An acidic amino acid cluster regulates the nucleolar localization and ribosome assembly of human ribosomal protein L22

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Abstract **The control of human ribosomal protein L22 (rpL22) to enter into the nucleolus and its ability to be assembled into the ribosome is regulated by its sequence. The nuclear import of rpL22 depends on a classical nuclear localization signal of four lysines at positions 13–16. RpL22 normally enters the nucleolus via a compulsory sequence of KKYLKK (I-domain, positions 88–93). An acidic residue cluster at the C-terminal end (C-domain) plays a nuclear retention role. The retention is concealed by the N-domain (positions 1–9) which weakly interacts with the C-domain as demonstrated in the yeast two-hybrid system. Once it reaches the nucleolus, the question of whether rpL22 is assembled into the ribosome depends upon the presence of the N-domain. This suggests that the N-domain, on dissociation from its interaction with the C-domain, binds to a specific region of the 28S rRNA for ribosome assembly. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

1. Introduction

The paradigm by which proteins are imported to the nucleus from the cytoplasm has been established [1–3]. Despite the fact that there is no precise consensus amino acid sequence for nuclear localization [4], the principles governing protein importation to the nucleus are well understood [5]. In contrast, little is known about the signal that directs a protein to the nucleolus after the protein enters the nucleus. The nucleolus contains many proteins and RNA molecules that participate in important functions in a eukaryotic cell [6]. The nucleolus is also the site of ribosome biogenesis and is where the ribosomal RNA interacts with the imported ribosomal proteins to make the pre-ribosomal particle [6]. Ribosomal proteins, like other nuclear proteins, are synthesized in the cytoplasm and are then required to pass through the nuclear pores [3] and nucleoplasm on their way to the nucleolus for the completion of ribosome assembly. Hence, the movement of ribosomal protein in the cellular compartment becomes a unique and attractive system for the study of the mechanism behind nuclear and nucleolar transportation.

It has been assumed that each ribosomal protein carries the signal sequence necessary for nuclear and nucleolar targeting [7,8]. Examination of the primary sequences of all eukaryotic ribosomal proteins available [9] allowed the tentative identification of the nuclear localization signal (NLS) of ribosomal proteins, the known consensus NLS [9]. Either a classic basic cluster sequence or a basic bipartite sequence [4] is commonly found with few exceptions in the sequences of most eukaryotic ribosomal protein [9]. On the other hand, identification of the nucleolar targeting sequence (NoS) of ribosomal proteins has not been reported. A few potential sequence motifs have been documented [8,10–15], but these lack a consensus sequence for the nucleolar targeting common to the ribosomal proteins or other nucleolar proteins [16–22].

In this report, we have attempted to identify the sequence that targets a ribosomal protein to the nucleolus and thence into the ribosome. As the first step in determining the mode of nucleolar entry, a human ribosomal protein L22 (rpL22) was selected because it contains three distinct structures [22]. The other reason for choosing rpL22 for this study, besides it being an intrinsic component of ribosome, is that rpL22 is known to be multi-functional. In a previous study, rpL22 was identified as EAP (the Epstein-Barr virus-encoded small nuclear RNA-associated protein) [23–25]. Subsequently, rpL22 was also found to be associated with the herpes simplex virus (HSV) regulatory proteins ICP-4 and ICP-22 [26–29]. These viral proteins are known to be involved in the transcription of the viral early genes in the nucleus. The presence of rpL22 regulates the expression of these viral early genes. Thus, the elucidation of the mechanism by which rpL22 targets the nucleus and shuttles itself between the nucleoplasm and nucleolus could help in understanding the important question as to how a cellular ribosomal protein possibly affects the activity of a virus in an infected cell.

2. Materials and methods

2.1. Plasmids, primers and polymerase chain reaction (PCR)

The gene coding for human rpL22 was obtained from the human liver cDNA library (kind gift of Dr. Peter Hsai, Institute of Genetics, National Yang-Ming University, Taiwan). It was amplified by PCR using primers identified from the published sequences of rat liver rpL22 (GenBank accession No. X78444) [23]. The primers were 5′-<u>catatg</u>gctcctgtgaaaaa-3′ (the N-primer, the sequence underlined was modified to create an *Nde*I site) and 5′-<u>gaattc</u>taatcctcgtcttcc-3′ (the C-primer, the sequence underlined was modified to create an *Eco*RI site). The PCR fragment was subsequently inserted into the vector pGEM-T to make the plasmid pGEM-T/rpL22. The plasmid pGEN-T/rpL22 was used as the template for constructions of wild type rpL22 and its truncated mutants which were inserted into the eukaryotic expression plasmid (pFLAG-CMV-2) (Eastman Kodak Co.) using a similar PCR strategy. These recombinant plasmids carried a CMV promoter and rpL22 gene or its mutant genes and these were tagged by a flag peptide at the amino terminal end of the coding

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sequence. The recombinant plasmids were prepared and transfected into HeLa cells to study the sub-cellular localization of the expressed recombinant proteins. All constructions have been verified by DNA sequencing.

2.2. Cell cultures and DNA transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum (Life Technologies), 5 mM L-glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin. Cells were seeded onto coverslips in a 60 mm petri dish. Eighteen h after plating, plasmid transfection was carried out by the calcium phosphate precipitation method. Ten μg of plasmid was used for each transfection. At different times of post-transfection, the coverslips were processed for indirect immunofluorescence staining, and examined using a confocal microscopy.

2.3. Confocal immunofluorescence microscopy

Cells grown on a coverslip were fixed at different times post-transfection with 3.7% formaldehyde in phosphate buffered saline (PBS), kept for 20 min at room temperature and then permeabilized in 0.1% Triton X-100 in PBS for 5 min. The coverslip was then incubated for 15 min at room temperature with a mouse monoclonal antiserum anti-flag peptide (M2, Eastman Kodak Co.). The antiserum was used at a 1:500 dilution in PBS containing 0.2% gelatin. The coverslip was washed with PBS containing 0.2% gelatin solution and then incubated for 15 min at room temperature with the fluorochrome conjugated secondary antibody. As the secondary antibody, an anti-mouse IgG (Fc-specific) conjugated with fluorescein isothiocyanate (FITC; Eastman Kodak Co.) was used at 1/500 dilution in PBS containing 2% gelatin. The coverslip was washed several times with PBS containing 0.2% gelatin and then mounted with Mowiol on a microscope slide. The cells were analyzed on a Leica confocal microscope (model TCSNT) using 450–490 nm fluorescent filters.

2.4. Yeast two-hybrid system

A reporter yeast strain was co-transformed with pAS2-1 (DNA BD plasmid) and pACT2 (AD plasmid) (Clontech Lab., USA). Two truncated rpL22 mutants were constructed for the two-hybrid system to detect the possible interaction between the N-domain and the C-domain of rpL22. One carried the first 87 residues of rpL22 (N87), and the other carried the last 65 amino acid residues (C65). These two clones were constructed by the same strategy of PCR amplification using pGEM-T/rpL22 as the template. The two clones, along with the gene coded for rpL22, and the genes encoding two other truncated rpL22 mutant genes (ΔN9 and ΔC8) were separately placed into DNA-BD plasmid (pAS2-1), and the GAL4 AD plasmid (pACT2). The recombinant plasmids were co-transformed into a reporter yeast strain (Y187 and CG1945) that possessed the His3 and LacZ genes under control of a GAL4 responsive element. The co-transformants were streaked on medium containing SD/−His/−Leu/−Trp/+5 mM 3-AT to assay for activation of the β-galactosidase reporter gene. The procedures for yeast cell transformation, growth in the selective medium and the β-galactosidase activity, including the o-nitrophenyl β-D-galactopyranoside (ONPG) assay for the measurement of the activity of β-galactosidase, followed those recommended in the Matchmaker system handbook (PT3024-1, Clontech Lab., USA).

2.5. Preparation of recombinant ribosomes and immunological ribosome dot blotting

HeLa cells were transfected by the different eukaryotic expression plasmids containing the wild type rpL22 gene or the truncated rpL22 genes. Twenty one h post-transfection, cells (1×10^7 cells) were broken by sonication in $T_{20}K_{50}M_{12.5}$ buffer (20 mM Tris–HCl, pH 7.6; 50 mM KCl; 12.5 mM MgCl$_2$ in 0.25 M sucrose. Ribosomes were prepared from cell lysate by differential centrifugation as described previously [30]. These ribosomes were made up of the ribosomes from non-transfected HeLa cells, the endogenous ribosomes from transfected HeLa cells, and the recombinant ribosomes that contained the recombinant rpL22 or the truncated mutant proteins if these recombinant proteins had been incorporated into ribosomes. To examine the presence of recombinant protein in the prepared ribosomes, total ribosomal proteins were extracted with 67% acetic acid and analyzed by Western blot using the anti-flag peptide antibody.

The ribosome immuno dot blotting assay [31] was used to determine the surface exposure of recombinant protein on the ribosome. In brief, one OD$_{260}$ unit of prepared ribosomes in 20 μl of $T_{20}K_{50}M_5$

buffer (20 mM Tris–HCl, pH 7.6; 50 mM KCl and 5 mM MgCl$_2$) was dotted on nitrocellulose paper. The nitrocellulose was blocked with bovine serum albumin and washed before being reacted with the anti-flag peptide antibody. Ribosomes that bound the anti-flag peptide antibodies were detected by a non-radioactive AP–biotin–avidin chromogen (Bio-Rad Co., USA).

3. Results

3.1. Construction and expression of genes coded for rpL22 and its mutants

The truncated mutant proteins of rpL22 were designed according to the characteristics of the primary structure of rpL22 (Fig. 1A,B) which shows the N-domain, the I-domain and the C-domain. These genes were cloned into eukaryotic expression vectors (pFLAG-CMV-rpL22). The expression of these genes in 21 h transfected HeLa cells was examined, and found to be quantitatively and qualitatively sound (Fig. 1C).

3.2. Sub-cellular localization of the flag tag proteins containing rpL22 and its mutant proteins

By indirect immunofluorescence with an anti-flag peptide antibody, the recombinant flag tagged rpL22 protein was located in the nucleolus of host HeLa cells (Fig. 2A). In the control, HeLa cells that carried only the flag peptide vector did not show any immunofluorescence. Following this, under the same conditions, the sub-cellular locations of transiently expressed deletion mutant proteins were examined. This showed that mutant protein ΔC8 was located in the nucleolus (Fig. 2B), but the mutant proteins that lacked the N-domain (ΔN9) remained in nucleoplasm (Fig. 2C). However, on deletion of both domains, the mutant protein (ΔN9/C8) entered the nucleolus (Fig. 2D). These results indicated that nucleolar entry of rpL22 must be controlled by both its N- and C-terminal regions.

By analysis of the amino acid sequence motifs of known nuclear or nucleolar targeting signals [4,9], it was determined that the protein rpL22 carried two potential signals, one around the N-domain and the other at the internal region (I-domain) (Fig. 1A). In order to define the possible involvement of these two regions in sub-cellular localization, indirect immunofluorescent localization with anti-flag antibody was carried out again with deletion mutants of ΔN15, ΔN15/C8 and Δ88–93. These results showed that the proteins without the first 15 residues at the NH$_2$-terminal end (ΔN15, or ΔN15/C8) resulted in a cytoplasmic rpL22 (Fig. 2E,F), implying that the sequence of KKKK (positions 10–14) was the NLS. Moreover, mutant protein Δ88–93 was retained in the nucleoplasm (Fig. 2G), suggesting that sequence KKYLKK (positions 88–93) was needed for nucleolar entry of rpL22.

3.3. Interaction between the N-domain and C-domain detected by the yeast two-hybrid system

The two-hybrid system was employed to study the possible interaction between the N-domain and the C-domain involved in entry into the nucleolus. Two deletion mutants were created specifically for this system. Mutant N87 carried only the first 87 amino acid residues of rpL22 and mutant C65 contained only the last 65 amino acid residues from its carboxyl terminus. Strains expressing both Lex-N87 and GAD-ΔN9 or Lex-C65 and GAD-C8 activated β-galactosidase expression of a LexAop-GAL-LacZ reporter gene (Fig. 3A). Activation depends on the presence of both the N- and C-domain because

A

N-domain

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HUMAN   ...MAPVKKLVVK G.....GKKK KQVLKFTLDC THPVEDGIMD AANFEQFLQE RIKVNGKAGN LGGGVVTIER
MOUSE   ...MAPVKKLVAK G.....GKKK KQVLKFTLDC THPVEDGIMD AANFEQFLQE RIKVNGKAGN LGGGVVTIER
_RAT    ...MAPVKKLVAK G.....GKKK KQVLKFTLDC THPVEDGIMD AANFEQFLQE RIKVNGKAGN LGGGVVTIER
_XENL   ...MAPVKKTVTK G.....SKKK KQLLKFTLDC THPVEDGIMD AANFEQFLHD RIKVNGKVGN LGGGVVSIER
_GADM   ...MAPVKK..Q G.....GKKK KQILKFTLDC THPVEDGIMD AANFEQFLQE RIKVNGKSGN LGNGVVSIER
TRIGR   ...MPGKTAQKG GRPSGKGKKK KQTLKFTIDC TLPVEDGIMD ASNFEQFLQE RIKVNGKTKN LTTNIV.IER
_CAEE   MVPKPHAKSAKK A.....LRKK KVHLKFNVEC KNPVEDGILR IEDLEAFLNE KIKVNGKTGH LAANNVKVEV
_YEAS   ...MAP.NTS...........RKQ KIAKTFTVDV SSPTENGVFD PASYAKYLID HIKVEGANGN LG.NAVTVTE
HBp15   ...MAPVKKLVAK G.....XKKK KQVLKFTLDX THPVEXXXXX XXXXXXXXXX XXXXXXKAGN LGGGVVTIER
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　　　　　　　　　　　　　I-domain　　　　　　　　　　　　C-domain

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HUMAN   SKSKITVTSE VPFSKRYLKY LTKKYLKKNN LRDWLRVVAN SKESYELRYF QINQDEEEEE DED.......
MOUSE   SKSKITVTSE VPFSKRYLKY LTKKYLKKNN LRDWLRVVAN SKESYELRYF QINQDEEEEE DED.......
_RAT    SKSKITVTSE EPFSKRYLKY LTKKYLKKNN LRDWLRVVAN SKESYELRYF QINQDEEEEE DED.......
_XENL   SKSKITVSSE VPFSKRYLKY LTKKYLKKNN LRDWLRVVAN SKESYELRYF QINQDEEEEE DED.......
_GADM   XXSKISVNSE VPFSKRYLKY LTKKYLKKNN LRDWLRVVAN TKESYELRYF QINQDEEEIH MTS.......
TRIGR   KKSKVTVTSE IAFSKRYLKY LTKKYLKKNQ LRDWLRVVAA NKESYELRYF QINQDEEEEE DD........
_CAEE   AKSKVSVVSE VPFSKRYLKY LTKKYLKRNS LRDWLRVVAV NKNTYEVRYF HINDGEDAGS DHE.......
_YEAS   DGTVVTVVST AKFSGKYLKY LTKKYLKKNQ LRDWIRFVST KTNEYRLAFY QVTPEEDEEE DEE.......
HBp15   SKSKITVTSE VPFSKRYLKY LTKKYLKKNN LRDWLRVVAN SKESYELRYF QINQDEEEEE E
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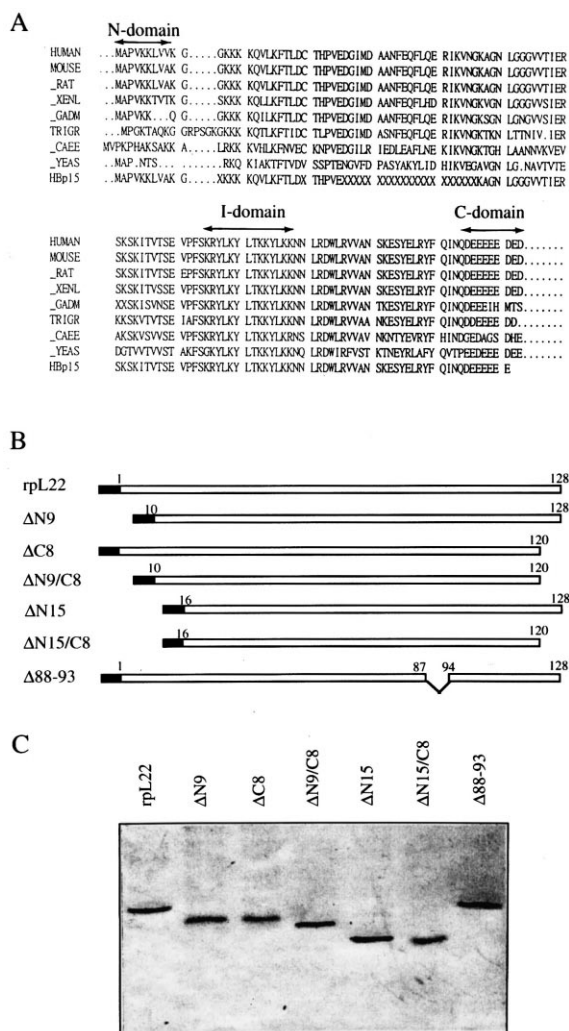
B

C

Fig. 1. Analysis of the expressed recombinant protein rpL22. A: The alignment of the primary structure of rpL22 with its homologous ribosomal proteins from other species and other related proteins. Regions of interest are indicated by a double arrowhead and marked 'N-domain', 'I-domain' and 'C-domain'. XENL, *Xenopus laevis*; GRDM, fish; TRIGR, sea urchin; CAEE, *Caenorhabditis elegans*; YEAS, *Saccharomyces cerevisiae*; HPp15, heparin binding protein 15. B: Schematic representation of rpL22 and its truncated mutants. The filled bar represents the sequence that carries the flag peptide. The open bars are the remaining portions (as indicated by number) of rpL22. ΔN9 (a mutant rpL22 with nine residues deleted from its amino terminal end); ΔN15 (a mutant rpL22 with 15 residues deleted from its amino terminal end); ΔC8 (a mutant rpL22 with eight residues deleted from its carboxyl terminal end); ΔN9/C8 (a mutant rpL22 with the nine and eight residues deleted from the amino terminal and the carboxyl end, respectively); ΔN15/C8 (a mutant rpL22 with the 15 and eight residues deleted from the amino terminal and the carboxyl end, respectively); Δ88–93 (a deletion of the I-domain which has lost amino acids 80–93). C: The expression of recombinant protein detected by Western blotting using an anti-flag peptide antibody. Analysis was done on a total cell lysate of HeLa cells that had been transfected with the vector for 21 h. Equal cell numbers (1.5×10^7 cells) were analyzed by sodium dodecyl sulfate-containing 15% polyacrylamide gel electrophoresis.

no activation was detected in the absence of either in the fusion proteins when expressed in combination with LexA or GAD (data not shown).

The results of the yeast two-hybrid system (Fig. 3A) indicated that the mutant N87 reacted positively to wild type L22

and ΔN9 (having the C-domain), but failed to react when N87 gene was used as either the donor or as the acceptor in the two-hybrid system (Fig. 3A). Similarly, mutant C65 was capable of interacting with ΔC8 (carrying the N-domain), but did not undergo self-interaction (Fig. 3A). The β-galactosidase activity was then measured by the ONPG assay (Fig. 3B). This result showed that the β-galactosidase activity due to the interaction between the N- and C-domain increased 3–4-fold when compared to the measurable β-galactosidase activity of a hybrid without either domain, suggesting that the N- and C-domain interaction was weak.

3.4. Assembly of rpL22 into ribosome

To see whether the recombinant flag tagged rpL22 or its mutant proteins after reaching the nucleolus were capable of being assembled into ribosomes, the recombinant flag tagged rpL22 was detected in prepared ribosomes using anti-flag peptide antibody. Also, the possible exposure of recombinant protein on the surface of ribosome particle was examined using a ribosome dot blot assay [31]. Ribosomes were prepared from 21 h post-transfection cells that had been transfected with the plasmid carrying the flag tagged rpL22 genes. These ribosomes comprised of native ribosomes from non-transfected HeLa cells plus native ribosomes from transfected HeLa cells and recombinant ribosomes which had incorporated the transiently expressed flag tagged rpL22. The ribosome dot blotting showed that the prepared mixed ribosomes from transfected cells immunologically reacted with the antibody (Fig. 4A), whereas ribosomes (wild type ribosomes) separately prepared from non-transfected HeLa cells did not (Fig. 4A). Using the same dot blot assay, the ribosomes prepared from HeLa cells that had been transfected with the different mutant genes of rpL22 were tested. The results (Fig. 4A) showed that the mutants of ΔN15, Δ88–93 (ID) and ΔN9/C8 produced negative results, while ΔC8 was positive. The negative findings for mutants of ΔN15 and Δ88–93 are understandable since these proteins have been shown to be unable to reach the nucleolus (Fig. 2G). However, the failure of mutant ΔN9/C8 to be incorporated into ribosomes was surprising because the protein was transported to the nucleolus (Fig. 2C). One possible reason was that the flag tag peptide of recombinant proteins was inaccessible to the antibody in assembled ribosomes. Therefore, we examined the presence of the recombinant mutant protein (ΔN9/C8) in a total ribosomal protein extract carried out by Western blotting using the anti-flag peptide antibody. The results as given in Fig. 4B showed that the recombinant ΔN9/C8 protein could not be detected, even when the amount of total ribosomal proteins was overloaded, supporting the fact that the ΔN9/C8 mutant protein was incapable of incorporation into ribosomes. In contrast, the recombinant wild type or the ΔC8 mutant proteins were incorporated into the ribosomes (Fig. 4B).

4. Discussion

rpL22 contains three distinct domains, namely, the N-domain, the I-domain and the C-domain (Fig. 1A). The N-domain is made up of the first nine amino acid residues at the NH₂-terminal end, and is hydrophobic in nature. The I-domain (positions 80–93) includes two basic cluster repeats of KKYLK. The C-domain (positions 120–128) is a stretch of nine acidic amino acid that is found in many nucleolar
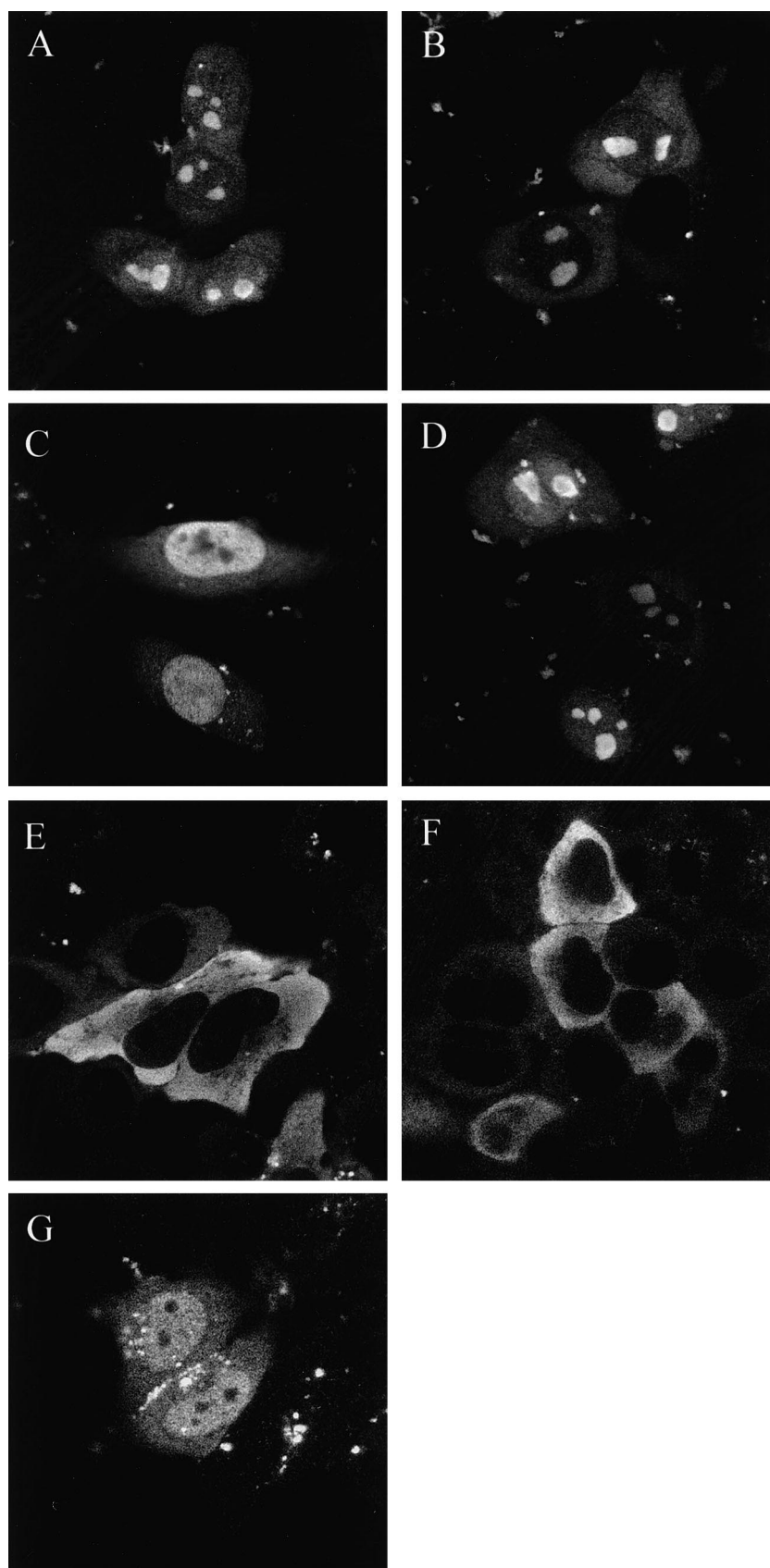
Fig. 2. Immunofluorescent localization of flag tagged rpL22 and its truncated mutant proteins. Expression of recombinant rpL22 and its truncated mutant proteins in HeLa cells transfected with the pCMV-promoter plasmids which carry the rpL22 gene and its mutant genes. After 21 h, the position of the expression of recombinant proteins rpL22 (A), ΔC8 (B), ΔN9 (C), ΔN9/C8 (D), ΔN15 (E), ΔN15/C8 (F), Δ88–93 (G) were located in the HeLa cells by indirect immunofluorescence using an anti-flag peptide antibody. The immunostaining was carried out by a second antibody, FITC-conjugated anti-mouse IgG antibody.

A

a   b   c

N87::rpL22   N87::ΔN9   C65::ΔC8

d   e   f

N87::C65   N87::N87   C65::C65

B
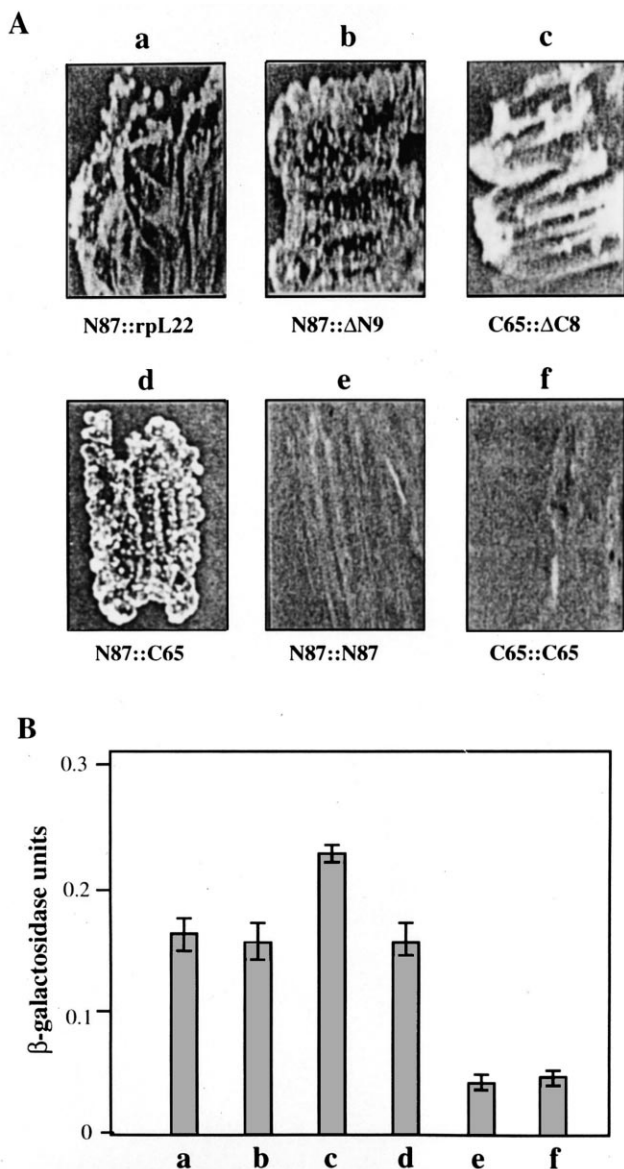
Fig. 3. The interaction between the N-domain and the C-domain in the yeast two-hybrid system. The mutant genes N87 and C67 along with the genes coded for wild type rpL22, ΔN9 and ΔC8, were placed separately into DNA-BD plasmid (pAS2–1) and GAL4 AD plasmid (pACT2). The recombinant plasmids were co-transformed into a reporter yeast strain (Y187 and CG1945), which possessed the His3 and LacZ genes under the control of a GAL4 responsive element. Co-transformants were streaked on SD/−His/−Leu/−Trp/ +5 mM 3-AT to assay for the activation of the β-galactosidase reporter gene. A: The panels present the interaction of (a) N87::wild type rpL22; (b) N87::ΔN9; (c) C65::ΔC8; (d) N87::C65; (e) N87::N87 and (f) C65::C65. The profuse growth in (a), (b), (c) and (d) indicated that these co-transformants contained interacting hybrid plasmids. B: The level of expression of β-galactosidase that was measured by the ONPG assay. These measurements indicated that the activity of β-galactosidase found in hybrids of N87-rpL22, N87-ΔN9, C65-ΔC8 and N87-C65 was increased 3–4-fold as compared with hybrids of N87::N87 or C65::C65.

proteins [6,19,22,32]. The importance of these domains in the targeting of the protein to the nucleus or the nucleolus and in the assembly of the ribosome is the focal point of this study.

The indirect immunofluorescent microscopy study confirms that the basic KKKK (residues 13–16) positioned near the N-

domain is the sequence responsible for nuclear entry of rpL22. This basic cluster is similar to the known consensus NLS [8,36], and the determined NLS of the ribosomal protein S7 [10]. The same experiments showed that the KKYLKK sequence (positions 88–93) in the I-domain was a necessary sequence for nucleolar entry of rpL22 since the deletion of this sequence resulted in nucleoplasmic rpL22 (Fig. 2G). The KKYLK sequence is repeated twice at the I-domain (KRYLK at positions 80–84) of the protein. Such a repeated sequence cannot be found in other eukaryotic ribosomal proteins (unpublished survey). An attempt to fuse the I-domain to green fluorescent protein to test for nucleolar entry failed (data not shown), suggesting that more than one sequence is needed for a cooperative nucleolar entry as suggested by another study [2].

Besides requiring the sequence KKYLKK, nucleolar entry of rpL22 may be regulated by an interaction between the N-domain and the C-domain. This is suggested because a protein without the N-domain became a nucleoplasmic protein, while a protein without both N- and C-domain does reach the nucleolus (Fig. 2D). These observations suggest that the acidic amino acid cluster may carry a nuclear retention role, and the regulation of such a retention role may depend upon its interaction with the N-domain. The yeast two-hybrid system confirmed the existence of the N- and C-domain interaction. However, because of the low level of β-galactosidase activity tested, we suggested that the contact between the N-domain and C-domain is weak but would seem to be sufficient to mask the nuclear retention effect of the C-domain.

The question needs to be proposed as to why a protein

A   **B**

ribosome   Nucleolar
dot blot   targeting

rpL22   +

ΔN16   −

ID   −

ΔN9/C8   +

ΔC8   +

w.t.
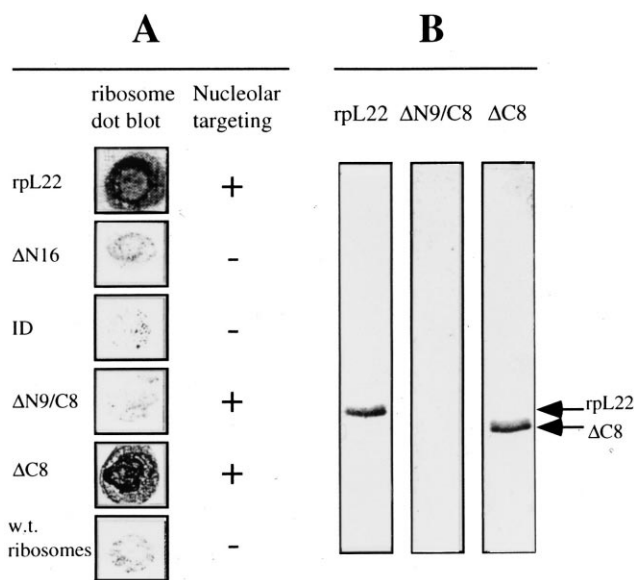ribosomes   −

rpL22 ΔN9/C8 ΔC8

→ rpL22
→ ΔC8

Fig. 4. Ribosome dot blotting analysis of the assembly of rpL22 into ribosomes. A: The detection of the incorporation of flag tagged rpL22 in ribosomes by dot blot assay. The assay detects ribosomes with a surface flag tagged recombinant protein. Each dot contains one OD_{260} unit of ribosomes that were isolated from a 21 h post-transfection HeLa cell. The nucleolar entry of each expressed recombinant protein is shown by markers of + (for the positive), and − (for the negative). ID represents mutant of Δ88–93. B: The Western blot of total protein extract from ribosomes 21 h post-transfected HeLa cells. The results from (A) and (B) were visualized by the immuno-cross-reactivity using the anti-flag peptide antibody.

needs two regulating elements to enter the nucleolus. One possible clue could be the dual function of rpL22 and its importance in viral infected cells. In recent studies, the host EAP protein that regulates the transcription of the early gene of EBV was identified to be the rpL22 [9,24]. Ironically, rpL22 was lately shown to be related to the major regulatory viral proteins of ICP4 or ICP22 of HSV [26–28]. There are reports that rpL22 participated with these regulatory proteins in the expression of early genes of HSV. Under these circumstances, it is reasonable to suggest that nuclear retention of rpL22 could be an inevitable step in virus-infected cells. Thus, breaking the weak interaction between the two domains would make rpL22 nucleoplasmic and available for transcription of the viral gene. The necessary role of an acidic cluster in the shuttling of rpL22 between nucleus and nucleoli is obvious.

It is noticeable that many nucleolar proteins, such as SSB-1 [6], Nop2 [30], NOP77p [32], p120 [21] and NOP4 [33] contain an acidic amino acid cluster. These proteins are known to be involved in the interaction of the ribosomal components during the ribosome biogenesis. Trapping by the fine structure of the nucleolus in order to retain these proteins has been suggested, but the role of the acidic amino acid cluster in these proteins has never been revealed. It can only be speculated that their acidic clusters are masked to allow these proteins to move into the nucleolus. In this study, we have assumed that the acidic cluster plays the same role of nuclear retention for rpL22, that is, it helps a nucleolar protein remain in the nucleus. This is also true for several nucleic acid associated proteins, such as heparin binding protein (HBp15) [34], the DNA binding protein of the high mobility group [35], and the transcription activator of Tat-SF1 [36]. These proteins which are retained in the nucleus characteristically carried a stretch of acidic clusters at their carboxyl-terminal region. Although, the role that the acidic cluster plays in these proteins has not been studied in detail, the same nuclear retention role is suggested.

A similar acidic amino acid cluster has been found in the structure of several eukaryotic proteins, namely L5, L31, P0, P1, P2, S8 and S9 (range of four to eight acidic residues) [9]. Whether these clusters play the same role needs to be studied. In fact, it may be more important if these ribosomal proteins have a dual function, a reality for these members of eukaryotic ribosomal proteins [9].

In this study, we posed the question as to whether the transiently expressed recombinant flag tagged ribosomal protein can be successfully assembled into ribosomes. We detected the recombinant flag tagged protein in isolated ribosomes. The detection relied on the immuno-reactivity between the flag tagged recombinant protein and the anti-flag peptide antibody. We also detected, using ribosome dot blotting [31], whether the flag tag peptide was on the outside of the ribosome particles and detected by Western blotting if the recombinant proteins were part of the total ribosomal proteins. In both assays, the recombinant flag tagged rpL22 was found to be present, indicating that the recombinant protein has successfully incorporated into ribosomes. Under such conditions, the mutant protein, without the C-domain (lacking of acidic cluster) which allowed it to reach the nucleolus, was also found in the ribosome. However, the mutant protein without both the N- and the C-domains could reach the nucleolus but was not assembled into ribosomes. These results clearly show that the binding of the N-domain to 28S rRNA is a prerequisite for rpL22 to be assembled into the

ribosomes. In a previous study [25], the nucleotide sequence from 302 to 317 of the 28S rRNA was found to be the binding site for rpL22. Thus, it should be expected that the interaction takes place between this nucleotide segment and the eight amino acid residues from the N-domain. The interaction should be strong enough to compete with the weak association of the N-domain and the C-domain once rpL22 enters the nucleolus. It can also be speculated that a similar competition might occur in the nucleoplasm when the cell is infected with virus. This needs to be determined.

References

[1] Silver, P.A. (1991) Cell 64, 489–497.
[2] Hicks, G.R. and Raikhel, N.V. (1995) Cell Dev. Biol. 11, 155–188.
[3] Gorlich, D. (1997) Curr. Opin. Cell Biol. 9, 412–419.
[4] Dingwall, C. and Laskey, R.A. (1991) Trends Biochem. Sci. 16, 478–481.
[5] Nigg, E.A., Baeuerle, P.A. and Luhrmann, R. (1991) Cell 66, 15–22.
[6] Meelese, T. and Xue, T. (1995) Curr. Opin. Cell Biol. 7, 319–324.
[7] Laskey, R.A. and Dingwall, C. (1993) Cell 74, 585–586.
[8] Rout, M.P., Blobel, G. and Aitchison, J.D. (1997) Cell 89, 715–725.
[9] Wool, I.G., Chan, Y.L. and Gluck, A. (1995) Biochem. Cell Biol. 73, 933–947.
[10] Schmidt, C., Lipsius, E. and Kruppa, J. (1995) Mol. Biol. Cell 6, 1875–1885.
[11] Michael, W.M. and Dreyfuss, G. (1996) J. Biol. Chem. 271, 11571–11574.
[12] Quaye, I.K.E., Toku, S. and Tanaka, T. (1996) Eur. J. Cell Biol. 69, 151–155.
[13] Annilo, T., Karis, A., Hoth, S., Rikk, T., Kruppa, J. and Metspalu, A. (1998) Biochem. Biophys. Res. Commun. 249, 759–766.
[14] Russo, G., Ricciardelli, G. and Pietropalo, C. (1997) J. Biol. Chem. 272, 5229–5235.
[15] Timmers, A.C., Stuger, R., Schaap, P.J., van't Riet, J. and Raue, H.A. (1999) FEBS Lett. 452, 335–340.
[16] Clark, M.W., Yip, M.L., Campbell, J. and Abelson, J. (1990) J. Cell Biol. 111, 1741–1751.
[17] Meier, U.T. and Blobel, G. (1992) Cell 70, 127–138.
[18] Schmidt-Zachmann, M.S. and Nigg, E.A. (1993) J. Cell Sci. 105, 799–806.
[19] De Beus, E., Brockenbrough, J.S., Hong, B. and Aris, J.P. (1994) J. Cell Biol. 127, 1799–1813.
[20] Girard, J-P., Bagni, C., Caizergues-Ferrer, M., Amalric, F. and Lapeyre, B. (1994) J. Biol. Chem. 269, 18499–18506.
[21] Valdez, B.C., Perlaky, L., Henning, D., Saijo, Y., Chan, P.K. and Busch, H. (1994) J. Biol. Chem. 269, 23776–23783.
[22] Antoine, M., Reimers, K., Dickson, C. and Kiefer, P. (1997) J. Biol. Chem. 272, 29475–29481.
[23] Chan, Y.L. and Wool, I.G. (1995) Biochim. Biophys. Acta 1260, 113–115.
[24] Toczyski, D.P.W. and Steitz, J.A. (1991) EMBO J. 10, 456–466.
[25] Dobbelstein, M. and Shenk, T. (1995) J. Virol. 69, 8027–8034.
[26] Faber, S.W. and Wilcox, K.W. (1986) Nucleic Acids Res. 14, 6067–6083.
[27] Leopardi, R. and Roizman, B. (1996) Proc. Natl. Acad. Sci. USA 93, 4572–4576.
[28] Leopardi, R., Ward, P.L., Ogle, W.O. and Roizman, B. (1997) J. Virol. 71, 1133–1139.
[29] Ng, T.I., Chang, Y.E. and Roizman, B. (1997) Virology 234, 226–234.

[30] Collatz, E., Wool, I.G., Lin, A. and Stoffler, G. (1976) J. Biol. Chem. 251, 1808–1816.

[31] Lin, E., Liu, S.R. and Lin, A. (1999) Biochem. J. 125, 1029–1035.

[32] Berges, T., Petfalski, E., Tollervey, D. and Hurt, E.C. (1994) EMBO J. 13, 3136–3148.

[33] Sun, C. and Woolford Jr., J.L. (1994) EMBO J. 13, 3127–3135.

[34] Fujita, Y., Okamoto, T., Noshiro, M., Mckeehan, W.L., Crabb, J.W., Whitney, R.G., Kato, Y., Sato, J.D. and Takada, K. (1994) Biochem. Biophys. Res. Commun. 199, 706–713.

[35] Grosschedl, R., Giese, K. and Pagel, J. (1994) Trends Genet. 10, 94–100.

[36] Zhou, Q. and Sharp, P.A. (1996) Science 274, 605–610.