ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 314 (2003) 591–600

www.elsevier.com/locate/yviro

Functional analysis of the interaction of the human immunodeficiency virus type 1 Rev nuclear export signal with its cofactors

A. Kiss,[1] L. Li,[1] T. Gettemeier, and L.K. Venkatesh*

Institute for Molecular Virology, Saint Louis University School of Medicine, 3681 Park Avenue, Saint Louis, MO 63108, USA

Received 7 March 2003; returned to author for revision 25 April 2003; accepted 23 June 2003

Abstract

Human immunodeficiency virus type 1 (HIV-1) Rev-mediated nuclear export of viral RNAs involves the interaction of its leucine-rich nuclear export sequence (NES) with nuclear cofactors. In yeast two-hybrid screens of a human lymph node derived cDNA expression library, we identified the human nucleoporin Nup98 as a highly specific and potent interactor of the Rev NES. Using an extensive panel of nuclear export positive and negative mutants of the functionally homologous NESs of the HIV-1 Rev, human T cell leukemia virus type 1 (HTLV-1) Rex, and equine infectious anemia virus (EIAV) Rev proteins, physiologically significant interaction of hNup98 with the various NESs was demonstrated. Missense mutations in the yeast nuclear export factor Crm1p that abrogated Rev NES interaction with the XXFG repeat-containing nucleoporin, Rab/hRIP, had minimal effects on the interaction of GLFG repeat-containing hNup98. Functional analysis of Nup98 domains required for nuclear localization demonstrated that the entire ORF was required for efficient incorporation into the nuclear envelope. A putative nuclear localization signal was identified downstream of the GLFG repeat region. Whereas overexpression of both full-length Nup98 and the amino-terminal GLFG repeat region, but not the unique carboxy-terminal region, induced significant suppression of HIV unspliced RNA export, lower levels of exogenous Nup98 expression resulted in a relatively modest increase in unspliced RNA export. These results suggest a physiological role for hNup98 in modulating Rev-dependent RNA export during HIV infection.

Keywords: HIV-1 Rev; Nup98; Rab; REBP; CRM1; Cofactor; RNA export

Introduction

The transport of genomic and partially spliced HIV mRNAs containing the *cis*-acting Rev response element (RRE) from their sites of synthesis within the nucleus to the cytoplasm is stringently dependent on the energy-requiring nucleocytoplasmic shuttling action of the viral *rev* gene product, Rev (reviewed in Cullen, 1998, 2000; Pollard and Malim, 1998). Bound to the highly structured RRE (Malim et al., 1989) in a multimeric state via its amino-terminal RNA binding domain (RBD), Rev mediates the transport of human immunodeficiency virus (HIV) RNA cargoes by the interaction of its carboxy-terminally located leucine-rich nuclear export signal (NES) (Malim et al., 1991; Venkatesh and Chinnadurai, 1990) with multiprotein export complexes in the nucleoplasm and nuclear envelope (Pollard and Malim, 1998, and references therein).

The Rev-mediated pathway of RNA transport (Fischer et al., 1995; Malim and Cullen, 1993) appears to be distinct from that utilized by cellular mRNAs (Bogerd et al., 1998; Cullen, 2000; Reed and Hurt, 2002) and involves the interaction of its NES with nucleoplasmic and nuclear pore resident (nucleoporin) cofactors (Bogerd et al., 1995; Fornerod et al., 1997a; Fritz et al., 1995; Fritz and Green, 1996; Neville et al., 1997; Stade et al., 1997; Stutz et al., 1995, 1996; Venkatesh et al., 2003). The nucleoporins described initially as cofactors for the Rev NES were the XXFG-repeat containing nucleoporin Rab/hRIP and a related yeast nucleoporin, Rip1p (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995). Rab/hRIP was also shown to interact with the functionally homologous NES regions (Hope et al., 1991; Mancuso et al., 1994) of the Rex protein of human T

* Corresponding author. Fax: +1-314-577-8406.
E-mail address: venkat@slu.edu (L.K. Venkatesh).
[1] These authors contributed equally to this work.

0042-6822/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0042-6822(03)00531-2

cell leukemia virus type 1 (HTLV-1) and the Rev protein of equine infectious anemia virus (EIAV). Subsequently, interaction of the leucine-rich Rev NES with several different nucleoporins was demonstrated (Fritz and Green, 1996; Neville et al., 1997; Stutz et al., 1996), implicating the involvement of the FG-repeat regions in such interactions. Genetic analyses in yeast have demonstrated the requirement of the homolog of the human Rev NES export factor, CRM1 (Fornerod et al., 1997b; Stade et al., 1997), for nucleoporin interaction (Neville et al., 1997) and in vitro biochemical interaction assays have suggested that CRM1 may mediate RanGTP-dependent interactions of the Rev NES with the FG-repeat regions of nucleoporins (Askjaer et al., 1999; Fornerod et al., 1997b; Floer and Blobel, 1999). Although certain members of the three different classes of FG-repeat nucleoporins, XXFG, FXFG, and GLFG, have been identified as putative Rev NES cofactors (Fritz and Green, 1996; Stutz et al., 1995, 1996) in the export pathway, not all nucleoporins interact with the Rev NES. The basis for nucleoporin selectivity and the physiological relevance of the observed nucleoporin interactions of the Rev NES and their contribution in vivo to nuclear export of RRE-containing HIV RNA remains uncertain.

We report here the results of an extensive series of experiments that identify human Nup98 as a particularly potent interactor of the Rev NES and a physiologically relevant component of Rev-dependent RNA transport. Human Nup98 is produced by two alternatively spliced transcripts, one encoding a 98-kDa protein that is autoproteolytically cleaved at F863 into an amino-terminal 90-kDa fragment; the other transcript produces an ~186-kDa Nup98–Nup96 precursor protein that is processed at F863 to yield an additional 96-kDa carboxy-terminal fragment (Fontoura et al., 1999; Rosenblum and Blobel, 1999). hNup98 has been localized to the nucleoplasmic face of the nuclear pore complex (NPC), where it is associated with the nuclear basket, the nucleolar periphery, and the intranuclear network of translocated promoter region (TPR) filaments (Fontoura et al., 2001). The primarily intranuclear location of hNup98 makes it an attractive nucleoporin target for Rev NES interaction during the initial phase of RRE RNA export during HIV infection.

Results

Genetic screen for Rev NES-interacting human cofactors

To screen for proteins interacting with the Rev NES, we used a bait protein, comprising the DNA binding domain of Gal4, Gal4(1–147), fused with HIV-1 Rev residues 59–116 encompassing the leucine-rich NES, expressed from the plasmid pMA424 (Venkatesh et al., 2003). The GAL4 activation domain-tagged cDNA expression library was derived from human lymph node which contains all the physiological cell targets (dendritic cells, macrophages, and $CD4^+$ T cells) for HIV infection. Three clones encoding the amino-terminal ~500 residues of human Nup98, which interacted strongly and specifically with the Rev NES, were obtained. Full-length Nup98 (920 aa) was cloned into the activation domain vector GAD10 as a carboxy-terminal fusion with the GAL4 activation domain (GAL4 AD) and used subsequently in further interaction analyses along with a VP16-activation domain-tagged Rab/hRIP fusion gene product (Bogerd et al., 1995) for simultaneous comparison of the patterns of interaction. As shown in Table 1, Nup98 failed to interact with nonspecific baits such as human foamy virus (HFV) Bel1 residues 56–227 and HIV-1 Tat residues 48–101, but reacted strongly with both Rev 59–116 and full-length Rev (residues 2–116). When a panel of Rev NES mutants (Venkatesh et al., 1990, 2003; Venkatesh and Chinnadurai, 1990) was tested for Nup98 reactivity, functionally defective NES mutants Rev 59–73, Rev 59–116/78–79s, and Rev 59–116/81s failed to interact, whereas functionally positive NES mutants Rev 59–98, Rev 59–116/76–77s, and Rev 59–116/80s showed evidence of strong reactivity. A similar pattern of interactions was observed with the same panel of NES mutants in the full-length Rev 2–116 background (data not shown). In addition, Nup98 also interacted strongly with the functionally equivalent HTLV-1 Rex NES (but not with a functionally inactive mutant, ΔLSLD, thereof) and EIAV Rev NES regions. Simultaneous comparative analysis of Rab/hRIP interaction demonstrated subtle differences in the reactivity of certain mutants. For example, Rab/hRIP failed to interact with the functionally positive NES domains HIV-1 Rev 59–116/76–77s and EIAV Rev 2–66, whereas hNup98 reacted efficiently. These differences may account for the particularly

Table 1
Interaction level of Gal4(1–147)-tagged baits, based on *lac*Z activity, with Gal4 AD-tagged prey in yeast two-hybrid assays

Gal4(1–147)-tagged bait	Rab/hRIP (full-length) interaction	Nup98 (full-length) interaction	NES function in mammalian cells
pMA424	−	−	−
HFV Bel 56–227	−	−	−
HIV-1 Tat 48–101	−	−	−
HIV-1 Rev 59–116	++++	++++	++++
Rev 59–73	−	−	−
Rev 59–98	++	+++	+++
Rev 59–116/76–77s	−	+++	+++
Rev 59–116/78–79s	−	−	−
Rev 59–116/80s	+++	+++	++++
Rev 59–116/81s	−	−	−
Rev 2–116	++++	++++	++++
Rev 2–116/81s	−	−	−
EIAV Rev 2–66	−/+	++	+++
HTLV-1 Rex 51–110	++++	++++	++++
Rex 51–110/ΔLSLD	−	−	−

Note. −, none; −/+, +, weak; ++/+++, strong; ++++, very strong. Only β-galactosidase activities indicated in a given column are comparable as the reactivity of Rev with Nup98 is three- to fivefold greater than with Rab/hRIP.

Table 2
Average levels of *lac*Z activity from pooled colonies triply transformed with pMA424 Rev 59–116, plasmid expressing AD-tagged Rev interactor, or GAD-GH (negative control), and pSH18–34 (*lac*Z reporter plasmid; Neville et al., 1997) followed by selection on appropriate SD media

Yeast Strain	REBP-y	Nup98	Rab
W303	5.2	89.0	89.0
crm1-1	2.0	27.5	1.90
crm1–2	1.6	29.1	1.6

Note. *Lac*Z activity for each transformed strain induced by the Rev NES interactor is expressed relative to the GAD-GH induced level assigned a value of 1.0.

strong reactivity of Nup98 (three- to fivefold greater than Rab/hRIP) with the Rev NES observed in the yeast two-hybrid assay. Collectively, the detailed mutational analyses of NES–hNup98 interaction presented in Table 1 are strongly supportive of a physiological role for hNup98 in the nuclear export function of the HIV-1 Rev leucine-rich NES.

Analysis of Crm1p requirement for Rev NES–cofactor interaction

We next investigated the requirement of the yeast homolog of the human Rev NES export factor, CRM1, for interaction of the Rev NES with the kinesin-like nuclear cofactor REBP (Venkatesh et al., 2003) and the nucleoporin cofactors, Rab/hRIP (Bogerd et al., 1995; Fritz et al., 1995) and Nup98. For this purpose, we examined the reactivity of wt and functionally inactive Rev NESs with REBP as well as Rab/hRIP and Nup98 in the *Saccharomyces cerevisiae* strain W303 (expressing wt Crm1p) or in W303 strains carrying viable missense mutations in the Crm1 gene, *crm1-1* and *crm1-2* (Neville et al., 1997; Yan et al., 1998) in a two-hybrid protein interaction-based *lac*Z reporter assay (Table 2). Whereas the reactivity of Rab/hRIP with the Rev NES was drastically abrogated in the mutant strains *crm1-1* and *crm1-2* by approximately 50-fold as previously reported (Neville et al., 1997), we observed that the reactivity of REBP-y (the Rev NES-interacting carboxy-terminal 75 aa region of REBP) and hNup98 was only marginally affected (~threefold). Since Rev has been shown to be functional in RRE RNA transport in yeast and in view of the high level of conservation between the *S. cerevisiae* and human CRM1 proteins (~48% identity and ~57% similarity), these results suggest that Crm1p mutations that strongly affect Rev NES interaction of certain nucleoporins such as the XXFG repeat-containing Rab/hRIP have relatively modest effects on the interaction of other NES cofactors such as REBP and the GLFG repeat-containing Nup98. The marginal reduction in the levels of interaction of REBP-y and Nup98 due to missense *crm1* mutations in *S. cerevisiae* may be reflective of modest global inhibitory effects on gene expression due to diminished Crm1p function in *crm1-1* and *crm1-2* yeast or of the requirement of other regions of Crm1p (than those affected by the missense mutations) for efficient REBP and Nup98 interaction.

Analysis of domain function in hNup98

Whereas most FG-repeat containing nucleoporins are symmetrically located on either side of the NPC, the Nup98 protein is located asymmetrically, being confined to the midsection of the nuclear basket and to an intranuclear filamentous network extending from the NPC to the nucleolar periphery (Fontoura et al., 2001). Protein sequence analysis of hNup98, using the Genetics Computer Group (GCG, Wisconsin) software, revealed a number of interesting protein signatures. These include an amino-terminal ~500 residue FG-repeat region that contains 13 GXFG repeats (eight of the GLFG type) between aa 15 and 459, a threonine-rich region between residues 14 and 397, a putative nuclear localization signal PKKEER (at residue 509), and a carboxy-terminal region that is devoid of readily recognizable motifs. To investigate the role of various Nup98 motifs/domains in the Rev export function, we constructed influenza hemagglutinin (HA)-tagged fusion genes (in the mammalian expression vector pcDNA3-HA), encoding the amino-terminal GLFG repeat-containing or the carboxy-terminal unique regions, inclusive of or without the putative NLS, and tested their properties with respect to subcellular localization and effects on RRE RNA transport. The HA-tagged constructs were designated Nup98N (aa 2–499), Nup98NA (aa 2–534, containing the putative NLS), Nup98C (aa 526–920), and Nup98AC (aa 493–920, putative NLS-containing).

The pcDNA3-HA-based Nup98 gene constructs were transfected into 293T cells and examined for levels of protein expression 48 h after transfection. As apparent from Fig. 1, expression of wt and the various mutant Nup98 proteins (Nup98-N, -NA, -C, -AC), including the GLFG-repeat and unique regions, was readily detectable in whole cell extracts, suggesting that the proteins were expressed at comparable levels. A single C-terminal protein lacking the putative NLS (Nup98C) was expressed at lower levels; overexpression of this protein segment was found to be significantly toxic in 293T and HeLa cells. These results demonstrate that the HA-tagged wt and mutant Nup98 proteins were expressed in transfected cells in a relatively stable manner.

Subcellular fractionation of wt and mutant Nup98 proteins in HeLa cells

We next examined the distribution of wt and mutant hNup98 proteins in HeLa cells by biochemical fractionation studies. Transfected HeLa, rather than 293T cells, were utilized for this purpose, since 293T cell nuclei are relatively fragile and refractory to handling for subcellular frac-

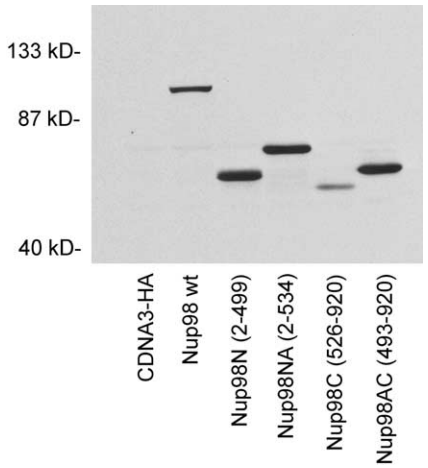

Fig. 1. Expression of wt and mutant Nup98 proteins. Subconfluent monolayers of 293T cells were transfected with 3 μg of the parental expression vector pcDNA3-HA or pcDNA3-HA-derived plasmids expressing wt and mutant hNup98 genes. Forty-eight hours posttransfection, equivalent amounts of cell lysates in 2× Laemmli's dissociation buffer were fractionated on SDS–10% polyacrylamide gels and analyzed for HA-tagged protein expression by Western blot analysis using the rabbit polyclonal anti-HA antibody Y-11. Protein molecular wt markers are indicated on the left.

tionation procedures. Initial experiments demonstrated very low levels of wt and mutant hNup98 expression in HeLa cells upon plasmid transfection. To enhance the levels of Nup98 protein expression and to facilitate their detection, a plasmid pCMV-Tag expressing the SV40 antigen was cotransfected to drive low-level replication of the pcDNA3-HA expression vector. Forty-eight hours after transfection, cells were fractionated into nuclear and cytoplasmic extracts as described under Materials and methods and probed for HA-tagged protein expression (Fig. 2). wt Nup98 was found to partition primarily (>70%) in the nuclear fraction. A higher level of the Nup98 NA protein (~65%), containing the putative NLS, could be found in the nuclear fraction compared to Nup98 N (~30%), which lacks the NLS. The carboxy-terminal fragment Nup98 AC was more efficiently localized in the nuclear fraction compared to Nup98 C, which lacks the putative NLS and partitions primarily with the cytosolic fraction (>80%). The GLFG repeat region is thus inherently capable of localizing to the nucleus; however, nuclear import of both the GLFG (N) and the C-terminal (C) Nup98 protein segments is significantly enhanced by the presence of the putative NLS region, as observed with the NA and AC proteins. The putative NLS of Nup98 displays the characteristics of an authentic NLS and may facilitate nuclear import of Nup98.

Subcellular localization of Nup98 proteins

The subcellular fractionation properties of the various Nup98 proteins were further substantiated by subcellular localization studies of HA-tagged wt and mutant Nup98 protein expression in transfected HeLa cells by indirect immunofluorescence, using anti-HA antibody and confocal laser scanning microscopy (Fig. 3). As expected from previous studies, wt Nup98 protein was expressed primarily in the nuclear envelope of transfected cells expressing low levels of the protein; however, cells expressing higher levels of the protein exhibited patchy nucleolar surface staining in addition to the nuclear envelope staining. Less frequently, cells expressing diffuse nucleoplasmic staining alone could be observed. The GLFG-repeat region proteins, NA and N (devoid of the NLS), exhibited both nuclear and cytoplasmic staining but lost the ability to localize efficiently in the nuclear envelope, as apparent from a lack of readily detectable nuclear rim staining; nevertheless, pronounced patchy nucleolar surface (peripheral) distribution was observed. In some nuclei, particularly with the NA protein, prominent subnuclear speckles of uncertain origin could also be observed. The carboxy-terminal AC and C fragments exhibited primarily cytoplasmic distribution with a more pronounced accumulation of the AC protein in the nucleoplasm. Both these proteins were, however, excluded from the nucleolar region and, unlike wt Nup98, did not exhibit readily discernible nuclear rim staining. These results suggest that in addition to the need for Nup98 processing near the carboxy-terminus for efficient nuclear envelope localization, as previously reported (Fontoura et al., 1999, 2001; Griffis et al., 2002), cooperative interactions between the amino-terminal GLFG-repeat region and the carboxy-terminal unique region are required for efficient association of Nup98 with the nuclear envelope.

Effects of wt and mutant Nup98 protein overexpression on Rev function in 293T and HeLa cells

To determine the contribution of various Nup98 domains to HIV RNA transport, we next examined the effects of

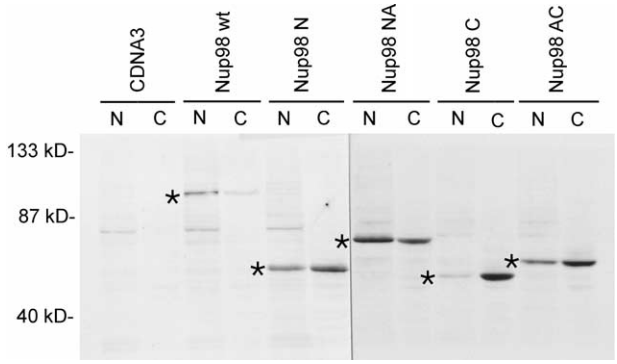

Fig. 2. Subcellular fractionation of wt and mutant Nup98 proteins expressed in HeLa cells. Subconfluent monolayers of HeLa cells were transfected with 3 μg of pCMV-Tag and 6 μg each of pcDNA3-HA vectors expressing Nup98 wt, N, NA, AC, and C proteins. Nuclear and cytosolic fractions were dissociated in equivalent amounts of 2× Laemmli's dissociation buffer for analysis of HA-tagged protein expression by SDS–PAGE and Western blot analysis, using rabbit polyclonal anti-HA Y-11 antibody. The migration of protein molecular wt markers is indicated on the left.

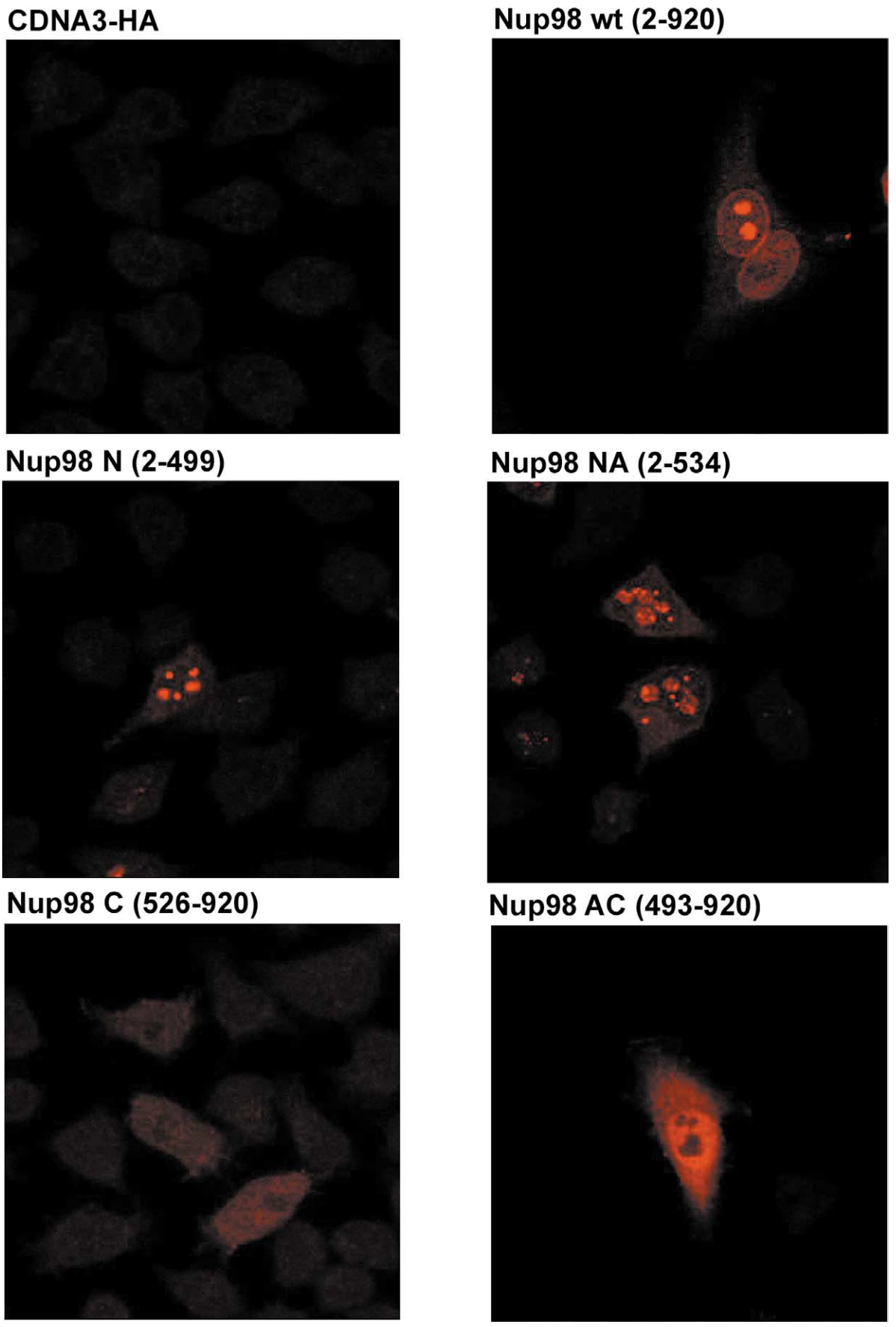

Fig. 3. Indirect immunofluorescence analysis of wt and mutant Nup98 expression. HeLa cells on glass coverslips were transfected with 1.5 μg of pcDNA3-HA or its derivative plasmids expressing Nup98 wt, N, NA, C, and AC proteins. Forty-eight hours after transfection, cells were permeabilized, fixed, and stained for detection of HA-tagged protein expression by laser scanning confocal microscopy as described under Materials and methods.

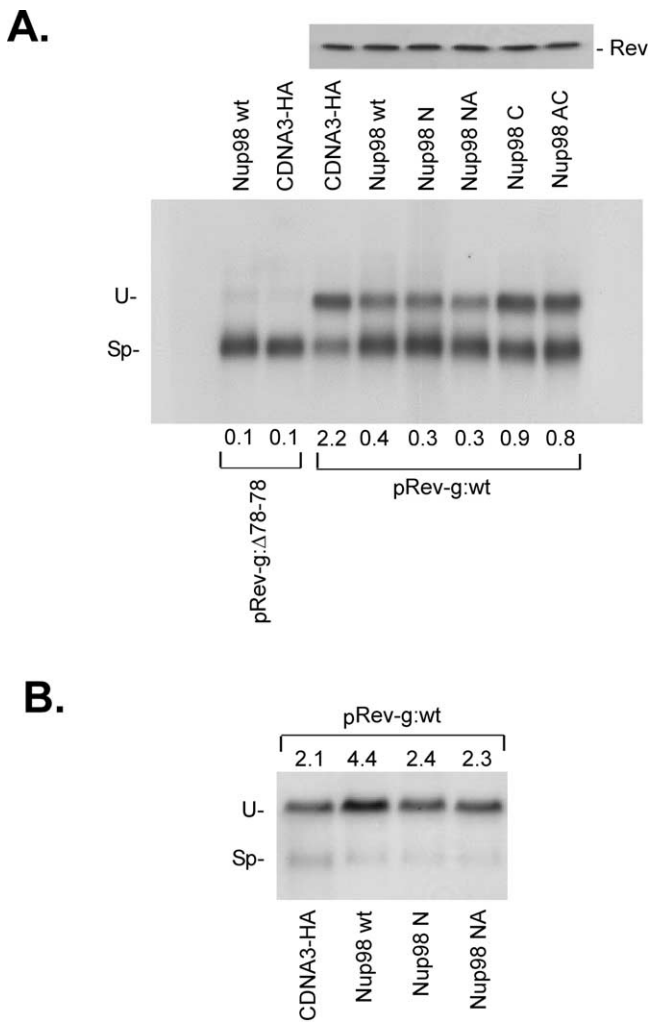

Fig. 4. Effect of wt and mutant Nup98 expression on Rev function. (A, Bottom) Approximately 1.5×10^6 293T cells were transfected with 1 μg of pRev-g:wt or pRev-g:Δ78–79 and 5 μg of the pcDNA3-HA or Nup98 expression plasmid as indicated. Forty-eight hours after transfection, cytoplasmic RNA was isolated and probed for levels of RRE-containing, Rev-dependent unspliced (U) and Rev-independent spliced (Sp) RNA expression as described under Materials and methods. (Top) Expression of Rev protein in a constant fraction of 293T cells harvested from each of the above transfections was characterized by Western blot analysis, using a polyclonal rabbit antibody to Rev residues 2–37. (B) HeLa cells were transfected with 0.5 μg of pRev-g:wt and 5.0 μg of either pcDNA3-HA or various Nup98 expression plasmids as indicated and analyzed for unspliced and spliced mRNA expression in cytoplasmic RNA samples.

overexpression of wt and mutant Nup98 proteins on Rev-mediated RRE RNA transport in human cells. For this purpose, we constructed HIV subgenomic RNA expression vectors that contain an HIV-1 *env* region segment capable of expressing biexonic wt (pRev-g:wt) or inactive NES mutant (pRev-g:Δ78–79s) *rev* genes. Transfection of these plasmids into 293T cells (that are highly efficient in mediating Rev-dependent RRE RNA transport) resulted in the expression of a spliced, ~700 bp (Rev-independent) and unspliced RRE-containing ~1500-bp RNA (Rev-dependent) species. In pRev-g:wt-transfected 293T cells (Fig. 4A, bottom), unspliced (U) and spliced (Sp) RNAs were readily detectable in cytoplasmic RNA preparations in an ~3:1 ratio (lane CDNA3-HA). As expected, in pRev-g:Δ78–79-transfected cells, only the spliced RNA species was readily detectable. In different experiments, coexpression of wt Nup98 or the GLFG-region N and NA proteins resulted in two- to fourfold suppression of the expression of unspliced RRE-containing mRNA species with a concomitant increase (about three- to fourfold) in the level of spliced mRNA in pRev-g:wt-transfected 293T cells. In contrast, both versions of the unique carboxy-terminal region of Nup98, AC and C, failed to suppress cytoplasmic expression of unspliced mRNA expression, which remained at the same level as in pRev-g:wt (lane CDNA3-HA) transfection. Analysis of cells in different transfections for levels of Rev expression demonstrated that Rev expression was comparable in all transfections (Fig 4A, top); thus, variations in the levels of Rev expressed from the spliced mRNAs in the various transfections cannot account for the varied levels of Rev-dependent unspliced mRNA expression in the cytoplasm. Interestingly, nuclear export of the spliced Rev-encoding mRNA species from pRev-g:wt was unaffected by overexpression of wt Nup98 or its mutants, being comparable to that expressed from the pRev-g:Δ78–79 vector.

These results clearly demonstrate that overexpression of the Rev NES-interacting GLFG-repeat region of Nup98 as well as wt Nup98 in 293T cells (that support pCDNA3 expression vector replication) results in pronounced suppression of Rev-dependent unspliced mRNA export. In contrast, expression of exogenous wt Nup98 but not the GLFG-repeat containing N or NA proteins at lower levels in HeLa (Fig. 4B) or in 293 (data not shown) cells, wherein pCDNA3 cannot replicate, consistently induced a small enhancement (~twofold) in the level of unspliced RNA export from cotransfected pRev-g:wt plasmid.

Discussion

The initial characterization of Rab/hRIP as a nucleoporin cofactor for the leucine-rich nuclear export signals of functionally homologous retroviral Rev proteins in yeast two-hybrid screens of human cDNA expression libraries led to the subsequent identification of a number of nucleoporins of the XXFG, FXFG, and GLFG classes, including Nup98 (Fritz and Green, 1996; Stutz et al., 1996), in random analyses of known FG-repeat containing nuclear pore proteins, as potential targets for Rev NES interaction during nucleocytoplasmic transport. Notably, however, certain yeast nuclear pore proteins such as the FXFG nucleoporins Nup1p and Nup116p as well as the GLFG nucleoporin Nup116p did not interact with the Rev NES in the yeast two-hybrid assay (Stutz et al., 1995). Therefore, the physiological relevance of and the biochemical mechanism(s) for the observed nucleoporin interactions of the Rev NES and their contribution to the RNA export function of Rev during

HIV infection have yet to be resolved unambiguously. Stepwise interactions of the Rev NES with the FG-repeat regions of a selective group of nucleoporins by a ratcheting mechanism during transit through the nuclear pore complex likely mediates nuclear egress of the RRE RNA-bound, Rev-associated multiprotein export complex (exportasome).

Results from our detailed analyses of the Rev NES–hNup98 interaction offer new insights into the properties of this intriguing nucleoporin and argue persuasively in support of a physiological role for Nup98 in Rev-mediated nucleocytoplasmic transport of RRE-containing HIV RNAs, as previously proposed (Zolotukhin and Felber, 1999). hNup98 was isolated as the sole and a particularly avid nucleoporin interactor of the Rev NES from a cDNA expression library derived from human lymph node, which contains all the relevant cellular targets (macrophages, dendritic cells, and $CD4^+$ T lymphocytes) for HIV infection in humans. A remarkably precise correlation was observed between the ability of the functionally homologous HIV-1 Rev, HTLV-1 Rex, and EIAV Rev proteins, as well as functionally positive and negative NES mutants thereof, to interact with hNup98 and their reported nuclear export capabilities. This may account for the very strong reactivity of hNup98, three- to fivefold greater than Rab/hRIP, with the Rev NES in yeast protein interaction assays. Thus, an initial high-affinity interaction with Nup98 may target the Rev exportasome to the inner NPC; subsequently, a series of variable affinity interactions (including relatively lower affinity interactions such as with Rab/hRIP) may serve to propel the export complex through the NPC. Interestingly, recent studies suggest that Nup98 is a mobile nucleoporin, moving between the nuclear interior and the NPC as well as between the nucleus and the cytoplasm (Griffis et al., 2002); this observation raises the possibility that the Rev–Nup98 complex alone may display potential, albeit limited, for RRE RNA export. However, our recent studies indicate that a Rev 1–116 (M10)–Nup98 fusion protein is incapable of promoting RRE RNA export under conditions where a Rev 1–116 (M10)–CRM1 fusion protein mediates efficient nuclear export of such RNAs (L. Li and L.K. Venkatesh, unpublished observation).

Despite the high degree of homology (~50%) in the amino acid sequences of the CRM1 proteins of *S. cerevisiae* and man, we observed that missense *crm1* mutations in yeast that strongly abrogated the Rab/hRIP–Rev NES interaction effected only a marginal reduction in the extent of Rev interaction with Nup98 or the kinesin-like cofactor REBP. Explanations for this observation include possibilities that the Rev–Nup98 interaction is direct, that regions of yeast Crm1p other than those affected by the *crm1* mutations participate in Nup98 interaction, or that distinct cofactors mediate the Nup98–Rev NES interaction. It has been argued, based on observations of the in vitro requirements of cofactors for the reactivity of the Rev NES with the FXFG protein Nup42, that the Rev NES-RanGTP-CRM1 ternary complex facilitates Nup42 interaction (Floer and Blobel, 1999). Therefore, the interaction of the GLFG-repeat nucleoporin Nup98, unlike that of the XXFG-repeat containing Rab/hRIP, with the Rev NES may also involve the coordinate action of other cofactors in the Rev multiprotein export complex. Interestingly, a recent study suggests that the accumulation of Rev-bound RRE RNAs at the NPC is not inhibited by leptomycin B (Cmarko et al., 2002), an inhibitor of the Rev NES–CRM1 interaction; this may be reflective of the potential for CRM1-independent association of the Rev-bound RRE RNA cargo with inner NPC components, such as Nup98, during the initial phase of nuclear export.

Our initial mutational analyses of the role of various hNup98 domains on subcellular localization of this nucleoporin in HeLa cells (when expressed from a nonreplicating plasmid) and modulation of Rev function in 293T and HeLa cells have revealed a number of interesting features. Nup98 has been previously reported to be localized to the intranuclear ring basket of the nuclear pore complex as well as in the nucleolar periphery. While the mechanism of Nup98 association with these structures remains obscure, autoproteolytic processing of Nup98 from the Nup98–Nup96 precursor between residues F863 and S864 is an essential prerequisite for localization of Nup98 to the nuclear envelope (Fontoura et al., 1999, 2001). At low levels of expression, Nup98 localized primarily at the nuclear envelope; at higher levels of expression, diffuse as well as speckled nucleoplasmic and patchy nucleolar fluorescence were readily discernible. The GLFG repeat region, independently as well as in conjunction with the putative NLS, appears sufficient for localization in the nucleoplasm (often in speckles) and patchy areas on the nucleolar surface but not for incorporation into the nuclear envelope. Thus whereas the primary determinant of nuclear envelope localization may reside in the unique carboxy-terminal region, in the region of autoproteolytic processing (Fontoura et al., 1999, 2001; Griffis et al., 2002), the expression of this domain (as apparent from the localization of the C and AC proteins which contain the region of autoproteolyic processing) by itself appears insufficient to induce nuclear rim (envelope) staining, despite significant nuclear accumulation. Thus, our results indicate that the integrity of the entire Nup98 ORF is required for nuclear envelope localization. Consistent with the requirement of the GLFG-repeat region for Rev NES interaction, we observed pronounced dominant negative inhibition of unspliced mRNA expression upon overexpression of this segment in 293T cells. Since the GLFG region cannot localize at the nuclear envelope, this inhibition is probably reflective of intranuclear sequestration of Rev–RRE RNA complexes at subnuclear sites by inappropriately localized GLFG region (or wt Nup98) protein upon high-level overexpression. In contrast, the carboxy-terminal unique region failed to suppress RRE RNA transport despite a significant level of nuclear expression. Whether this region plays an important role in Rev transport, other than ensuring appropriate Nup98 localization, remains to be de-

termined. Interestingly, when exogenous wt Nup98 was expressed at lower levels, as in HeLa cells, significant increase of Rev-dependent RRE RNA export was detectable. A likely explanation for the discrepancy in the observed effects of exogenously introduced wt Nup98 on Rev-dependent unspliced RNA export in 293T (which supports expression vector replication) versus HeLa (wherein the expression vector cannot replicate) cells is the propensity of highly overexpressed exogenous Nup98 (in 293T cells) to localize predominantly in intranuclear, and perhaps inappropriate, structures rather than at the nuclear envelope; in contrast, lower level overexpression of wt Nup98, as in HeLa cells, results in primarily nuclear envelope localization of the exogenous protein. Another intriguing observation is the failure of wt as well as FG-repeat containing mutant Nup98 proteins to suppress spliced (Rev-expressing) RNA export in 293T cells in these experiments despite the demonstrated involvement of Nup98 in export of spliced cellular mRNAs through the TAP-p15 pathway (Reed and Hurt, 2002). The role of the Nup98-interacting Nup96 protein (Fontoura et al., 1999), the proteolytically derived carboxy-terminal fragment of the 186-kDa Nup98–96 precursor protein, in modulating Nup98 localization and in facilitating RRE RNA transport awaits resolution.

The localization of hNup98 to subnuclear structures and the innerface of the NPC makes it a plausible target for Rev NES interaction during the initial phase of RRE RNA export. It has been recently demonstrated that increased Nup98 and Nup96 expression relieves vesicular stomatitis virus M protein-mediated inhibition of mRNA export (Enninga et al., 2002). Whether similar mechanisms are operational during HIV infection for the facilitated nuclear export of RRE-containing RNAs remains to be investigated.

Materials and methods

Plasmids, yeast, and bacterial strains

The bait expression plasmid pMA-Rev:59–116, encoding a fusion of GAL4(1–147) with Rev residues 59–116, used in the yeast two-hybrid protein interaction trap, as well as other pMA 424-based constructs encoding functionally positive and negative mutant NESs of various retroviral Rev proteins, have been described (Venkatesh et al., 1990, 2003; Venkatesh and Chinnadurai, 1990). The human lymph node derived activation domain-tagged cDNA expression library constructed in the vector pGAD10 was purchased from Clontech (No. HL4023AB). pGAD-Nup98 was constructed by cloning PCR-derived full-length hNup98 ORF DNA (920 aa) as an *Xho*I-*Eco*RI fragment downstream of the GAL4 activation domain II in the plasmid GAD10.

The plasmid pCMV-Nup98 was constructed by cloning a PCR-derived *Bam*HI-*Eco*RI DNA fragment comprising residues 2–920 of the hNup98 ORF downstream of the influenza hemagglutinin epitope in the plasmid pcDNA3-HA. hNup98 ORF regions corresponding to amino acids 2–499 (N), 2–534 (NA), 493–920 (AC), and 526–920 (C) were similarly cloned as HA-tagged proteins to yield plasmids pCMV-Nup98N, -Nup98NA, -Nup98AC, and -Nup98C. The plasmids pRev-g:wt and pRev-g:Δ78–79 express a subgenomic HIV-1 *env* region segment (derived from pgREV; Malim et al., 1989) encompassing the biexonic *rev* gene or its functionally inactive NES mutant *rev*Δ78–79s (M10; Malim et al., 1991), respectively, and inclusive of the *tat/rev* 5′ and 3′ splice sites as well as the intronic RRE, under the control of the CMV-IE promoter and the IL-2 poly(A) addition signals. The HIV-1 segment utilized for these genomic Rev-expressing constructs was a *Sal*I-*Xho*I fragment (HXB-3 nt 5367–8475; Malim et al., 1989) carrying deletions of the *Sal*I-*Mst*II and *Kpn*I-*Bgl*II regions in pgREV.

The yeast indicator strain *S. cerevisiae* GGY1::171, containing an integrated copy of the bait–prey protein interaction-dependent inducible *lac*Z gene, and *Escherichia coli* strain MH4 (*leuB*), used for recovery of library plasmids encoding Rev NES-interacting cDNA fusion gene products have been described (Fields and Song, 1989; Venkatesh et al., 2003). *S. cerevisiae* strains W303 (*crm1 wt*) as well as *crm1-1* and *crm1-2* (missense mutants) have been described (Neville et al., 1997; Yan et al., 1998).

Genetic screening of human lymph node derived cDNA expression library

Approximately 2×10^6 his^+leu^+ yeast cotransformant colonies derived from simultaneous transformation of competent GGY::171 cells with pMA-Rev:59–116 and human lymph node derived cDNA expression library plasmid DNA were screened for induction of *lac*Z activity as described (Venkatesh et al., 2003). Of several colonies that turned blue, only three yielded library plasmids that encoded proteins interacting specifically with GAL4(1–147)-Rev:59–116, as determined by retesting for protein interaction-dependent induction of β-gal activity in GGY1::171. All three plasmids were found to contain cDNA inserts of identical size (~1500 bp) and sequence corresponding to the amino-terminal GLFG-repeat region of hNup98.

Western blot analysis of protein expression

For determination of the steady-state levels of expression of the wt and mutant Nup98 proteins, approximately 1.5×10^6 293T cells were transfected with 3 μg of each pcDNA3 vector expressing HA-tagged wt or mutant Nup98 using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals). Forty-eight hours posttransfection, cells were washed with phosphate-buffered saline (PBS) and lysed in 2× Laemmli's protein dissociation buffer. Equal volumes of extracts were fractionated by SDS–PAGE (12% polyacrylamide) and transferred to 0.45 μm nitrocellulose for 1 h at 100 V. The membrane was incubated in blocking

buffer (1% casein in PBS) for 1 h at room temperature and then treated sequentially with a 1:500 dilution of rabbit polyclonal anti-HA antibody Y-11 (Santa Cruz Biotechnology) and a 1:2000 dilution of horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG (Amersham). HA antibody-bound proteins on the membrane were detected using HRP-reactive chemiluminescent substrates supplied in the ECL detection reagents system (Amersham Pharmacia Biotech).

To study the distribution of wt and mutant Nup98 proteins in nuclear and cytoplasmic fractions of cells, subconfluent monolayers of HeLa cells in 75^2-cm flasks were transfected with 3 μg of pCMV-Tag (expressing the SV40 T antigen) and 6 μg of Nup98-expressing plasmid, using FuGENE 6 Transfection Reagent. Forty-eight hours posttransfection, cells were harvested and washed with ice-cold PBS. All subsequent operations were performed at 4°C. Washed cells were swollen in 400 μl of hypotonic buffer (10 mM Tris–HCl, pH 7.60, 10 mM KCl, 1.5 mM $MgCl_2$) containing a protease inhibitor cocktail (Complete Mini, Roche Molecular Biochemicals) and lysed by the addition of an equal volume of hypotonic buffer containing 0.2% NP-40. Lysed cells were centrifuged at 1000 *g* for 5 min to sediment nuclei; the cytosolic supernatant was clarified by centrifugation at 18,000 *g* for 10 min and mixed with an equal volume of 2× Laemmli's protein dissociation buffer. The nuclei were washed three times in hypotonic buffer and suspended in two cytoplasmic lysate volumes of 2× Laemmli's buffer. Protein samples were boiled and equal volumes of nuclear and cytoplasmic protein extracts were fractionated by SDS–PAGE (10% polyacrylamide) for detection of HA-tagged protein expression by Western blot analysis essentially as described above.

Expression of HIV-1 Rev in pRev-g:wt-transfected 293T cell lysates was detected with a rabbit polyclonal anti-Rev serum (raised against Rev residues 2–37) diluted 1:2000, using a Western blot protocol similar to that utilized for HA-tagged protein detection.

Indirect immunofluorescence

HeLa cells on glass coverslips were transfected with 1.5 μg of pcDNA3 plasmids expressing HA-tagged Nup98 wt, Nup98N, Nup98NA, Nup98AC, and Nup98C proteins. Forty-eight hours after transfection, cells were washed in PBS, fixed in 3.7% paraformaldehyde/PBS for 10 min at room temperature, and permeabilized with acetone at −20°C for 3 min. Permeabilized cells were blocked with 1% BSA in PBS and then incubated sequentially with rabbit polyclonal anti-HA antibody Y-11 (1:100 dilution) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes No. A-11011, diluted 1:250) for 30 min each at 30°C. After repeated washes in PBS, cells were mounted in Prolong Antifade mounting medium (Molecular Probes) and analyzed with a Bio-Rad 1024 laser scanning confocal microscope.

RNA analysis

For analysis of the levels of unspliced and spliced mRNAs in cytoplasmic RNA preparations, 293T cells were transfected with 1 μg of pRev-g:wt or pRev-g:Δ78–79 alone or in conjunction with 5 μg of a hNup98 expression plasmid. Forty-eight hours posttransfection, cytoplasmic RNA was isolated in the presence of rRNAsin RNAse Inhibitor (Promega). The RNA samples were treated with RQ1 DNAse (Promega) to remove contaminating DNA. Ten micrograms of RNA from each transfection was denatured, fractionated on a 1.4% formaldehyde-agarose gel, and transferred to 0.45-μm NYTRAN SuPerCharge nylon transfer membrane (Schleicher & Schuell) by capillary transfer with 20× SSC. RNA was immobilized by UV-crosslinking and the membrane was then probed with a denatured 422-bp *Bam*HI-*Xho*I (HIV-1 HXB-3 nt 8060–8475) digoxigenin-dUTP-labeled DNA probe. The hybridized membrane was then treated with alkaline phosphatase conjugated antidigoxigenin sheep Fab fragments for chemiluminescent detection of unspliced and spliced mRNAs in the presence of CSPD substrate, exactly as described in the DIG High Prime DNA Labeling and Detection Kit (Roche Molecular Biochemicals).

Acknowledgments

This study was supported by a grant from the National Institutes of Health, CA-73474, to L.K.V. The authors thank Julian Borrow (Massachusetts Institute of Technology, Cambridge), Jean-Pierre de Villartay (Hospital Necker, Paris), and Bryan Cullen (Duke University, Durham) for gifts of hNup98, CMV-SV40 T Ag, and Rab-VP16 plasmids, respectively, and Michael Rosbash (Brandeis University, Waltham) for W303 and *crm1* mutant yeast.

References

Askjaer, P., Bachi, A., Wilm, M., Bischoff, F.R., Weeks, D.L., Ogniewski, V., Ohno, M., Niehrs, C., Kjems, J., Mattaj, I.W., Fornerod, M., 1999. RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase. Mol. Cell. Biol. 19, 6276–6285.

Bogerd, H.P., Echarri, A., Ross, T.M., Cullen, B.R., 1998. Inhibition of human immunodeficiency virus Rev and human T-cell leukemia virus Rex function, but not Mason–Pfizer monkey virus constitutive transport element activity, by a mutant human nucleoporin targeted to CRM1. J. Virol. 72, 8627–8635.

Bogerd, H.P., Fridell, R.A., Madore, S., Cullen, B.R., 1995. Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. Cell 82, 485–494.

Cmarko, D., Bøe, S.-O., Scassellati, C., Szilvay, A.M., Davanger, S., Fu, X.-D., Haukenes, G., Kalland, K.-H., Fakan, S., 2002. Rev inhibition strongly affects intracellular distribution of human immunodeficiency virus type 1 RNAs. J. Virol. 76, 10473–10484.

Cullen, B.R., 1998. Retroviruses as model systems for the study of nuclear RNA export pathways. Virology 249, 203–210.

Cullen, B.R., 2000. Nuclear RNA export pathways. Mol. Cell. Biol. 20, 4181–4187.

Enninga, J., Levy, D.E., Blobel, G., Fontoura, B.M.A., 2002. Role of nucleoporin induction in releasing an mRNA nuclear export block. Science 295, 1523–1525.

Fields, S., Song, O.K., 1989. A novel genetic system to detect protein-protein interactions. Nature 340, 245–246.

Fischer, U., Huber, J., Boelens, W.B., Mattaj, I.W., Luhrmann, R., 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell 82, 475–483.

Floer, M., Blobel, G., 1999. Putative reaction intermediates in Crm1-mediated nuclear protein export. J. Biol. Chem. 274, 16279–16286.

Fontoura, B., Blobel, G., Matunis, M.J., 1999. A conserved biogenesis pathway for nucleoporins: proteolytic processing of a 186 kD precursor generates Nup98 and the novel nucleoporin, Nup96. J. Cell. Biol. 144, 1097–1112.

Fontoura, B., Dales, S., Blobel, G., Zhong, H., 2001. The nucleoporin Nup98 associates with the intranuclear filamentous protein network of TPR. Proc. Natl. Acad. Sci. USA 98, 3208–3213.

Fornerod, M., Ohno, M., Yoshida, M., Mattaj, I.W., 1997a. Crm1 is an export receptor for leucine-rich nuclear export signals. Cell 90, 1051–1060.

Fornerod, M., van Deursen, J., van Baal, S., Reynolds, A., Davis, D., Murti, K.G., Fransen, J., Grosveld, G., 1997b. The human homolog of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. EMBO J. 16, 807–816.

Fritz, C.C., Green, M.R., 1996. HIV Rev uses a conserved cellular protein export pathway for the nucleocytoplasmic transport of viral RNAs. Curr. Biol. 6, 848–854.

Fritz, C.C., Zapp, M.L., Green, M.G., 1995. A human nucleoporin-like protein that specifically interacts with HIV Rev. Nature 376, 530–533.

Griffis, E.R., Altan, N., Lippincort-Schwartz, J., Powers, M.A., 2002. Nup98 is a mobile nucleoporin with transcription-dependent dynamics. Mol. Biol. Cell 13, 1282–1297.

Hope, T.J., Bond, B.L., McDonald, D., Klein, N.P., Parslow, T.G., 1991. Effector domains of human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type 1 Rex are functionally interchangeable and share an essential peptide motif. J. Virol. 65, 6001–6007.

Malim, M.H., Cullen, B.R., 1993. Rev and the fate of pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes. Mol. Cell. Biol. 13, 6180–6189.

Malim, M.H., Hauber, J., Le, S.-Y., Maizel, J.V., Cullen, B.R., 1989. The HIV-1 *rev trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature 338, 254–257.

Malim, M.H., McCarn, D.F., Tiley, L.S., Cullen, B.R., 1991. Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. J. Virol. 65, 4248–4254.

Mancuso, V.A., Hope, T.J., Zhu, L., Derse, D., Philips, T., Parslow, T.G., 1994. Posttranscriptional effector domains in the Rev proteins of feline immunodeficiency virus and equine infectious anemia virus. J. Virol. 68, 1998–2001.

Neville, M., Stutz, F., Lee, L., Davis, L.I., Rosbash, M., 1997. The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. Curr. Biol. 7, 767–775.

Pollard, V.W., Malim, M.H., 1998. The HIV-1 Rev protein. Ann. Rev. Microbiol. 52, 491–532.

Reed, R., Hurt, E., 2002. A conserved mRNA export machinery coupled to pre-mRNA splicing. Cell 108, 523–531.

Rosenblum, J.S., Blobel, G., 1999. Autoproteolysis in nucleoporin biogenesis. Proc. Natl. Acad. Sci. USA 96, 11370–11375.

Stade, K., Ford, C.S., Guthrie, C., Weis, K., 1997. Exportin 1 (Crm1p) is an essential nuclear export factor. Cell 90, 1041–1050.

Stutz, F., Izaurralde, E., Mattaj, I.W., Rosbash, M., 1996. A role for nucleoporin FG repeat domains in export of human immunodeficiency virus type 1 Rev protein and RNA from the nucleus. Mol. Cell. Biol. 16, 7144–7150.

Stutz, F., Neville, M., Rosbash, M., 1995. Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 protein in yeast. Cell 82, 495–506.

Venkatesh, L.K., Chinnadurai, G., 1990. Mutants in a conserved region near the carboxy-terminus of HIV-1 Rev identify functionally important residues and exhibit a dominant negative phenotype. Virology 178, 327–330.

Venkatesh, L.K., Gettemeier, T., Chinnadurai, G., 2003. A nuclear kinesin-like protein interacts with and stimulates the activity of the leucine-rich nuclear export signal of the human immunodeficiency virus type 1 Rev protein. J. Virol. 77, 7236–7243.

Venkatesh, L.K., Mohammed, S., Chinnadurai, G., 1990. Functional domains of the HIV-1 rev gene required for *trans*-regulation and subcellular localization. Virology 176, 39–47.

Yan, C., Lee, L.H., Davis, L.I., 1998. Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. EMBO J. 17, 7416–7429.

Zolotukhin, A.S., Felber, B.K., 1999. Nucleoporins nup98 and nup214 participate in nuclear export of human immunodeficiency virus type 1 Rev. J. Virol. 73, 120–127.