Nucleolar protein p120 contains an arginine-rich domain that binds to ribosomal RNA

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Human proliferation-associated protein p120 has previously been shown to localize to the nucleolus, and several functional domains of p120 have been elucidated. By using a nitrocellulose filter binding assay and a Northwestern blotting procedure this study shows that recombinant p120 binds to an rRNA fragment *in vitro* with a dissociation constant of 4 nM. The specific RNA-binding region of p120 (residues 1–57) was identified with glutathione S-transferase-fused p120 deletion constructs and Northwestern blotting procedures. This RNA-binding region of p120, which includes the nucleolar localization signal of p120, is similar to the arginine-rich RNA-binding regions found in other RNA-binding proteins such as HIV Rev and Tat. Experiments *in vivo* with HeLa cell nucleolar extracts showed that p120 was associated with the 60–80 S pre-ribosomal particles. This association is disrupted by treatment with either RNase A or buffer of high ionic strength. These results suggest that p120 might be involved in rRNA/ribosome maturation, consistent with the role of the yeast homologue Nop2p in rRNA biogenesis.

INTRODUCTION

The nucleolus of eukaryotic cells is the organelle mainly responsible for rRNA synthesis and ribosome biogenesis [1,2]. Nucleolar pleomorphism is exhibited during periods of increased cell proliferation, reflecting the need for greater ribosome biogenesis necessary for cell division [1]. Several proteins have been shown to localize specifically to the nucleolus, including RNA polymerase I [3], fibrillarin [4], nucleolin/C23 [5], nucleophosmin/B23 [5], and p120 [6].

Protein p120 was originally identified in human tumour cells by the use of monoclonal antibodies. The specific nucleolar localization of p120 was shown by immunofluorescent staining [6]. The level of p120 expression increased during cell proliferation [7–9] and the yeast homologue of protein p120 (Nop2p) was shown to be essential for cell growth and viability in yeast [10]. The increased expression of p120, along with its nucleolar localization, suggest a possible role for p120 in rRNA processing. Deletion of the Nop2p gene in yeast results in the decreased methylation of specific regions of 27 S rRNA, and processing of 27 S pre-rRNA to mature 25 S rRNA is blocked [11].

The sequences of the p120 gene and its cDNA have been elucidated [12,13] and several functional domains have been defined for the cDNA-derived protein sequence. By using *Escherichia coli*-expressed p120 deletion mutants as glutathione S-transferase (GST) fusion proteins, the nuclear localization signal (NAPRGKKRPAPG, residues 99–110) was defined as well as the nucleolar localization signal (residues 40–57, SKRLSSRARKRAAKRRLG) [14]. A p120 binding domain for nucleolar protein B23 was also identified (p120 residues 24–56 [14]). Protein B23 is a proposed 'shuttle protein' [15–17] that carries other nucleolar proteins from the cytoplasm to the nucleolus. In that protein B23 binds to the nucleolar localization signal of p120, it has been suggested that B23 might be involved in the nucleolar localization of p120 [14].

Protein p120 contains an arginine-rich region that overlaps the nucleolar localization signal and is similar to that of other RNA-binding proteins [18]. This report demonstrates that this arginine-rich region of p120 is an rRNA-binding domain that might account for the ribosomal RNA–protein complex (rRNP) binding of p120 *in vivo*.

MATERIALS AND METHODS

Extraction of p120 from HeLa cell nucleoli

All procedures were performed at 4 °C. Nucleoli were prepared from 10 g of HeLa cells as described [19]. The nucleolar pellets were extracted for 2 h with 0.01 M KCl in extraction buffer [50 mM Tris/HCl (pH 8.0)/5 mM dithiothreitol/1 mM EDTA/0.15% deoxycholate/1 μg/ml leupeptin/1 μg/ml aprotinin/1 mM PMSF], yielding approx. 18% of the total extractable p120 as determined by ELISA assays. The sample was centrifuged for 10 min at 10000 ***g*** and the resulting pellet was then extracted for 2 h with 0.3 M KCl in extraction buffer and recentrifuged; approx. 25% of the total extractable p120 was present in this extract. The final pellet was extracted for 16 h with 0.4 M KCl in extraction buffer, yielding approx. 57% of the total extractable p120. The three extracts were stored at 4 °C for further use.

Expression and purification of recombinant p120

Recombinant p120 protein, overexpressed in Sf9 insect cells in the baculovirus system, was prepared from nucleolar pellets that were serially extracted by the procedure described above for HeLa nucleoli, yielding a p120 purity of 91%. Recombinant GST fusion proteins of p120 from *E. coli* were purified as described previously [14]. Recombinant whole p120 construct

Abbreviations used: GST, glutathione S-transferase; (r)RNP, (ribosomal) RNA–protein complex.

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was expressed in *E. coli* as described by Valdez et al. [20]. Extracts were made by boiling whole *E. coli* cells in Laemmli sample buffer [21].

Site-directed mutagenesis

A human p120 cDNA fragment encoding residues 19–167 was amplified by PCR and subcloned into the *Bam*HI–*Xho*I sites of pGEX 4T-3 vector (Pharmacia Biotech). The resulting construct was used as a template for site-directed mutations by inverse PCR with appropriate synthetic oligonucleotide primers [22].

Transcription *in vitro* and ^{32}P labelling of rRNA fragments

Rat ribosomal DNA constructs were in plasmid form (a gift from Dr. P. K. Chan, Department of Pharmacology, Baylor College of Medicine, Houston, TX, U.S.A.). These plasmids were linearized and transcribed with the MEGAscript *In Vitro* Transcription Kit or the MAXIscript *In Vitro* Transcription Kit (Ambion) with [α-^{32}P]UTP (Amersham). The synthesized transcripts included a 1.4 kb probe (*Bam*H1 fragment from the 5′ half of the 28 S rRNA), a 1.5 kb 28 S probe (*Bam*H1–*Eco*RI fragment from the 3′ half of the 28 S rRNA) and a 1.1 kb probe (*Bam*H1–*Eco*R1 fragment of 18 S rRNA).

Western blot analysis

The samples were subjected to electrophoresis on 7.5 % (w/v) polyacrylamide gels containing 0.1 % SDS [21]. The proteins were transferred to nitrocellulose, which was then blocked with 3 % (w/v) BSA and 10 % (v/v) goat serum. Reaction with anti-p120 monoclonal antibody was done, followed by alkaline phosphatase-conjugated goat anti-(mouse IgG) and colour development with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium (Promega).

Northwestern blot analysis

The Northwestern blot RNA binding assay was done as described by Schiff et al. [23]. Samples of highly purified recombinant p120 from baculovirus-infected Sf9 insect cells, *E. coli* cell lysate or GST-purified p120 constructs from *E. coli* were separated by SDS/PAGE and transferred electrophoretically to nitrocellulose membranes. The membranes were blocked overnight at room temperature in Northwestern buffer [10 mM Tris/HCl (pH 6.8)/25 mM NaCl/1 mM EDTA/0.04 % BSA/0.04 % Ficoll 400/0.04 % poly(vinyl pyrrolidone)-40]. The blots were probed with ^{32}P-labelled transcripts (1.5×10^5 c.p.m./ml) at room temperature for 2 h. The blots were then washed three times (10 min per wash) with Northwestern buffer to remove unbound or non-specifically bound RNA. After air-drying for 15 min, autoradiography was performed to detect rRNA-binding proteins.

Protein–RNA nitrocellulose filter binding assay

Filter binding assays [24–26] were done with recombinant p120 purified from Sf9 insect cells. An aliquot of purified recombinant p120 was diluted serially in binding buffer [10 mM Tris/HCl (pH 8.0)/10 mM NaCl/50 μg/ml BSA/0.2 mM dithiothreitol/5 mM $MgCl_2$] and 10 μl of each dilution was added to 50 μl of binding buffer containing approx. 100000 c.p.m./ml of ^{32}P-labelled rRNA fragments synthesized *in vitro*. The assay mix was incubated at room temperature for 20 min and then vacuum filtered through nitrocellulose filters presoaked in filtration buffer [10 mM Tris/HCl (pH 8.0)/10 mM NaCl]. Finally, the filters were washed once with 300 μl of filtration buffer, air-dried for 10 min and then placed in 5 ml of scintillation fluid for counting.

Sucrose gradient ultracentrifugation

Nucleoli from HeLa cells were extracted with 10 mM Tris/HCl (pH 8.0)/10 mM KCl/0.15 % deoxycholate/0.5 mM $MgCl_2$/1 mM PMSF/1 μg/ml leupeptin/1 μg/ml aprotinin. The nucleolar extracts were layered on 5–45 % (w/v) sucrose density gradients and centrifuged at 100000 ***g*** for 17 h in a Beckham SW41 rotor. Fractions (0.9 ml) were collected and analysed by ELISA. Aliquots (50 μl) of the gradient fractions were bound to Immulon-2 microtitre plates (Dynatech Laboratories) overnight at 4 °C. Wells were blocked with 3 % (w/v) BSA/10 % (v/v) goat serum, incubated with anti-p120 monoclonal antibodies, goat anti-(mouse IgG) coupled to peroxidase and, finally, substrate 2,2′-azino-di-3-ethylbenzthiozoline sulphonic acid (Boehringer-Mannheim). Colour development was measured by absorbance at 405 nm.

RESULTS

Protein p120 binds to rRNA

Recombinant protein p120 was expressed and isolated from the baculovirus/Sf9 insect cell system and subjected to SDS/PAGE. Protein transferred to nitrocellulose paper was probed with the ^{32}P-labelled 1.5 kb transcript of the 3′ half of the 28 S rRNA. Strong binding was observed to full-length p120 protein and two of its major degradation products as verified by a corresponding immunoblot (Figures 1A to 1C). Similar binding was also shown by this assay with the 1.4 kb transcript of the 5′ half of the 28 S rRNA sequence and the 1.1 kb transcript of the 18 S rRNA (results not shown). No RNA binding was observed for molecular mass marker proteins (β-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, ovalbumin, lactate dehydrogenase, triose phosphate isomerase) present on the same blot (Figure 1A, lane M). The rRNA probes also bound to recombinant p120 and its major degradation products expressed in *E coli* (Figures 1D and 1E) but not to any proteins from non-transformed *E. coli* cells (Figure 1D).

rRNA-binding domain of p120

GST–p120 deletion fusion proteins were expressed and purified [14]. After SDS/PAGE, proteins were transferred to nitrocellulose and incubated with the ^{32}P-labelled 1.5 kb 28 S rRNA transcript. Only the p120 constructs containing the nucleolar localization signal (residues 40–57) strongly bound to rRNA (pG116 and pG115 in Figure 2). The rRNA probe did not bind to p120 residues 87–196 containing the nuclear localization signal (pG119) or to p120 residues 1–23 and 168–658 (pG114 and pGAK2 in Figure 2). These results show that the 1.5 kb fragment of rRNA binds specifically to a region of p120 at the N-terminus (residues 1–57) that includes the nucleolar localization signal (residues 40–57). The probe did not bind to other regions of p120 or to other proteins such as GST (Figure 2), up to the standard molecular mass markers (see Figure 1).

To determine whether the p120 nucleolar localization signal specifically interacts with the rRNA probe, the sequence R^{46}ARKRAAKRRL^{56}, in a GST fusion protein containing residues 19–167 or p120, was mutated to G^{46}AGILAANGGL^{56}. The wild-type fusion protein, but not the mutant, bound to the ^{32}P-labelled 1.5 kb 28 S rRNA probe (Figure 3).

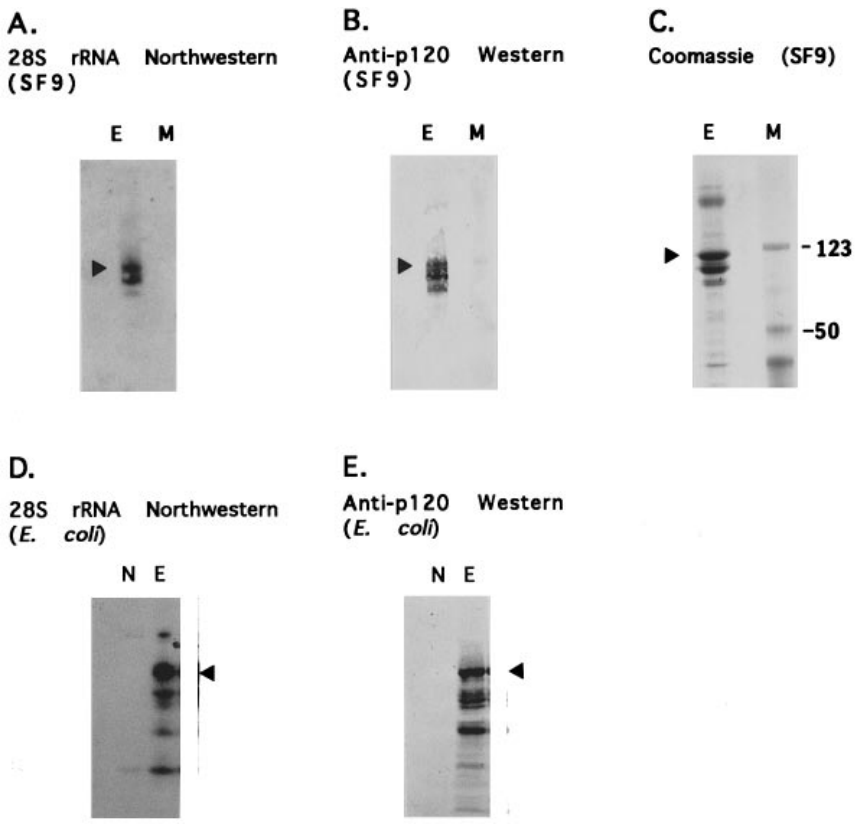

Figure 1 Binding of the 1.5 kb 28 S (3′ half) rRNA transcript to p120

Recombinant p120 was overexpressed by using baculovirus/Sf9 insect cells, and nucleoli were isolated and serially extracted. The 0.4 M extract was subjected to SDS/PAGE (7.5% gel) and then transferred to nitrocellulose and probed with ^{32}P-labelled 1.5 kb 28 S rRNA (**A**), transferred to nitrocellulose and probed with monoclonal p120 antibodies for Western blot analysis (**B**), or stained with Coomassie Blue (**C**). The migrations of β-galactosidase (123 kDa) and ovalbumin (50 kDa) are indicated. Recombinant p120 protein was also expressed in *E. coli*. Cells were boiled in Laemmli buffer, subjected to SDS/PAGE, transferred to nitrocellulose and then probed with either 1.5 kb 28 S 3′ half rRNA transcript (**D**) or monoclonal p120 antibodies for Western blotting (**E**). Abbreviations: E, extract from p120-containing bacterial cells; N, non-transformed *E. coli* lysate; M, molecular mass standards. Arrowheads point to p120.

p120–rRNA binding characteristics

The p120–rRNA interaction in the liquid phase was characterized by the two-step procedure previously used to determine the Tat–TAR interaction [27]. To determine the binding affinity of the p120–rRNA interaction, increasing amounts of p120 protein were incubated with a fixed amount of the labelled 1.5 kb 28 S rRNA fragment (Figure 4). By fitting a hyperbolic function (SigmaPlot), a K_d of 4 nM p120 was derived. The 1.1 kb 18 S rRNA fragment exhibited a similar binding affinity for p120 (results not shown).

The specificity of binding was determined by competition with unlabelled tRNA and poly(A)$^+$RNA in a nitrocellulose filter binding assay (Table 1). Unlabelled 1.5 kb 28 S rRNA fragment (10-fold excess) decreased the binding of labelled probe by 73%, whereas tRNA and poly(A)$^+$RNA (10-fold excess) resulted in only 7% and 13% decreases in binding respectively. A 1.5 kb fragment of 28 S rRNA in 3-fold excess over labelled 28 S rRNA decreased the binding of labelled probe by 50%, whereas a 100-fold excess of tRNA (on a nucleotide basis) did not decrease binding. A maximum 40% decrease in binding of labelled probe was achieved with a 20-fold excess of poly(A)$^+$RNA, indicating that p120 exhibits a specificity for rRNA *in vitro*.

When the p120–rRNA binding efficiency was determined at various ionic strengths from 0.01 to 0.5 M NaCl, decreased binding occurred with increasing ionic strength; at 0.4 M NaCl, for example, binding efficiency was only 50% of that observed at 10 mM NaCl (results not shown).

Association of p120 with pre-rRNP particles *in vivo*

Protein p120 was extracted from nucleoli of HeLa cells as described in the Materials and methods section. The extraction buffer, without deoxycholate, has previously been used for the extraction of pre-rRNP particles or small nuclear RNP particles [28,29]. Sucrose density gradient ultracentrifugation of the nucleolar extract was done under conditions that permitted the separation of pre-rRNP particles. Immunodetection by ELISA assays or Western blot showed that a major fraction of protein p120 sedimented with 60–80 S particles, and a minor fraction sedimented more slowly at 20–40 S (Figure 5).

Pretreatment of nucleolar extracts with solutions of increasing ionic strength resulted in the sedimentation of p120 near the top of the gradient, suggesting that p120 was dissociated from the pre-ribosomal particles. Complete dissociation occurred with 0.35 M NaCl or 1 M KCl, and partial dissociation was found with 0.12 M KCl (Figure 6). These results suggest that p120 is associated with pre-rRNP particles by ionic bonds. When nucleolar extracts were treated with RNase A before sucrose

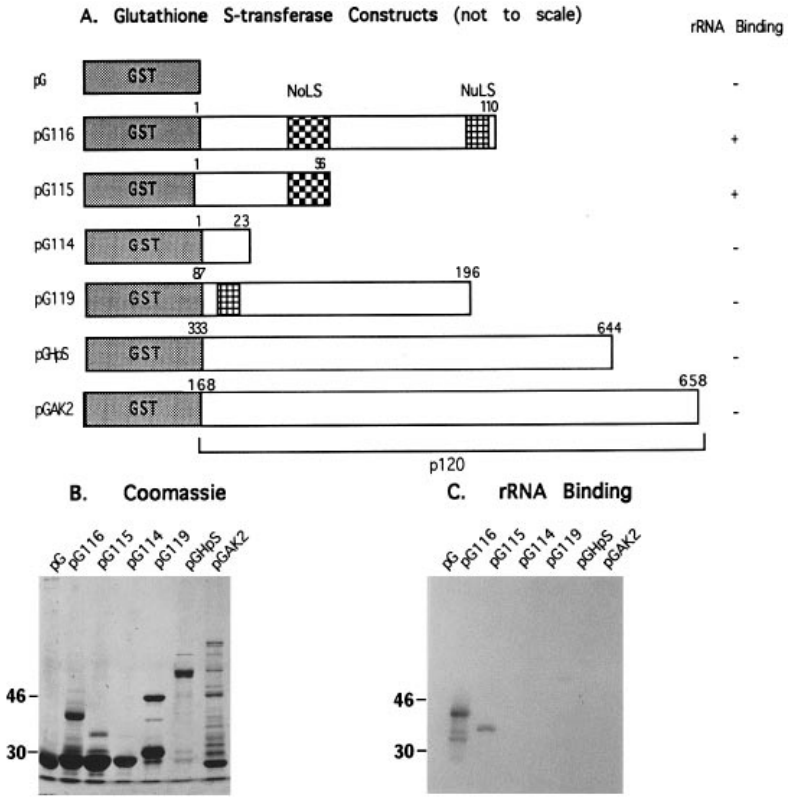

Figure 2 Binding of the 1.5 kb rRNA probe to p120 nucleolar localization signal

(**A**) The GST gene was fused in frame with p120 cDNA fragments. Amino acid residues of p120 are indicated above the constructs. Abbreviations: NoLS, nucleolar localization signal; NuLS, nuclear localization signal. Fusion proteins were expressed in *E. coli* and purified with glutathione–Sepharose beads. rRNA binding activity is indicated. (**B**, **C**) Fusion proteins were subjected to SDS/PAGE (10% gel) and stained with Coomassie Blue (**B**) or transferred to nitrocellulose and probed with ^{32}P-labelled 1.5 kb 28 S rRNA and then subjected to autoradiography (**C**). Molecular masses (in kDa) of standards are indicated at the left.

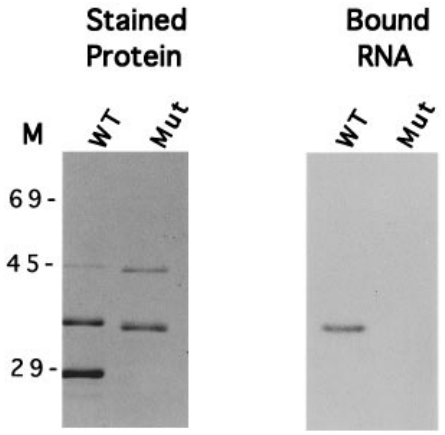

Figure 3 Binding of rRNA probe to mutated p120 nucleolar localization signal

The RNA binding activities of GST fusion proteins with wild-type (WT) or mutant (Mut) p120 nucleolar localization signal were determined as described in the legend to Figure 2. The sequence R^{46}ARKRAAKRRL^{56} was mutated to G^{46}AGILAANGGL^{56}. Molecular masses (in kDa) of standards are indicated at the left.

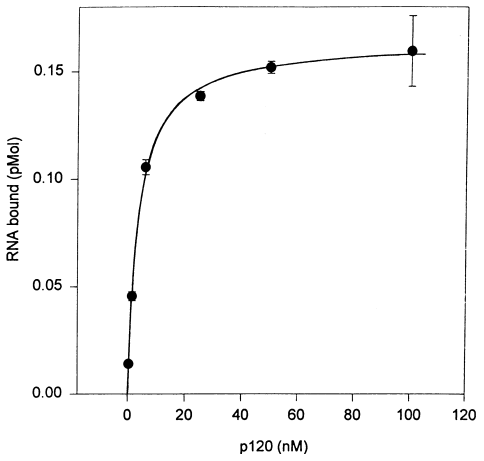

Figure 4 Kinetics of RNA–p120 binding

^{32}P-labelled 1.5 kb 28 S rRNA (0.3 pmol) was incubated with increasing amounts of recombinant p120 at 25 °C for 20 min, filtered through nitrocellulose paper and analysed by scintillation counting as described in the Materials and methods section. A SigmaPlot program was used to determine K_d.

density centrifugation, p120 moved towards the top of the gradient (Figure 7). DNase had no effect on p120 sedimentation behaviour (Figure 7), indicating that the association of p120 with RNP particles depends on ionic interactions with rRNA and that p120 is not bound to DNA. Interestingly, 5–10-fold more p120 was detected by ELISA after RNase treatment than in control

Table 1 Competition nitrocellulose filter binding assay

A ^{32}P-labelled 1.5 kb 28 S rRNA probe was, either directly (no competitor) or after incubation with a 10-fold excess (on a nucleotide basis) of unlabelled 1.5 kb 28 S rRNA, tRNA, or poly(A)$^+$RNA, incubated with recombinant p120 (91% pure, isolated from Sf9 cells) for 30 min. p120–rRNA binding was detected by nitrocellulose filtration and scintillation counting.

Competitor RNA	rRNA binding (% of control)
None	100
rRNA	27
tRNA	93
Poly(A)$^+$	87

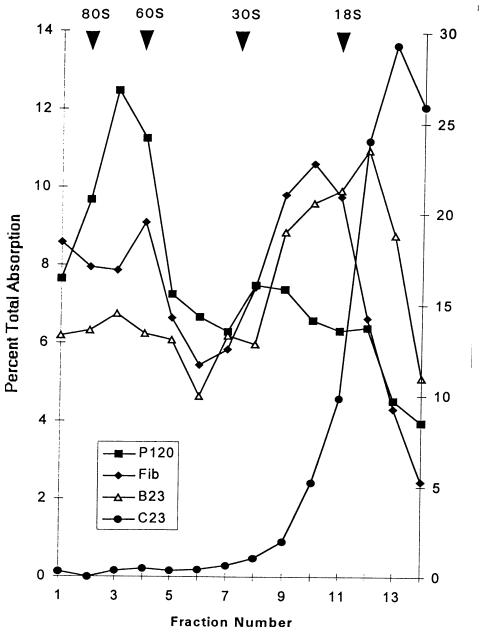

Figure 5 Fractionation of nucleolar extract by sucrose density gradient ultracentrifugation

Nucleolar extracts were centrifuged for 17 h on a 5–45% (w/v) sucrose density gradient at 80000 g_{avg} in a Beckman SW41 rotor at 4 °C. Fraction numbers are from bottom to top. Aliquots (50 μl) of each fraction were analysed for immunoreactivity by ELISA assays with monoclonal antibodies against p120, C23, B23 and human autoimmune patient serum that recognizes fibrillarin (Fib).

samples, which might reflect the release of p120 from the RNP particles by RNase. The epitope might be masked when p120 is bound to the particles and becomes accessible to the antibody when the RNA is digested. No change in antigenic reactivity was observed on RNase treatment of pre-rRNPs when measured on SDS-denatured immunoblots (results not shown).

DISCUSSION

The localization of p120 to the nucleolus and its co-sedimentation with pre-rRNPs on sucrose density gradients suggest that p120 might participate in rRNA processing. The shift in sedimentation pattern of p120 to a higher position on sucrose density gradients when the extract is treated either with RNase or high-ionic-strength buffer provides evidence for the binding of p120 to

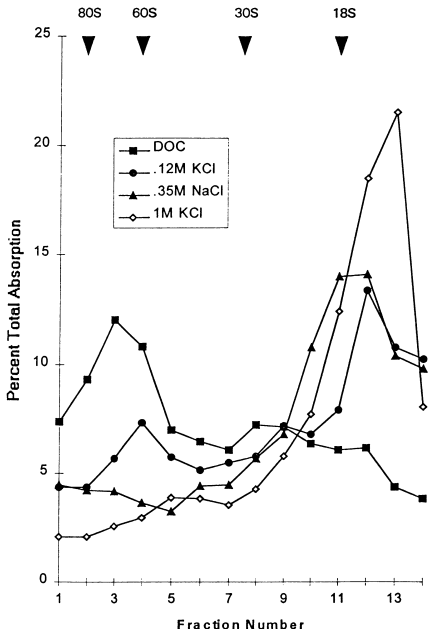

Figure 6 Sedimentation patterns of p120 at different ionic strengths

Nucleolar extracts were subjected to sucrose density gradient ultracentrifugation in the presence of extraction buffer [10 mM Tris (pH 8.0)/10 mM KCl/0.5 mM $MgCl_2$/0.15% deoxycholate (DOC)] and increasing salt (0.12 M KCl, 0.35 M NaCl and 1 M KCl). The amount of p120 was determined as described in the legend to Figure 5.

rRNA. This sedimentation change reflects the release of p120 from the RNP under these conditions. The interaction of p120 with rRNA was investigated with RNA binding studies *in vitro*.

Ribosomal RNA underwent binding to nucleolar protein p120 with a dissociation constant of 4 nM. The ionic strength required for 50% dissociation of p120 *in vitro* from the 1.5 kb 3′ half of the 28 S rRNA transcript (0.4 M NaCl) is similar to the ionic strength necessary for disruption of p120 bound to rRNP particles *in vivo* (0.35 M NaCl; Figure 7) and is also similar to the ionic strength used to extract free p120 from nucleoli (see the Materials and methods section).

We have identified the region of p120 that binds to rRNA as an arginine/lysine-rich region coinciding with the nucleolar localization signal of p120. Mutations of some of these arginine/lysine residues to uncharged residues (Figure 3) inhibited the RNA-binding activity of the N-terminal region of p120. Several RNA-binding proteins have been identified that contain similar arginine-rich domains; this family of proteins has been partly characterized. The members of this family, in general, do not show overall structural similarities between one another [18]. Arginine-rich RNA-binding domains are found in HIV Tat and Rev proteins [27,30,31], and phage λ N, ϕ21 N and P22 N [32] gene product proteins. The arginine-rich domain of HIV Rev protein is most similar to that of p120 (Figure 8). HIV Rev binds to specific Rev response elements in viral mRNA and might be important for viral mRNA transport or splicing [33]. It is also noteworthy that Rev, like p120, has been shown to bind specifically to nucleolar protein B23 [34]. A proposed mechanism to explain the arginine-rich domain binding to RNA is that the positive charge of the arginine residues non-specifically attracts the RNA-binding protein to the phosphate backbone of the RNA [18]. Such non-specific binding allows the arginine-rich domain to search the local RNA structural conformation for a

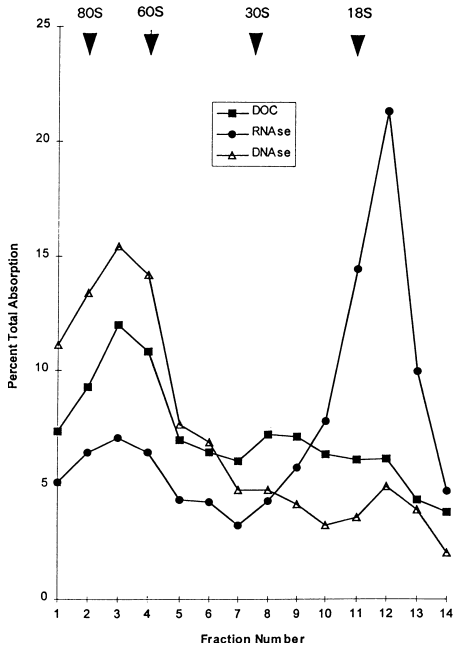

Figure 7 Sedimentation patterns of p120 after RNase or DNase treatment

Nucleolar extracts were incubated with RNase (Boehringer, DNase-free; 10 μg/ml) or DNase (Promega, RQ1 RNase-free; 20 units/ml) for 30 min at room temperature and subsequently loaded on a sucrose gradient. After ultracentrifugation, fractions were analysed for the amount of p120 by ELISA as described in the legend to Figure 5. Abbreviation: DOC, deoxycholate.

Protein	Arginine Rich RNA-binding Motif
p120	K R L S S R A R K R A A K R R L G
HIV Rev	T R Q A R R N R R R R W R E R Q
HIV Tat	A L G I S Y G R K K R R Q R R R P
λN	M D A Q T R R R E R R A E K Q A Q W
ϕ21N	G T A K S R Y K A R R A E L I A E R
P22N	G N A K T R R H E R R R K L A I E R

Figure 8 Comparison of the arginine-rich RNA-binding domain of p120 with similar regions in other proteins

Sequences of the arginine-rich RNA-binding domains for several proteins are listed. Sequences outside these regions are required for full RNA-binding activity. Arginine residues are highlighted. Proteins are HIV Tat, HIV Rev and N gene product proteins from the three phages λ, ϕ21 and P22.

high-affinity binding site (possibly one with an exposed major groove [35]) with which the arginine residues can then specifically form hydrogen bonds. Because the rRNA ligands used in our experiments were synthesized *in vitro*, they do not have post-transcriptional modification such as pseudo-uridine formation or base methylations. Therefore the spatial structure of the rRNA synthesized *in vitro* is probably different from that assumed by the rRNA *in vivo*. This might explain the broad specificity of p120's interaction with rRNA *in vitro*.

The binding of p120 to nucleolar protein B23 also involves the nucleolar localization signal of p120 [14]. It has been hypothesized that B23 binds p120 in the cytoplasm [14] and acts as a shuttle protein to transport p120 from the cytoplasm to the nucleolus. On reaching the nucleolus, the high affinity of p120 for pre-rRNA results in the displacement of B23 from the p120 nucleolar localization signal and p120, bound to rRNA, remains localized to the nucleolus.

The results presented in this paper on the interaction of human p120 with rRNA are consistent with the involvement of yeast Nop2p (the yeast homologue of p120) in ribosome biogenesis. Specifically, depletion of Nop2p decreased the levels of the large ribosomal subunit and blocked processing of the yeast 27 S precursor rRNA to mature 25 S rRNA [11]. Methylation of the conserved site $UmGm\Psi UC_{2922}$, a late methylation event in 27 S pre-rRNA, was low after depletion of Nop2p protein, indicating a close link between methylation and processing of 27 S pre-rRNA [11]. Studies are in progress to address how closely the mammalian p120 mimics the role of the yeast Nop2p in ribosome biogenesis.

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