Nuclear Trafficking of La Protein Depends on a Newly Identified Nucleolar Localization Signal and the Ability to Bind RNA*

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The human La protein (hLa) is a 47-kDa phosphoprotein predominantly localized in the nucleus. It was first discovered as an autoantigen recognized by antibodies present in the sera of patients suffering from systemic lupus erythematosus and Sjogren’s syndrome (1, 2). The La protein is a member of a large group of RNA-binding proteins containing RNA recognition motifs (RRMs) (3–7) and interacts with a variety of small RNAs (for reviews see Refs. 8–10). The La protein is implicated in several aspects of the RNA metabolism, including processing of precursor transcripts occurring in various subnuclear compartments. Among these, precursor transcripts of certain RNAs are located in the nucleoplasm or nucleolus. Here we examined which functional domains of hLa are involved in its nuclear trafficking. By using green fluorescent-hLa fusion proteins, we discovered a nucleolar localization signal and demonstrated its functionality in a heterologous context. In addition, we revealed that the RRM2 motif of hLa is essential both for its RNA binding competence in vitro and in vivo and its exit from the nucleolus. Our data imply that hLa traffics between different subnuclear compartments, which depend decisively on a functional nucleolar localization signal as well as on RNA binding. Directed trafficking of hLa is fully consistent with its function in the maturation of precursor RNAs located in different subnuclear compartments.

The human La protein (hLa) is a 47-kDa phosphoprotein predominantly localized in the nucleus. It was first discovered as an autoantigen recognized by antibodies present in the sera of patients suffering from systemic lupus erythematosus and Sjogren’s syndrome (1, 2). The La protein is a member of a large group of RNA-binding proteins containing RNA recognition motifs (RRMs) (3–7) and interacts with a variety of small RNAs (for reviews see Refs. 8–10). The La protein is implicated in several aspects of the RNA metabolism, including processing of precursors of tRNAs, U3 snoRNA, U1 RNA, U6 snRNA (11–20), and stabilization of viral (21–23) as well as cellular RNAs (24). The network of functional interactions between La and precursor RNAs is essential for RNA binding (42). Recently, we have shown that the RNP-2 of RRM2 in hLa is required for binding of hepatitis B virus RNA (43), and it seems reasonable to assume that this motif is also required for the binding of cellular precursor transcripts. La interacts with RNA polymerase III products by binding the poly(U) stretch at the 3’-end common to this class of transcripts (44) or, in the case of the RNA polymerase II product U3 snoRNA, after partial processing exposing a poly(U) stretch (17).

Because elements for nucleolar localization have been described for other proteins (45–52), it is conceivable to assume that hLa bears such a signal to ensure its interaction with pre-tRNAs, for example, in the nucleolus besides the binding of pre-U3 snoRNA or U6 snRNA in the nucleoplasm. In this report we identified a yet unknown nucleolar localization signal (NoLS) in the C-terminal region of hLa, and we show that it functions in a heterologous context. In addition, we demonstrate the accumulation of hLa mutants deficient in interaction with pre-tRNA in vivo in the nucleolus. Based on these and additional data, we propose a model for the dynamic intranuclear distribution of hLa which is consistent with the complex network of functional interactions between La and precursor transcripts occurring in various subnuclear compartments.

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‡ The abbreviations used are: hLa, human La autoantigen; FRAP, fluorescence recovery after photobleaching analysis; GFP, green fluorescent protein; EGFP, enhanced GFP; RNP, ribonucleoprotein particles; RRM, RNA recognition motif; WAM, Walker-A-motif; WT, wild type; NoLS, nucleolar localization signal; snoRNA, small nuclear RNA; snRNA, small nuclear RNA; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole.
Plasmid Constructs and Mutagenesis—Expression plasmid pET28a(+) hLa-D2 was described previously (43). Plasmids described here were produced with the same strategy. Oligonucleotide synthesis was as follows: for hLa-M63, 5′-TTG GTC TTC TAT TAT TTT CTT TGC CTC-3′ and 5′-GTA CAG TTT CAG GGC AAG-3′; for hLa-F118A, 5′-GTA AAA AAG AGA CTG TGT TAT ATT AAA CGG GCC CCA ACT GAT GCA ACT C-3′ and 5′-GAG TAT CAT CAG TGT GGG CGC TCT TAA TAT AAA CAC ATG TGT TTA C-3′. For expression of hLa mutants, respective hLa DNAs were amplified from the pET28a(+) mutants as GFP fusion proteins by a eukaryotic expression vector, the pEGFP-C1 vector (Clontech). The respective hLa DNAs were amplified from the pET28a(+) plasmids mentioned above via PCR by using the primers 5′-AGA TCG GTA CCA ATG GAA AAT GAA TCC CTA AAC AAA TGG-3′ (containing a HindIII restriction site (underlined), respectively) and Sacl and HindIII restriction sites (underlined), respectively. Purified PCR products were digested with the appropriate restriction enzymes and cloned into the HindIII and Sacl linearized vector pEGFP-C1 (Clontech) by using the rapid ligation kit (Roche Applied Science). Finally, in-frame cloning was controlled by sequencing.

For fusion of hLa amino acids 323–354 to the N terminus (hLa-NoLS-PTB-GFP) or C terminus (PTB-hLa-NoLS-GFP) of PTB cloned into the eukaryotic expression vector EGFP-N1 (Clontech), pEGFP-P, the respective hLa sequence was amplified by PCR from the pET-28a(+) hLa plasmid using the following primers: 5′-CCT ACA CTC GAG GCC ACC ATG CA GAA TCC CTA AAC AAA TGG-3′ (containing an Xhol restriction site (underlined), a Kozak sequence (underlined), and a start codon (boldface), respectively) and 5′-CCT ACA AAG CTT TTT TCT TCC TTT ACC AGA CCC AGG-3′ (containing a HindIII restriction site (underlined), a Kozak sequence (underlined), 5′-CCT ACA CTC GAG GCC ACC ATG CA GAA TCC CTA AAC AAA TGG-3′ (containing an EcoRI restriction site (underlined) and 5′-CAC ACA GCC GCT CTT CCT CTA CAA GCC CAG GGC-3′ (containing a Sacl restriction site (underlined)). PCR was performed, and purified products were digested with Xhol and HindIII for N-terminal fusion to PTB-GFP cDNA or with EcoRI and Sacl for C-terminal fusion and cloned into the linearized, respective PTB-GFP vector. In the case of PTB-hLa-NoLS-GFP an extra alanine or leucine was added for in-frame cloning at both fusion sites.

Expression and Purification of Recombinant Proteins—His-tagged recombinant WT and hLa mutant proteins were purified and analyzed as described previously (43). Immunoblot signals were quantified with the Fluor-S MultiImager system (Bio-Rad) to ensure that equal amounts were used in subsequent experiments. For all experiments at least 2 to 3 different protein preparations were used.

In Vitro Transcription and UV Cross-linking Assays—DNA templates used for in vitro transcription of pre-tRNA were produced by PCR as described previously (22). Plasmid HRV1 (a kind gift of H. Beier, Institute for Biochemistry, University of Wurzburg, Germany (53)) containing the template tRNA sequence served as template, and the primers used for generation of pre-tRNA DNA template were 5′-CCA TCG ATT AAC AGC ACT CAC CTT AGT ATG CTT CCC TAG TGT AGT GG-3′ containing a ClaI restriction site (underlined) and the T7 RNA polymerase promoter (boldface), and 5′-AAA GCG ACT CTC TTT GTC TTC CTT-3′. In vitro transcription was performed as mentioned elsewhere (22). For UV cross-links, recombinant hLa proteins (amounts as indicated in Fig. 2A) were incubated for 10 min at room temperature with excess amounts of Ap-labeled (300,000 cpm) and in vitro transcribed pre-tRNA in 20 μl reactions containing 10 μm Tris-HCl, pH 7.4, 100 μm NaCl, 0.5% Nonidet P-40, 3 μm MgCl2, 0.5 mM EDTA, and 2 μg of poly(rC) (Sigma). Afterward, cross-linking occurred with 0.6 J in a Stratalinker-1800 (Stratagene) followed by RNAse A (5 μg), treatment for 20 min at 40 °C, addition of loading buffer, incubation at 60 °C for 5 min, and electrophoretic separation in 12.5% SDS-polyacrylamide gels, stained with Coomassie brilliant blue R250 for 20 min at 50 °C with addition of 0.1% SDS; (ii) 20 μg of each glycine (Roche Applied Science) was added for precipitation. RNAs were separated on 12% polyacrylamide, 7 M urea gels, blotted to Hybond-N membranes (Amersham Bioscience), and detected with radioactively end-labeled oligonucleotide probes (pre-tRNA, 5′-GG TAT CTC CTT CTT AAG TAG CTA C-3′; mature tRNA, 5′-GGA TGT CTC CTT CTT AAG TAG CTA C-3′). Signals were quantified using a Fuji-X BAS 2000 Phosphorimaging system (Fuji). Note, the estimated transfection efficiency of 293T cells was ~20% in contrast to ~15% for HeLa/Huh7 cells because of different cell line characteristics and transfection procedures.

Fluorescence Recovery after Photobleaching (FRAP) Analysis—HeLa cells were plated at a density of 4 × 10^5 cells in Lab-Tek double chamber systems (Nunc, Germany) and transfected as mentioned above, and FRAP analysis was performed 1 day after transfection on a Leica TCS-SP2 confocal microscope (Leica, Germany) using the 488 nm laser line of the built-in argon laser (nominal output 65 milliwatts). A time resolution of 657 msec intervals was chosen, and images were taken before bleaching, followed by a triple bleach pulse at 488 nm in the indicated area and subsequent image collection for at least 30 s. Quantification of the fluorescence intensity detected in a region of interest was normalized to the loss in total fluorescence because of bleaching and imaging as described by others (55) using the Scion Image Beta 4.0.2 software. Consistent results were obtained in three independent experiments with at least 10 cells measured for each of the described hLa proteins. Results shown indicate representative experiments. As a control, the temperature was decreased to 24 °C before and during analysis (not shown).
Recently, we have reported (43) that the RNP-2 consensus sequence of RRM2 is essential for hepatitis B virus RNA binding. Here we tested the ability of this RNP-2 deletion mutant (referred to as hLa-Δ2, Fig. 1A) and of a mutant protein with a single amino acid substitution (referred to as hLa-F118A; Fig. 1A) to bind pre-tRNA<sub>Val</sub> <i>in vitro</i> (Fig. 2A). As expected, incubation of recombinant hLa-WT with labeled pre-tRNA<sub>Val</sub> followed by UV cross-linking and electrophoretic separation led to reduction of RNA binding activity, because pre-tRNA<sub>Val</sub> (as well as processing intermediates thereof) but not mature tRNA<sub>Val</sub> can be co-precipitated as would be expected for endogenous hLa. Concerning the hLa mutants, this experiment revealed that GFP-hLa-WT but not GFP-hLa-Δ2.0 was associated with pre-tRNA<sub>Val</sub> <i>in vitro</i>, thereby reflecting the results obtained in UV cross-links. These data signify that deletion of the RNP-2 motif abolishes the interaction of hLa with pre-tRNA<sub>Val</sub> and point out the importance of RRM2 in hLa for pre-tRNA binding <i>in vivo</i>. In the case of co-immunoprecipitations with hLa-Δ2 or IgG control, where virtually no RNA was precipitated signal intensities of pre-tRNAs in supernatants were unexpectedly weaker than those in starting material and probably result from minor degradation during the experiment.

Next, the subcellular localization of hLa mutants GFP-hLa-WT, GFP-hLa-Δ2, and GFP-hLa-F118A were analyzed in human cell lines HuH7, HeLa, or U2OS by transient transfection and fluorescence microscopy. The GFP-hLa-WT signal was compared with immunostained endogenous hLa in nontransfected cells. In both cases a diffuse and a minor granular nuclear distribution pattern was obtained for all three cell lines (representatively shown for HuH7 cells, Fig. 3A). Of note, to reduce the risk of artifacts caused by overexpression, we chose cells with low expression levels of GFP fusion proteins; however, we observed comparable phenotypes (see below) in all cases. We conclude that the nuclear distribution of endogenous hLa is indistinguishable from exogenously overexpressed GFP-hLa fusion protein.

In order to analyze the effect of RNA binding activity of La on its cellular localization, distribution of GFP-hLa-Δ2 and GFP-hLa-F118A was monitored. Merging the GFP-hLa-Δ2 and GFP-hLa-F118A signals with that of nucleolus specific NOH61 showed that both mutants accumulate in nucleoli (Fig. 3B). To show that ongoing transcription has no detectable effect on the subnuclear distribution of hLa, we treated GFP-hLa-WT transfected cells with actinomycin D (2.5 and 5 μg per ml) for up to 8 h. No change in the distribution of the GFP-hLa proteins within the nucleus compared with nontreated cells was observed (data not shown). In addition to the prominent nucleolar localization, GFP-hLa-Δ2 and GFP-hLa-F118A were to a minor extent visible in the cytoplasm (Fig. 3B), which might indicate that RNA binding contributes to an efficient nuclear import (or retention) of hLa.

We conclude from these data that hLa mutants with a reduced or lacking pre-tRNA binding activity <i>in vitro</i> and <i>in vivo</i> (Fig. 2, A and B) accumulate or are retained in the nucleolus, which argues for a role of the RNA binding competence of hLa for maintenance of the predominant nuclear diffuse distribution pattern.

**Identification of the NoLS of hLa**—The C-terminal part of La contains the following functional motifs: a proposed dimerization domain (43, 56), a Walker-A motif (WAM (57)), nuclear retention signals, and a nuclear localization signal (58, 59). To investigate the contribution of the C-terminal part of hLa to its subcellular localization, several deletions were introduced into this region of the corresponding cDNA insert of pet28hLa and subsequently cloned in-frame with the EGFP protein. The cor-
responding GFP-hLa expression plasmids were transiently transfected into the human hepatoma cell line Huh7 or into HeLa or U2OS cells, and subcellular distribution was monitored. Analysis of one of these mutants, namely GFP-hLa-H90046.3 (amino acids 323–354, outlined in Fig. 1) by immunofluorescence and living cell microscopy (see below) revealed a diffuse nuclear localization except for distinct areas in which no or little GFP signal was observed (Fig. 4A). Merging the GFP signal of hLa-H90046.3 with the NOH61 staining illustrates that these areas represent nucleoli. We conclude that these 32 amino acids spanning a basic region and the WAM are mediating the recruitment of hLa into nucleoli. Therefore, we refer to this region as the hLa nucleolar localization signal (hLa-NoLS).

To show the functionality of the hLa-NoLS in a heterologous context, this signal was fused to an unrelated protein, which is normally absent from nucleoli. For this purpose, the 32-amino acid-long hLa-NoLS was fused at two different positions to a PTB-GFP fusion protein (Polypyrimidine-tract-binding protein; referred to as hLa-NoLS-PTB-GFP and PTB-hLa-NoLS-GFP, respectively), and these proteins were subsequently expressed in HeLa cells and analyzed by immunofluorescence staining. Consistent with a previous study (60) the PTB-GFP fusion protein localized evenly in the nucleoplasm and was...
absent from nucleoli (Fig. 4B, left panel). However, fusion of amino acids 323–354 of hLa to the N terminus of PTB-GFP targeted hLa-NoLS-PTB-GFP to nucleoli (Fig. 4B, right panel). In addition, a fusion protein containing the hLa-NoLS between the PTB and GFP sequences (PTB-hLa-NoLS-GFP) also localized to nucleoli (data not shown) and therefore underlines the functionality of the newly identified NoLS.

Because the C-terminal region including the sequence of the hLa-NoLS (amino acids 323–354) is implicated in the interaction of hLa with the 5′-end of pre-tRNAs (8), we tested the ability of hLaΔ6.3 to bind pre-tRNA in vitro by UV cross-linking assays (as well as electrophoretic mobility shift assays and BIAcore experiments, data not shown) and in vivo by co-immunoprecipitations. In vitro, hLaΔ6.3 formed complexes with pre-tRNAVal (Fig. 2A, lower right panel) that were less intense when compared with hLa-WT (Fig. 2A, upper left panel), indicating that residues 323–354 contribute to but are not necessary for RNA binding. Concordantly, similar results were obtained from co-immunoprecipitation experiments (Fig. 2B) where pre-tRNATyr can be co-precipitated with GFP-hLaΔ6.3, even though in reduced amounts when compared with GFP-hLa-WT, as expected from our in vitro results.

These observations imply that the amino acids 323–354, in contrast to the RNP-2 motif in RRM2, are not crucial for a stable interaction between hLa and pre-tRNAVal, although this region contributes to pre-tRNA binding. In summary, entry of hLa into nucleoli is mediated by the NoLS obviously in an RNA binding independent fashion, whereas exit from nucleoli appears to be facilitated by an RRM2-mediated interaction with RNA.

**A Diffusion-like Mobility of Human La in the Nucleus of Eukaryotic Cells**—We analyzed the mobility of GFP-hLa-WT and mutants in living cells by FRAP experiments. To rule out that nucleolar exclusion or accumulation was because of a reduced mobility or attributable to saturation of possible transport events caused by overexpression, we chose cells with low expression levels of GFP-hLa fusion proteins. One day after transfection of these different GFP-hLa expression vectors, a defined area within the nucleus of individual living cells was bleached by three repetitive high powered laser pulses. The recovery of fluorescence signal in the bleached area because of the laser pulses and the imaging process and probably because of a small fraction of immobile protein, as reported for others (61). This rate of recovery is similar to other nuclear proteins described to move by free diffusion (55, 61–63), indi-
cating that this applies also to GFP-hLa-WT. This assumption is further supported by data on the recovery rate of GFP-hLa-WT measured at room temperature (data not shown). Under these conditions the recovery rate was not prolonged, indicating that GFP-hLa-WT moves most likely by an energy-independent mechanism, a conclusion drawn on the basis of similar experiments for HMG-17, SF2/ASF, and fibrillarin (55). Next, the mobility of GFP-hLa-Δ2.0, GFP-hLa-F118A, and GFP-hLa-Δ6.3 was recorded. GFP-hLa-Δ2.0 showed no nucleolar GFP signal as examined by confocal microscopy (Fig. 5), which is consistent with immunofluorescence analysis of this mutant (Fig. 4A). Furthermore, no GFP signal was detected within the nucleolar compartment during the recovery process at any time, verifying that GFP-hLa-Δ6.3 was incapable of entering the nucleolus. The recovery rate of this protein was similar to GFP-hLa-WT, requiring approximately 3 s for 50% recovery. Furthermore, analysis of GFP-hLa-Δ2.0 and GFP-hLa-F118A revealed a comparable recovery rate, demonstrating a similar mobility of these mutants and the wild-type protein. Note, in the case of GFP-hLa-WT, recovery of signals appeared from nucleolar and nucleoplasmic regions simultaneously. Conversely, in the case of GFP-hLa-Δ2, recovering GFP signals arose from unbleached nucleoplasmic regions and barely from unbleached nucleoli (Fig. 5). These data support our conclusion that nucleolar exit of GFP-hLa-Δ2 is hampered.

Taken together, no significant differences in the recovery rates were obtained between all mutants and GFP-hLa-WT. Apparently, neither the internal deletions nor the amino acid substitution altered the mobility of the analyzed GFP-tagged hLa proteins. By choosing cells with low expression of the transfected GFP-tagged proteins for FRAP assays and fluorescence microscopy, we further minimized the possibility that overexpression of a protein leads to an apparently higher mobility because of saturation of interacting factors. Therefore, the observed differences in subnuclear localization of wild-type and mutant hLa protein are presumably caused by hindered interactions with other factors and not by altered mobilities.

**Fig. 5.** FRAP analysis of nuclear GFP-hLa-WT and mutants suggests diffusion-like mobility. HeLa cells were transfected with GFP-hLa-WT, GFP-hLa-Δ2, GFP-hLa-F118A, or GFP-hLa-Δ6.3 (as indicated to the left of the figures), and FRAP experiments were performed with confocal microscopy. As indicated above, cells were imaged before (prebleach) and during recovery (bleached and end) with a resolution of 657 ms before and after bleaching of the areas designated by squares in the figures. Scale bar, 10 μm. Quantification of recovery is shown to the right (as indicated above) for all GFP-hLa proteins, and intensity in the bleached region was measured and expressed as relative ratios. Recovery of the fluorescence signal was rapid in all cases and reached 50% after ~3 s and a plateau after ~25 s. Fluorescence intensity was measured for 55 s in all cases to ensure stable plateau establishment (not completely shown). Reduction of temperature to 24 °C had no effect on recovery (not shown). Note, faint cytoplasmic signals of GFP-hLa-Δ2 or GFP-hLa-F118A as seen in Fig. 3B are not visible as we had chosen cells with very low GFP expression levels for FRAP assays.
Subnuclear Localization of hLa

DISCUSSION

In this report, we provide evidence for the dynamic nuclear trafficking of the human La protein. We demonstrate that the RNA binding activity and an NoLS act in concert to connect its assigned functions in different subnuclear compartments. The hLa protein forms ribonucleoprotein particles (RNPs) containing both RNA polymerase II and III transcripts (as reviewed in Refs. 8 and 10). The detection of La protein and pre-tRNA molecules as well as RNase P in the nucleolus (9, 39, 40) supports the possibility that La interacts with pre-tRNAs in the nucleolus during the first step of pre-tRNA maturation. However, Intine et al. (37) recently showed that nonphosphorylated La localized to nucleoli and did not associate with pre-tRNAs, in contrast to nucleoplasmic phosphorylated La. Moreover, La was reported to interact with precursors of U3 snoRNA (17, 20) and U6 snRNA (64) in the nucleoplasm rather than in the nucleolus. Hence, the La protein has to be present both in the nucleolus and in the nucleoplasm in order to fulfill its support functions in processing and stabilization of transcript precursors.

The subnuclear localization of proteins is either established because of specific signal sequences or is determined via interaction with specific molecules. If the localization depends on the interaction with other factors, the on- and off-rates of the interaction are critical for the intracellular localization of the corresponding protein. Nuclear proteins find their targets either by free diffusion or by an active transport (for review see Refs. 65–68). For instance, it has been shown that fibrillarin moves by free diffusion through the nucleoplasm and accumulates within nucleoli (55). This implicates that its steady state level in nucleoli depends on faster on-rates and/or slower off-rates between fibrillarin and associated factors within this compartment compared with those in the nucleoplasm.

In the case of endogenous hLa or GFP-hLa-WT, a rather homogenous nuclear distribution was observed (Fig. 3A). Within the limits and sensitivity of the techniques used, our data indicate that hLa-WT does not accumulate predominantly in distinct nuclear areas. Therefore, the on- and off-rates between hLa-WT and its interaction partners are probably very similar in different subnuclear compartments. To gain information about the subcellular compartments hLa passes through, one strategy might be to change the specific on- and off-rates in order to increase or diminish GFP signals in a specific subnuclear compartment. In line with this approach, analysis of hLa mutants led to the discovery of functional domains mediating the nucleolar trafficking of hLa.

For several proteins it has been shown that subnuclear localization may depend on glycine/arginine-rich segments, threonine/proline repeats, and/or RNA binding domains, for example (52, 69–71). Although the hLa RRMs are not very typical in respect to the consensus sequences (6), deletion of the RNP-2 signature of RRM2 clearly showed that this motif is essential for pre-tRNA binding (in vitro and in vivo) (hLa-Δ2.0, Fig. 2). Recently, we have shown the requirement of this domain for an interaction with hepatitis B virus RNA (43), suggesting a general function of RRM2 in RNA binding. The RNP-2 and RNP-1 sequences assemble an RNA-binding surface in which aromatic residues are proposed to be required for the contact with RNA (42). The decrease in RNA binding after substitution of the phenylalanine 118 by an alanine in the RNP-2 of RRM2 (hLa-F118A, Fig. 2A) is in line with this method of binding.

The hLa mutants with abolished or reduced RNA binding activity (hLa-Δ2.0 and hLa-F118A, respectively) are able to move into the nucleolus. For this reason, RRM2-mediated binding of pre-tRNAs or other RNA species is not required for nucleolar entry but does not exclude a role for hLa as nucleolar import factor for precursor-tRNA, for example. However, we cannot rule out that hLa is associated with other RNA molecules via RRM1, RRM3, the NoLS, or yet unidentified RNA-binding surfaces. Furthermore, a minor contribution of the RRM2 for nuclear import of hLa is not excluded by our data and is conceivable, as a small increase in cytosolic GFP-hLa signals was observed with the corresponding hLa mutant proteins GFP-hLa-Δ2.0 and GFP-hLa-F118A (Fig. 3B). Therefore, RNA binding may contribute to nuclear import of hLa, but to our knowledge only one publication has shown that La might be involved in the nuclear import of RNA molecules (72).

In addition to the importance of RRM2 in nucleolar trafficking, we found that hLa contains a nucleolar localization signal mediating the entry of hLa into the nucleolus (Fig. 4). Notably, this signal was functional in a heterologous context. The presence of the NoLS clearly argues for an important function of La in the nucleolus, leading to the question why La is not concentrated in this compartment as it was observed for the fusion protein hLa-NoLS-PTB-GFP. An explanation comes from the analysis of the RNA-binding defective hLa mutants. These proteins accumulate in the nucleolus, strongly suggesting that specific RNA binding of hLa is required to exit this compartment. This could also apply to hLa-NoLS-PTB-GFP, as this fusion protein may not leave the nucleolus because of the absence of specific RNA interaction partners.

We believe that our data are in line with following assumptions: (i) hLa contains an NoLS for nucleolar import of hLa/pre-tRNPs, for example. (ii) hLa supports processing of pre-tRNAs in the nucleolus. (iii) The RRM2-mediated formation of hLa-RNPs leads to nucleolar exit. But how is the hLa-dependent processing of precursors of U3 snoRNA or U6 snRNA in the nucleoplasm mediated, if hLa is transported to the nucleolus? One potential explanation would be that hLa-RNPs exported from the nucleolus dissociate in the nucleoplasm and that a certain amount of free hLa is able to bind U3 snoRNA or U6 snRNA precursors. Another explanation might be that as soon as hLa is released from a potential factor tethering hLa in the nucleolus, hLa freely diffuses through the nucleus and interacts with U3 snoRNA or U6 snRNA precursors. At this time we cannot discriminate between these two options, but future work will be focused on the identification of cellular factors interacting with the hLa-NoLS.

The analysis of the protein composition of the nucleolus did not lead to the identification of a general nucleolar targeting signal (41, 73, 74). By looking for sequential similarities between the hLa-NoLS described here and other published sequences mediating nucleolar localization of different human proteins (45–51, 75–78), we aligned those sequences with the hLa-NoLS (Fig. 4C). In accordance with a previous report (79), the alignment revealed a (R/K)/R/K/X/K/R motif appearing as single or multiple copies in a region enriched with basic and hydrophilic amino acids. As predicted, these residues tend to be externally oriented with respect to their structural position, thereby allowing for the interaction of the hLa-NoLS with other molecules, which direct or tether hLa in the nucleolus. The data shown here imply that RNP-2 of RRM2 (amino acids 113–118) and the hLa-NoLS (amino acids 323 to 354) are molecular interfaces for the interaction with other molecules and are key features for subnuclear localization of hLa.

At first glance, the localization of GFP-hLa-Δ6.3 may appear inconsistent with published data concerning the nuclear retention element. Sequence elements required for nuclear retention of hLa were mapped between the amino acids 266–269, 312–337 (58), and 316–332 (59). With our mutant GFP-hLa-Δ6.3 (amino acids 323–354), the latter of these sequence elements was partially removed, which caused no increase in cytoplasm-
mic GFP signals. Therefore, it seems likely that additional sequences exist that play a role in nuclear retention of hLa or that a nuclear export element was deleted. However, these studies indicate that several important elements are located within the C-terminal part of hLa.

In conclusion, both the NoLS and the association with RNA holds the balance between nucleolar and nucleoplasmic localization of hLa. Accordingly, hLa enters the nucleus via its NoLS and may serve as a nucleolar import factor for pre-tRNAs, whereas RNA binding via RRM2 appears to mediate nucleolar export. Yet we cannot discriminate whether hLa exits the nucleus as an RNP (e.g., associated with pre-tRNA) or if its ability to interact with precursor U3 snoRNA localized in the nucleoplasm mediates this trafficking. We consider a model wherein the RNA binding activity and the nucleolar localization signal of human La guaranties the dynamic trafficking essential to accomplish its functions in different subnuclear compartments.

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