cells led to decreased levels of S9 protein paralleling the lower levels in B23 (Fig. 5F and supplemental Fig. S3). To explore the consequence of a high level of S9 in cell growth, we generated stable cell lines expressing GFP or FLAG-tagged S9. Interestingly, ectopic expression of S9 did not significantly affect cell growth and proliferation (data not shown), but suppressed endogenous S9 expression (Fig. 5G), suggesting that there exists a feedback regulatory mechanism in cells to maintain a constant level of S9 protein. Together, these results indicate that B23 facilitates stabilization of S9, whose cellular level appears to be critical for cell growth and proliferation, and is maintained by a feedback mechanism.

**B23 Facilitates Nucleolar Storage of S9**—In search for a putative explanation regarding the increased levels of S9 protein caused by B23 expression, we first analyzed the subcellular localization of S9. To determine this we fused S9 with GFP and analyzed its localization in living cells. S9-GFP predominantly localized in the nucleolus and the cytoplasm (Fig. 6A), as is the case for most other r-proteins (39–42). Infecting S9-GFP cells with a B23 virus led to an increase in S9-GFP nucleolar fluorescence.
ence compared with mock infected cells (Fig. 6B, upper panel), indicating that B23 promotes nucleolar localization of S9. In contrast, knocking down B23 using siRNA led to a decrease of S9-GFP nucleolar signal (Fig. 6B, lower panel). To confirm this, we used our specific S9 antiserum and found that the subcellular distribution of endogenous S9 was similar to GFP-S9 (cytoplasm and nucleolus). We also found that the impact of knocking down or overexpressing B23 had similar effects on endogenous S9 (supplemental Fig. S3). To investigate whether B23 increases both cytoplasmic (C) and nuclear (N) levels of S9, we fractionated stable U2OS S9-FLAG cells that had been infected with B23 or GFP viruses. As shown in Fig. 6C, B23 but not GFP promoted an increase in nuclear S9-FLAG (Fig. 6C, lane 3 versus lane 4), whereas the cytoplasmic S9-FLAG level remained at the same level (Fig. 6C, lane 1 versus lane 2). From these experiments we concluded that B23 has the ability to modulate nucleolar levels of S9 protein.

DISCUSSION

B23 is well known for its use as a nucleolar localization marker and its involvement in many forms of tumors (2, 43). When present in the nucleolus B23 facilitates ribosome biogenesis by multiple mechanisms: (a) B23 has a direct function as an endoribonuclease in the processing of 47S rRNA as have been shown in vitro and in living cells (13, 44). (b) B23 influences rDNA transcription by acting as a nucleolar histone chaperone (7). (c) B23 plays a role in the nuclear export of ribosome components such as L5 or the ribosome itself (18, 45). (d) B23 could have a role in ribosome assembly by binding to and stabilizing r-proteins and supporting their interaction with rRNA.

We were interested in finding novel binding partners of B23 that could be involved in mediating these effects. Using co-IP and mass spectrometry we found that B23 is associated with a protein complex composed of r-proteins and RNA helicases. B23 is known to associate with both small and large ribosomal subunits (18). Upon disruption of these complexes with RNase A, a major interaction with ribosomal protein S9 (also known as S4 in prokaryotes) was uncovered. In bacteria, S4 is one of the first proteins to bind the maturing rRNA where it preferentially binds to the 5’/H11032 end of the 16S rRNA (46). Mutation in Escherichia coli S4 is associated with high frequency of translation errors. The crystal structure of bacterial S4 revealed a two-domain molecule (47). One of them, the “S4 domain,” which probably mediates binding to RNA is a small domain consisting of 60–65 amino acid residues that was also detected in two families of pseudouridine synthases and a novel family of predicted RNA methylases among other proteins (48). Although S4/S9 is considered to be a primary rRNA-binding protein the S9-B23 interaction was not negatively affected by RNase treatment. The exact function of mammalian S9 in the ribosome is not entirely clear but from studies in bacteria and yeast S9 appears to have essential functions both in ribosome biogenesis and mature ribosome function. A recent study shows that yeast S9 is located at the entrance tunnel of mRNA in the ribosomes and is involved in regulation of mRNA translation, possibly translation termination (49). Very little is known about putative extraribosomal functions but S9 is induced during ischemia in brain, where it may play a protective role against oxidative stress-induced cell death (50).

Using siRNA-mediated knockdown we could determine that S9 is required for normal cell growth and proliferation as depletion of S9 resulted in decreased protein synthesis. The decrease in protein synthesis was also associated with a G1 cell cycle arrest and increased p21 levels. This finding is in agreement with the role of B23 in maintaining nucleolar stability and protein synthesis.
with recent studies suggesting that p53 becomes activated after nucleolar stress, as have been seen after depletion of ribosomal protein L23 (30) or S6 (51). Exactly how the cell senses a deficiency in ribosome biogenesis or ribosome function is unclear, but it could possibly be mediated by other r-proteins that become released and then bind to MDM2 followed by activation of p53. Examples of those r-proteins include L11, L5, L23, and more recently S7 (30, 52–54).

We established that both ectopic and endogenous S9 localize to both nucleoli and cytoplasm with only minor amounts being detectable in the nucleoplasm, as is the case for most other r-proteins (40). A recent study of a subset of ribosomal proteins including S9 showed that they are exclusively located within the granular component of the nucleolus (40). B23 can shuttle between the nucleus and cytoplasm and may act to recruit ribosomal or other nucleolar proteins from their site of synthesis in the cytoplasm and then to the nucleolus or anchor them in the nucleolus (55, 56). B23 has been shown to bind peptides with nuclear localization signal-rich regions of the SV40 LT type and stimulate their nuclear import in vitro (57). Interestingly, S9 is a basic lysine-rich protein with three putative predicted nuclear localization signal, including two overlapping bipartite nuclear localization signals that could be one cause for the strong interaction seen between S9 and B23. A plausible mechanism could be that B23 binds S9 in the cytoplasm and escorts it to the nucleolus. An alternative explanation could be that B23 serves as an anchor or storage platform for S9 in the nucleolus. B23 has been shown to bind peptides with various nuclear localization signal-rich regions of the SV40 LT type and stimulate their nuclear import.

We could determine that under normal conditions a significant fraction of S9 is metabolically stable, most likely because of its incorporation into ribosomes, whereas in the absence of ongoing ribosome biogenesis the newly synthesized S9 protein is unstable. In our hands, B23 promoted S9 nucleolar localization and storage. Recently, Hsp90 was shown to interact with free ribosomal protein S3 and to protect it from ubiquitination and proteasome-dependent degradation (58), suggesting that r-proteins use different cellular chaperones when not bound within the ribosome. It will be of interest in the future to further investigate the exact ribosomal and possible extraribosomal function of S9.

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