Nucleolin Interacts with Several Ribosomal Proteins through Its RGG Domain*

(Received for publication, March 12, 1998, and in revised form, May 18, 1998)

Philippe Bouvet‡§, Jean-Jacques Diaz¶, Karine Kindbeiter∥, Jean-Jacques Madjar¶, and François Amalric‡

From the ¶Laboratoire de Biologie Moléculaire Eucaryote, Institut de Biologie Cellulaire et de Génétique du CNRS, UPR 90096, 118 route de Narbonne, 31062 Toulouse Cedex, France and Immuno-Virologie Moléculaire et Cellulaire CNRS UMR5537, Faculté de Médecine Lyon-R.T.H. Laennec, Rue Guillaume Paradis, 69372 Lyon Cedex 08, France

Nucleolin is one of the major nonribosomal proteins of the nucleolus. Through its four RNA-binding domains, nucleolin interacts specifically with pre-rRNA as soon as synthesis begins, but it is not found in mature cytoplasmic ribosomes. Nucleolin is able to shuttle between the cytoplasm and the nucleus. These data suggest that nucleolin might be involved in the nucleolar import of cytoplasmic components and in the assembly of pre-ribosomal particles. Here we show, using two-dimensional blots in a ligand blotting assay, that nucleolin interacts with 18 ribosomal proteins from rat (14 and 4 from the large and small subunit, respectively). The C-terminal domain of nucleolin (p50) interacts with 10 of these identified ribosomal proteins. In vitro binding assays show that the glycine-arginine rich domain of nucleolin (RGG domain) is sufficient for the interaction with one of these proteins. Interestingly, most of the proteins that interact with p50 belong to the core ribosomal proteins, which are resistant to extraction with high salt concentration. These findings suggest that nucleolin might be involved in the nucleolar targeting of some ribosomal proteins and in their assembly within pre-ribosomal particles.

Ribosome biogenesis is a complex process that takes place in a specialized region of the cell nucleus called the nucleolus. Within this structure, ribosomal RNA (rRNA) is synthesized by RNA polymerase I, then processed and assembled with ribosomal proteins to form pre-ribosomal particles, which are then exported to the cytoplasm. The ribosome is probably one of the most complex ribonucleoparticles, involving interaction of a large number of ribosomal proteins (33 and 47 for the small and large subunits, respectively) with four rRNA molecules (1). The molecular mechanisms that direct the assembly of rRNA with ribosomal proteins within the nucleolus and the pathways involved in nuclear and nucleolar import of ribosomal proteins are still largely unknown.

The presence of a nuclear localization signal is necessary and sufficient to determine nuclear targeting of proteins (2, 3). Other signals are required for nucleolar targeting, sometimes overlapping the nuclear localization signal. It has been proposed that short highly basic sequences could be defined as nuclear localization signals for HTLV-I Rex and HIV Tat and Rev proteins (4–6). Nucleolin, NO38, NSR1, and GAR1 are imported from the cytoplasm to the nucleolus where they participate in the formation of pre-ribosomal particles, which are then exported to the cytoplasm (7–11). These nucleolar proteins exhibit sequences or protein domains required for nucleolar targeting. However these sequences are not sufficient by themselves to confer such localization when they are added to a cytoplasmic protein. Similarly, ribosomal proteins S6, L5, L7a, and L31 exhibit domains that have been localized within the protein and are required but not sufficient to confer nucleolar localization (12–15). It was therefore concluded that nucleolar targeting was not because of the sole presence of a signal within the protein, but rather was proceeding from the cooperation of several domains and/or from the functional interactions of such domains with other molecules present in the nucleolus.

Nucleolin is one of the most abundant nucleolar proteins (16). The nucleolin sequence exhibits a modular organization in functional domains (17). The N-terminal part of the molecule contains multiple phosphorylation sites (18) linked on both sides to stretches of acidic amino acids. Four RNA-binding domains (RBD)³ constitute the nucleolin central domain. The first two RBD are involved in the recognition of a small RNA stem-loop structure (19–21) present in the 5′ external transcribed spacer of rRNA, but also in the 3′ external transcribed spacer, in the internal transcribed spacer and in the 18S and 28S RNA sequence (19, 22). We have recently shown that the specific interaction of nucleolin with 5′ external transcribed spacer sequences is required for the first step of ribosomal RNA processing in vitro (23). The C-terminal domain (RGG domain) is unusually rich in glycine, arginine, and phenylalanine. The RGG domain of nucleolin binds RNA nonspecifically and with low affinity (24). This domain has been initially described as an RNA-binding motif in hnRNP U (25). In hnRNP A1, this domain is not only involved in interaction with RNA (26, 27) but also in protein-protein interaction (28).

Nucleolin interacts only transiently with rRNA and pre-ribosomal particles (19, 29) and is not detectable in mature cytoplasmic ribosomes. This suggests that nucleolin is removed during the assembly and/or the processing steps of pre-ribosomal particles. In addition, one remarkable characteristic of nucleolin is that it shuttles constantly between the nucleolus and the cytoplasm (30). These observations led us to postulate that nucleolin could act as a carrier for ribosomal proteins from the cytoplasm to the nucleolus and as an adaptor for specific binding of ribosomal proteins to rRNA. This functional model

* This work was supported by the CNRS and Université Claude Bernard Lyon 1 (to J.-J. M.) and by grants from the CNRS, University Paul Sabatier, and Association pour la Recherche contre le Cancer (ARC) (to P. B and F. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 33-5-61-33-59-52; Fax: 33-5-61-33-58-86; E-mail: bouvet@ibcg.biotoul.fr.

³ The abbreviations used are: RBD, RNA-binding domain(s); PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid.
Nucleolin Interacts with Ribosomal Proteins

**MATERIALS AND METHODS**

**Proteins Preparation and Purification**—Nuclei were prepared from Chinese hamster ovary cells (Computer Cells, Belgium) and lysed in low ionic-strength buffer. The resulting extract was applied to a heparin-Sepharose column (Pharmacia) as described previously (43). Fractions containing nucleolin were then subjected to anion-exchange chromatography (Mono Q, Amersham Pharmacia Biotech). Nucleolin was eluted with a 0.2–1 M KCl gradient made in 50 mM Tris-HCl, pH 7.9, containing 30% glycerol. Valuable fractions were analyzed by SDS-PAGE. Expression of recombinant proteins in *Escherichia coli* and purification were performed as described previously (20).

**Far-Western Blot Analysis**—Two-dimensional-PAGE of ribosomal proteins was performed as described previously, using the electrophoretic system II (basic-SDS) (32). After separation, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell), and to the membrane were incubated with purified hamster nucleolin (8.6 in the presence of 8 M urea for the first dimension and from top to bottom in the presence of 0.1% SDS in a 12.5% polyacrylamide gel for the second dimension. After transfer on a nitrocellulose membrane (A1 and B1), proteins were stained with Ponceau S dye. Ribosomal proteins bound to the membrane were incubated with purified hamster nucleolin (A2 and B2). After extensive washing, nucleolin was detected using an antibody raised against the C-terminal domain of nucleolin (p50) and revealed using ECL (Amersham Pharmacia Biotech). The autoradiograms of panels A2 and B2 were superimposed to the corresponding blot shown in panels A1 and B1 to identify each protein spot (A3 and B3). Spot positions of ribosomal proteins were identified according to previously published blots (32, 40).

**RESULTS**

Interaction of Nucleolin with a Subset of Ribosomal Proteins—Direct interaction of nucleolin with ribosomal proteins was evaluated using a ligand blotting assay (far-Western).

suggests a possible direct interaction between nucleolin and ribosomal proteins.

In this report, we show that nucleolin interacts through its RGG C-terminal domain with a subset of ribosomal proteins. This suggests that nucleolin may have a role in the assembly of the ribosomal subunits by bringing together ribosomal proteins and rRNA.

**Protein-Protein Interaction Assays**—Purified nucleolin was biotinylated as described previously (19). Biotinylated nucleolin-L3 complexes were recovered with magnetic streptavidin beads (Dynal). Beads were first extensively washed with binding buffer made of 15 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 150 mM NaCl, 0.1% Tween containing 0.1% bovine serum albumin. In a standard experiment, 1 μl of in vitro synthesized radiolabeled L3 was incubated with the indicated amount of nucleolin in 100 μl of binding buffer for 60 min at 4 °C. Beads were then extensively washed with binding buffer for a complete removal of unbound L3, then resuspended in electrophoresis buffer and separated by SDS-PAGE (44). L3 bound to nucleolin peptides fused to the histidine tag was recovered using Ni²⁺-NTA-agarose beads (Qiagen) and subjected to Western blot analysis. Membranes were washed twice in phosphate-buffered saline for 10 min, then for another 15 min in 10 mM Tris-HCl, pH 7.5, 150 mM KCl, and 0.1% Tween 20. Membranes were then incubated for 90 min at room temperature in buffer A (15 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1% Nonidet P-40) for 20 min at 4 °C. Tagged RGG protein was then added to the beads and incubated for 30 min at 4 °C with shaking. After two washes with NT2 buffer, labeled L3 proteins were added to the beads and incubated for 1 h at 4 °C. Bound proteins were removed by three washes with NT2 and precipitated with trichloroacetic acid (20% final volume). Bound and unbound proteins were analyzed by SDS-PAGE.

**FIG. 1. Interaction of nucleolin with a subset of ribosomal proteins.** Total proteins extracted from rat liver (panels A) and from human Hela cells (panels B) ribosomes were analyzed by two-dimensional-PAGE (System II;32). Migration was from left (anode) to right (cathode) at pH 8.6 in the presence of 8 M urea for the first dimension and from top to bottom in the presence of 0.1% SDS in a 12.5% polyacrylamide gel for the second dimension. After separation, proteins were transferred to nitrocellulose membrane (A1 and B1), proteins were stained with Ponceau S dye. Ribosomal proteins bound to the membrane were incubated with purified hamster nucleolin (A2 and B2). After extensive washing, nucleolin was detected using an antibody raised against the C-terminal domain of nucleolin (p50) and revealed using ECL (Amersham Pharmacia Biotech). The autoradiograms of panels A2 and B2 were superimposed to the corresponding blot shown in panels A1 and B1 to identify each protein spot (A3 and B3). Spot positions of ribosomal proteins were identified according to previously published blots (32, 40).
Nucleolin Interacts with Ribosomal Proteins

Ribosomal proteins were extracted from rat liver ribosomes and separated by two-dimensional-PAGE allowing separation of most of the 80 ribosomal proteins and their unambiguous identification (32). After separation, proteins were transferred to a nitrocellulose membrane and stained with Ponceau red (Fig. 1, panels A1 and B1). For far-Western blotting, transferred proteins were renatured and incubated with purified hamster nucleolin. After extensive washing, the presence of nucleolin bound to ribosomal proteins was revealed with a polyclonal anti-nucleolin antibody raised against the recombinant nucleolin bound to ribosomal proteins was revealed with a hamster nucleolin. After extensive washing, the presence of nucleolin bound to ribosomal proteins was revealed with a polyclonal anti-nucleolin antibody raised against the recombinant nucleolin C-terminal domain (Fig. 1, panel A2). Superposition of the spots obtained after detection of nucleolin bound to ribosomal proteins (Fig. 1, panel A2) with those on the membrane stained with Ponceau red used for far-Western blotting (Fig. 1, panel A1) allowed the identification of ribosomal proteins that interacted with nucleolin (Fig. 1, panel A3). This experiment revealed that 14 proteins of the large ribosomal subunit and 4 of the small one interacted with hamster nucleolin. These proteins are listed in Table I. As controls, two experiments were performed. First, the anti-nucleolin antibody was directly incubated with ribosomal proteins previously separated by two-dimensional-PAGE and transferred to another membrane, similar to that used in the previous experiment. Under these conditions no signal was obtained, indicating that anti-nucleolin antibody did not recognize ribosomal proteins (data not shown). This confirms the already published specificity of these polyclonal antibodies raised against nucleolin (19).

In another ligand blotting assay, total rat nucleolar proteins were used instead of purified ribosomal proteins. In these conditions, only two nuclear proteins were found to interact strongly with nucleolin (data not shown). Although these interactions have not been further characterized, one of them could represent histone H1, which has been previously shown to interact with nucleolin (31). The amino acid sequences of most ribosomal proteins are extremely well conserved in mammals about 95–100% between human and rat ribosomal proteins (1). Therefore, to further confirm the specificity of the interaction between nucleolin and ribosomal proteins, a ligand-blotting assay similar to that described above was performed with human ribosomal proteins purified from HeLa cell ribosomes (Fig. 1, panels B1–B3). In these conditions, 16 human ribosomal proteins of the 18 homologous rat proteins were found to interact with hamster nucleolin (Table I). L10 and L15, which were poorly detected on the far-Western blot performed with rat ribosomal proteins, were not detected at all with human proteins. Two additional proteins, S26 and L35a, were detected with hamster nucleolin when human instead of rat ribosomal proteins were used. The fact that most of the rat ribosomal proteins found to interact with hamster nucleolin were identical to those from HeLa cells indicates that the interaction is specific. Independent confirmation of these results was obtained from an in vitro binding assay. Biotinylated hamster nucleolin was incubated with rat 35S-labeled ribosomal protein L3 synthesized in vitro. L3 was found to interact with nucleolin in both types of far-Western experiments described above. Bound proteins were recovered on streptavidin magnetic beads and analyzed by SDS-PAGE (Fig. 2). L3 was recovered on the beads only in the presence of nucleolin (lane 3) showing that in vitro synthesized L3 interacted with nucleolin.

**Interaction of Ribosomal Proteins with the Nucleolin RGG Domain**—To identify the nucleolin domain responsible for the interaction with the ribosomal proteins, the ligand blotting assay was repeated using a truncated recombinant nucleolin, p50, comprised of the C-terminal part of hamster nucleolin only (see Fig. 3A). The p50 protein contains the four RBD and one RGG domain. Both the full-length nucleolin and the deleted p50 proteins interact through their RBD with the same RNA target (20). In a far-Western experiment, 10 rat ribosomal proteins were found to interact with p50 (Table I). Remarkably, all these proteins were also detected when full-length nucleolin was used to probe rat ribosomal proteins. 8 human homologous proteins of these 10 rat proteins were also found to interact with p50 (not shown). The rat ribosomal proteins that did not react with p50 (L5, L9, L13a, L28, L37a, S3a, S9, and S11) might therefore interact with nucleolin through its N-terminal end.

To further determine which domain of p50 interacts with these ribosomal proteins several recombinants proteins were used that were constituted of different domains of p50 (Fig. 3A). These recombinant proteins (RBD 1–2, RBD 3–4, RBD...
Nucleolin Interacts with Ribosomal Proteins

3–4-RGG) were designed to preserve the structural integrity of each RNA-binding domain (20). A histidine tag was fused to the N-terminal end of each recombinant protein. After incubation with 35S-labeled L3 synthesized in vitro, proteins bound to the nucleolin peptides were recovered on Ni2+ agarose beads (Fig. 3). Nucleolin peptides were recovered on Ni2+ with either 0.25 M imidazole (lane 1) or 1 M imidazole (lane 2), where the RBD 3–4 alone was unable to bind L3, the RGG domain appeared to be the binding site for L3. In contrast, a protein containing only RBD 1–2 or RBD 3–4 were bound to the beads, indicating that this kind of interaction is probably not a general feature of all RGG domains. Furthermore, in our experimental conditions, nucleolin was never found to interact with ribosomal proteins containing only RBD 1–2 or RBD 3–4 (lane 3). The mixture was immunoprecipitated with a monoclonal anti-T7-Tag antibody to pull down proteins associated with the recombinant RGG domain. As shown in Fig. 3B (lanes 10–11), the nucleolin RGG domain alone was sufficient to recover L3 efficiently. This result indicates that the RGG C-terminal domain of nucleolin is the minimal domain required for interaction of nucleolin with ribosomal protein L3.

**DISCUSSION**

In this report, we show that nucleolin interacts with a subset of ribosomal proteins. These interactions are observed between hamster nucleolin and rat or human ribosomal proteins. The C-terminal RGG domain of nucleolin is responsible for the interaction with the L3 protein and is also very likely responsible for the interaction with all ribosomal proteins detected with p50. The ability of nucleolin to interact specifically with both ribosomal RNA sequences through its RNA-binding domains (19, 20, 22) and with ribosomal proteins strongly supports the hypothesis that nucleolin is involved in ribosome assembly.

We found that the C-terminal domain of nucleolin (p50; Table I) mediates the interaction of hamster nucleolin with 10 of 18 rat ribosomal proteins. It is unlikely that these interactions are based simply on the basicity of these ribosomal proteins since L3 (pI: 10.63) and S8 (pI: 10.73), which interact with p50, and L17 (pI: 10.63), S2 (pI: 10.66), and S15 (pI: 10.81), which do not interact with this nucleolin domain, have comparable basic pIs. The N-terminal domain of nucleolin, which contains acidic motifs, is a candidate for electrostatic interactions with basic regions of the 8 ribosomal proteins not interacting with the C-terminal part of nucleolin. These interactions have not been further characterized.

The RGG motif was initially described as an RNA-binding motif in hnRNP U, hnRNP A1, and nucleolin (24–26) and is usually found in association with other RNA-binding domains (33). The interaction of the RGG domain with RNA does not seem to require a specific RNA sequence and involves low RNA binding affinity (24–27). Although the amino acid composition of the RGG domain found in different proteins is very similar, their RNA binding properties are quite different. The RGG domain of hnRNP U binds single-stranded DNA and homopolymeric poly(G) and poly(U) in high salt conditions, whereas the RGG domain of hnRNP A1 does not (25). In nucleolin, the RGG domain binds RNA nonspecifically and with low affinity. Furthermore, it is not involved in determining the nucleolin RNA binding specificity (20, 24). Studies on the RNA binding properties of hnRNP A1 have implicated its RGG domain in both direct interaction with nucleic acid and in the cooperative binding of A1 to RNA (26, 27). This suggests that this domain is involved in protein-protein interactions. More recently, the RGG domain of hnRNP A1 was shown to interact in vitro in a nucleic acid-independent way with both other hnRNPs and with itself (28). We were unable to detect any homomorphic interactions of the nucleolin RGG domain (data not shown), indicating that this kind of interaction is probably not a general feature of all RGG domains. Furthermore, in our experimental conditions, nucleolin was never found to interact with ribosomal protein S2, which contains a RGG motif (34). Our finding that the RGG domain of nucleolin is involved in protein-protein interactions, similarly to hnRNP A1 (28), suggests that one general function of proteins that contain an RGG domain in association with an RBD motif might be to direct the assembly of protein complexes on specific RNA sequences.

The RGG domain of hnRNP A1, fibrillarin, and nucleolin contain the modified amino acid N6-Ac-dimethylarginine (35–37). The significance of this posttranslational modification on RNA binding is not known. A recombinant RGG domain produced in E. coli interacts as efficiently as purified nucleolin with in vitro translated L3 ribosomal protein. This suggests
that N<sup>G</sup>N<sup>G</sup>-dimethylarginines are not absolutely required for this interaction.

The nuclear localization of nucleolin requires at least two RBD or one RBD together with the RGG domain in addition to the nuclear localization sequence (8, 9). This suggests that nucleolin accumulates within the nucleolus by virtue of binding to ribosomal RNA. It has been shown that nucleolin shuttles between nucleus and cytoplasm (30), and it was speculated that nucleolin could play a role in the transport of ribosomal components from the cytoplasm to the nucleus and nucleolus. The demonstration that nucleolin interacts directly with ribosomal proteins supports this hypothesis. It has been recently proposed that a specific pathway (45, 46) mediates the import of ribosomal proteins into the nucleus. It would be interesting to determine if nucleolin is involved in this import pathway.

Nucleolin purified from hamster cells interacts with 16 homologous ribosomal proteins from either rat or human origin. In addition to these proteins, hamster nucleolin interacts with rat L10 and L15 and with human S26 and L35a. The sequence of rat L10 was found to display a 99% amino acid identity with a putative tumor suppressor human protein, QM, and 94% with a chicken protein, Jif-1 (38). The sequence of HeLa cell L10 is not available nor are those of S26 and L15. A comparison between the electrophoretic behavior of proteins extracted from rat liver and from HeLa cell ribosomes indicates that rat L15 should be somewhat different than its HeLa cell counterpart (see Refs. 32, 39, and 40). The differences in the binding ability of nucleolin to human and rat L15, S26, and L10 could therefore reflect some differences between these proteins. However it remains to be proved that the binding of nucleolin to these proteins is indeed significant.

It is remarkable that 5 of the 10 proteins that interact with p50 (L3, L4, L6, L18a, and S8) cannot be extracted from ribosomes with 3 M LiCl, whereas 4 other proteins (L7, L8, L15, and p50 (L3, L4, L6, L18a, and S8) cannot be extracted from ribosomal proteins is indeed significant.

References—We are grateful to Dr. Yuen-Ling Chan and Ira G. Wool (Chicago) for the generous gift of pcD L3-1.

REFERENCES

1. Wool, I. G., Chan, Y.-L., and Gluck, A. (1996) in Translational Control (Hershey, J., Mathews, M., and Sonenberg, N., eds), pp. 685–732, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY