Export and transport of tRNA are coupled to a multi-protein complex

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Vigilin is a ubiquitous multi heterogeneous nuclear ribonucleoprotein (hnRNP) K homologous (KH)-domain protein. Here we demonstrate that purified recombinant human vigilin binds tRNA molecules with high affinity, although with limited specificity. Nuclear microinjection experiments revealed for the first time that the immuno-affinity-purified nuclear vigilin core complex (VCC_N) as well as recombinant vigilin accelerate tRNA export from the nucleus in human cells. The nuclear tRNA receptor exportin-t is part of the VCC_N. Elongation factor (EF)-1α is enriched in VCC_N and its cytoplasmic counterpart VCC_C, whereas EF-1β, EF-1γ and EF-1δ are basically confined to the VCC_C. Our results suggest further that vigilin and exportin-t might interact during tRNA export, provide evidence that the channeled tRNA cycle is already initiated in the nucleus, and illustrate that intracellular tRNA trafficking is associated with discrete changes in the composition of cellular cytoplasmic multi-protein complexes containing tRNA.

Key words: elongation factor, human cells, KH domain, translation, vigilin.

INTRODUCTION

Recent investigations have shown that RNA export is coupled to protein import and the RNA species pass the nuclear envelope as ribonucleoprotein complexes (RNPs) [1,2]. These complexes leave the nucleus via nuclear pore complexes [3], which is an energy-dependent process mediated by specific saturable factors [4–6]. The small Ras-like GTPase RanGTP is the energy source in many of the characterized RNA export mechanisms [2,7,8]; however, Ran-independent export mechanisms have also been identified [7,9]. Various export and import mechanisms for the different classes of RNA and protein have been reviewed recently [1,10]. Nuclear export of tRNA seems to follow routes distinct from other RNA species because it does not require most of the export factors characterized [5,11] and is not dependent on RanGTP hydrolysis [2]. However, exportin-t [or exportin(tRNA)], the recently described tRNA export receptor lacking known RNA-binding domains, requires RanGTP for tRNA binding [12,13] and, unlike other RNA export receptors, can bind tRNA directly and not as part of an RNP [8]. However, the gene encoding the putative yeast analogue of exportin-t, LOS 1p, has been inactivated without significant influence on the viability of yeast cells [14]. It therefore seems likely that tRNA can leave the nucleus by more than one mechanism in yeast and possibly in higher eukaryotic cells.

A string of evidence has suggested that the ubiquitous multi heterogeneous nuclear ribonucleoprotein (hnRNP) K homologous (KH)-domain protein vigilin might have a role in tRNA export, particularly under conditions of high activity of protein synthesis. (1) We have demonstrated that vigilin is present in both the nucleus and the cytoplasm of human HEp-2 cells [15,16]. (2) Vigilin large composition [cytoplasmic (VLC_C) and nuclear (VLC_N)], containing large amounts of tightly bound tRNA, were isolated by immunoaffinity purification with monospecific anti-vigilin antibodies [16,17] from cytoplasmic and nuclear soluble protein fractions enriched in export factors [3]. A wash at high salt concentration releases the endogenous tRNA from immobilized VLC_C and VLC_N, resulting in tRNA-depleted vigilin core complexes (VCC_C and VCC_N). These complexes were shown to be capable of selectively rebinding tRNA from a nuclear RNA pool [16,17]. (3) Cellular levels of vigilin are increased in cells displaying a high rate of protein synthesis [17]. (4) The putative vigilin homologue in yeast is Scp 160p. A deletion mutant of this protein resulted in a decrease in viability [18], indicating that vigilin is essential to yeast cell metabolism.

As a continuation of our previous work, we further investigated the RNA-binding properties of vigilin and demonstrate here that recombinant human vigilin alone can bind directly to a variety of tRNA species. For lysine-specific tRNA we determined an overall apparent equilibrium dissociation constant, K_d (app), of 20 nM. Because the finding of a nuclear tRNA-containing vigilin complex has suggested a role for vigilin in nuclear tRNA export, we microinjected fluorescently labelled tRNA species into the nuclei of human HEp-2 cells and followed the time course of tRNA export by fluorescence microscopy as a function of co-injected vigilin or tRNA-depleted VCC_N. The human protein exportin-t has recently been identified as a tRNA-specific nuclear export receptor [12,13]. We therefore analysed whether exportin-t might be part of the VCC_N and followed the time course of vigilin and exportin-t levels in primary human fibroblasts with decreasing protein synthesis activity. Finally we looked for compositional differences between the VCC_C and the VCC_N. The composition of the VCC_N reveals possible links between different aspects of tRNA export; its conversion to the VCC_C sheds first light on how tRNA species might enter the cytoplasmic channelled tRNA cycle.

Abbreviations used: EF-1, elongation factor 1; HAV IA, hepatitis A virus 5′ non-transcribed region IA; KH, heterogeneous nuclear ribonucleoprotein K homologous; NTR, non-translated region; RNP, ribonucleoprotein complex; tRNP, tRNA-binding protein complex; VCC, vigilin core complex; VLC, vigilin large composition.

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EXPERIMENTAL

Recombinant expression and purification of vigilin

Vigilin cDNA was amplified by PCR with the sense primer 5′-CATGCCATGGGGAGTTCCGTTGCAGTTTTGACC-3′ and the anti-sense primer 5′-GAAGATCTTCGTTTGGGGC-CCCAAGGGAG-3′, introducing *Nco*I and *Bgl*II restriction sites (underlined) for insertion of the PCR product into the expression vector pQE 60 (Qiagen). The identity of the cloned gene was verified by DNA sequencing. The expression of recombinant vigilin, carrying an N-terminal His-tag, in *Escherichia coli* C 600$^-$ [19] was induced at 30 °C for 3 h by incubation in Luria–Bertani broth containing 0.5 mM isopropyl β-D-thiogalactoside. Harvested cells were sonicated in 50 mM NaH_2PO_4 (pH 6.0)/300 mM NaCl; the lysate was loaded on a Ni^{2+}-nitrilotriacetate superflow column (Qiagen). After being washed with 30 column vol. of wash buffer [50 mM NaH_2PO_4 (pH 8.0)/1 M NaCl] and 10 column vol. of wash buffer containing 40 mM imidazole, the protein was eluted in the wash buffer containing 80 mM imidazole. Western blot analysis of the purified recombinant protein with the vigilin-specific antiserum FP III (see below) revealed a protein of exactly the same molecular mass as native vigilin from HEp-2 cell lysates.

Preparation of ^{32}P-labelled RNA species

RNA species were prepared *in vitro* by run-off transcription from plasmids pTFMa (yeast $tRNA^{Asp}$) digested with Mva I [20], pUC19$tRNA^{Met}$ and pUC19$tRNA^{Met}$G57-U (human initiator $tRNA^{Met}$ and a mutant thereof; see below) [5], from plasmid pT7HAV1 linearized with *Nco*I [hepatitis A virus 5′ non-transcribed region Ia RNA (HAV Ia RNA)] [21] and from the following PCR-generated templates: T7-vitellogenin-3′ non-translated region (NTR): sense primer, 5′-TAATACGACTC-ACTATAGGG-3′; anti-sense primer, 5′-TTGAGATCAGTT-TATCATCATCAGTGACCCTATAGTGAGTCGTATTA-3′, comprising the vigilin core binding site (27 nt) of the 3′ non-transcribed region of *Xenopus* vitellogenin mRNA [22]; T7-Lys3, sense primer, 5′-TAATACGACTCACTATAGGCCCGGATA-GCTCAGTC-3′; anti-sense primer, 5′-TGGCGCCCGAACA-GGGAC-3′, using plasmid ptRNALys3 [23] encoding human $tRNA^{Lys}$ (UUU) as a template. Except for HAV Ia RNA (transcribed with SP6 RNA polymerase), transcripts were synthesized with T7 RNA polymerase, and RNA species were internally labelled to equal specific molar activities with [α-^{32}P]CTP (NEN). After transcription, RNA species were purified by denaturing 8–10 % (w/v) PAGE with 8.3 M urea/1 × TBE (Tris/borate/EDTA), eluted from gel slices by incubation at 4 °C overnight in 200 mM Tris/HCl (pH 6.5)/0.1 mM EDTA, precipitated with ethanol and redissolved in water.

T7 transcription and fluorescence labelling

Human initiator-$tRNA^{Met}$ and $tRNA^{Met}$ G57-U, and *Xenopus laevis* U1ΔSm and U6Δss RNA species were synthesized by T7 transcription from plasmid constructs described previously [5,24,25], linearized with *Bfa*I ($tRNA^{Met}$ and $tRNA^{Met}$G57-U), *Bam*HI (U1ΔSm RNA) or *Dra*I (U6Δss RNA). Mature $tRNA^{Gly}$ from *Thermus thermophilus* [26] was transcribed after digestion with *Bam*H1 from a plasmid carrying the T7 promoter and the mature $tRNA^{Gly}$ coding sequence followed by a *cis*-hammerhead. Self-cleavage of transcripts resulted in mature $tRNA^{Gly}$ with the CCA 3′ end extended by the trinucleotide GUC. Transcription assays were incubated overnight at 37 °C in 80 mM Hepes (pH 7.5)/22 mM $MgCl_2$/1 mM spermidine/5 mM dithiothreitol/120 μg/ml BSA containing each NTP at 3.75 mM, 15 mM GMP as initiator nucleotide, approx. 40 μg/ml of linearized plasmid DNA, 5 units of pyrophosphatase (purified from *Sulfolobus acidocaldarius*) and 2000 units of T7 RNA polymerase (MBI Fermentas) for 1 ml reaction assays. The template DNA species were then digested by adding 8 μl of DNase I (10 units/μl; Boehringer Mannheim) to a 1 ml transcription mixture and incubation at 37 °C for 30 min, followed by extractions with phenol/chloroform (1:1, v/v) and chloroform, desalting by Sephadex G-50 gel filtration and precipitation with ethanol in the presence of 75 mM sodium acetate, pH 6.7. RNA species were then purified by 8 % (w/v) PAGE with 8 M urea, localized by UV shadowing, excised from the gel, eluted overnight at 4 °C in 200 mM Tris/HCl (pH 7.0)/1 mM EDTA and precipitated with ethanol. RNA species were dissolved in water and concentrations were determined by A_{260} (1 A_{260} unit = 37 μg).

3′-Dephosphorylation of $tRNA^{Gly}$CCAGUC p

Mature $tRNA^{Gly}$CCAGUC p, carrying a 2′,3′-cyclic phosphate at the 3′ end owing to hammerhead self-cleavage, was 3′-dephosphorylated with T4 polynucleotide kinase (MBI Fermentas) essentially as described [27]. Assays, containing 0.1 mM ATP, 100 mM imidazole/HCl, pH 6.0, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol 20 μg/ml BSA and 1 unit of T4 polynucleotide kinase per 86 pmol of RNA, were incubated for 6 h at 37 °C, followed by extractions with phenol/chloroform (1:1, v/v) and chloroform, desalting on Sephadex G-50 columns, precipitation with ethanol in the presence of 75 mM sodium acetate, pH 6.7, and dissolving the RNA in water.

Fluorescence labelling of RNA

For oxidation of 3′-terminal riboses with periodate, 20 nmol of RNA were incubated for 50 min at 0 °C in a volume of 400 μl containing 2.5 mM $NaIO_4$ and 100 mM sodium acetate, pH 5.0, followed by precipitation with ethanol, washing of the pellet with 70 % (v/v) ethanol and drying in air. The oxidized RNA was dissolved in 400 μl of 100 mM sodium acetate (pH 5.0)/1 mM fluorescein-5-thiosemicarbazide (Sigma) (200 mM stock solution in dimethylformamide). The coupling reaction was performed overnight at 0 °C and RNA species were concentrated by precipitation with ethanol as above and redissolved in 50 μl of water. RNA species were then purified on 8 % (w/v) polyacrylamide/urea gels to remove unreacted dye and to separate modified from unmodified RNA. The latter two RNA fractions exhibited slightly different gel mobilities, which were distinguishable by comparison of RNA-associated fluorescence before and after staining with ethidium bromide.

Gel retardation assays

Samples were incubated in 10 μl of 1 × shift buffer [50 mM glycine (pH 7.5)/20 mM NH_4Cl/10 mM $MgCl_2$/5 mM dithiothreitol/0.2 mM EDTA] for 7 min at room temperature. After addition of 0.1 vol. of sample buffer [50 % (v/v) glycerol/1 μM EDTA (pH 8.0)/0.05 % Bromophenol Blue], samples were subjected to electrophoresis on 5 % (w/v) non-denaturing polyacrylamide gels in 1 × TBE buffer for 45 min at room temperature. Detection and quantification were performed with a Bio-Imaging Analyser BAS-1000 (Fujifilm). The percentage of complex formation (percentage shift) was calculated as the proportion of radioactivity in the complex divided by the total radioactivity in the lane (corresponding to 100 %). The smear above bands corresponding to the tRNA probe in Figure 1(C) (e.g. lanes 4 and 5) was assigned to the complex. K_d (app) was defined as the free vigilin concentration at which half of the

binding-competent fraction of the tRNA probe (approx. 80 %) was complexed with the protein.

Immunostaining of human HEp-2 cells

In brief, $(2–4) \times 10^5$ cells were grown on round coverslips (diameter 22 mm) for 48 h, washed twice with PBS [137 mM NaCl/8.5 mM Na_2HPO_4/1.5 mM KH_2PO_4/3 mM KCl (pH 7.3)], fixed with methanol for 10 min and permeabilized at −20 °C with acetone/water (1:1, v/v) for 5 min, acetone for 5 min and acetone/water (1:1, v/v) for 5 min. Cells were washed again twice with PBS. Endogenous peroxidase was blocked by incubation in PBS containing 0.5 % (v/v) H_2O_2 for 15 min. Permeabilized cells were first incubated for 1 h at 37 °C with rabbit anti-vigilin antibodies (FP III, dilution 1:1000 in PBS containing 0.1 % BSA). For staining of cells with the ABC immunoperoxidase-staining technique, the Vectastain Elite ABC Kit (Camon) was used in accordance with the instructions of the manufacturer. Diaminobenzidine served as the substrate.

Cell culture and preparation of cell extracts

The established cell line HEp-2 (ATCC CCL23), derived from human epithelial larynx carcinoma and human primary fibroblasts. was grown as described previously [17,28]. Fibroblasts were harvested 2 and 6 days after seeding. For SDS/PAGE, 10^6 cells were lysed for at least 1 h [17]. The cell extract was centrifuged and the supernatant was immediately used or stored at −70 °C. For preparation of native nuclear and cytosolic fractions, we used a modification [15] of the method originally described by Dignam et al. [29].

Electrophoresis and immunoblotting

Supernatants from centrifuged HEp-2 cell lysates (derived from 10^6 cells) [15] as well as VCC_N and VCC_C were analysed by SDS/PAGE [5–15 % (w/v) gradient gel]; polypeptides were stained with a silver staining kit (Bio-Rad) in accordance with the manufacturer's protocol or revealed by immunodetection. Before gel loading, samples were boiled in Laemmli sample buffer in the presence of 0.1 mM 2-mercaptoethanol.

Affinity chromatography and characterization of vigilin-containing fractions

Affinity-purified anti-vigilin antibodies were coupled to an affinity membrane chromatography cartridge (Millipore). The cartridge was operated with the ConSep LC 100 elution and monitoring system (Millipore) with a pressure of $(4.83–5.52) \times 10^5$ Pa. Non-specific adsorption was excluded as described previously [17]. The cartridge was loaded with either the cytoplasmic or the nuclear fraction of HEp-2 cell extracts and washed with water until A_{280} decreased to the baseline. The cartridge was subsequently subjected to the following procedures: (1) elution of weakly bound proteins by application of a 0–0.8 M NaCl gradient, retaining only the VLCs (containing vigilin, core proteins and bound RNA species) on the cartridge; (2) elution of nucleic acids tightly bound to the VLC by applying a 0.8–1 M NaCl gradient, leaving only the vigilin core complexes on the cartridge; (3) elution of the VCC with a PBS/glycine (pH 1.5, 0–1 M) gradient.

Microinjection

Fluorescence-labelled RNA species transcribed *in vitro* were incubated with different protein samples (see Figure 2) at room temperature for 15 min and then filtered through a syringe filter (0.45 μm pore size). RNAs were injected into HEp-2 cell nuclei at 20–50 pmol/μl with protein concentrations of 0.05–0.1 μg/μl, by using the microinjector Transjector 5246 and PatchMan from Eppendorf and Sterile Femtotips (Eppendorf) with a pressure of 60 hPa for 0.5 s, in accordance with the protocol of the manufacturer. Fluorescent nuclei were then scanned at different times after injection (0, 6 and 10 min) and the relative decline in nuclear fluorescence intensity was analysed statistically (n = 7–20) with the program SigmaPlot (Jandel Scientific).

Vigilin overlay assay

Vigilin was transcribed and translated from plasmid pTM1Vig in a coupled rabbit reticulocyte lysate system (TNT, Promega) in the presence of [^{35}S]methionine (Amersham), as described previously [17]. Vigilin overlay assays were performed as described by Hatzfeld et al. [30]. Protein fractions were separated on 4–15 % (w/v) polyacrylamide gradient gels and blotted to nitrocellulose membranes. Blots were incubated for 2 h in probing buffer [10 mM Tris/HCl (pH 7.5)/50 mM NaCl/1 mM EDTA/0.02 % BSA/0.02 % polyvinylpyrrolidone/0.02 % Ficoll] at room temperature to renature blotted proteins, followed by incubation for 2 h at room temperature in 5 ml of probing buffer containing 5×10^5 c.p.m. of [^{35}S]methionine-labelled vigilin and 6 μg of BSA. The blot was washed (each step for 5 min at room temperature) three times with probing buffer and three times with probing buffer containing 300 mM NaCl. The membrane was air-dried and exposure to X-ray film (Fujifilm) was performed for 16 h at −80 °C with an intensifying screen. Aliquots from the same protein fraction were also blotted to PVDF membranes after separation and stained with antibodies specific for exportin-t.

RESULTS

tRNA binding to vigilin

Binding of various RNA species to recombinant human vigilin (see the Experimental section) was analysed by gel retardation. Figure 1(A) shows that purified vigilin was capable of binding to tRNA and, in consistency with a previous report [31], to the 3′ NTR of *Xenopus* vitellogenin mRNA, whereas it was unable to form a gel-resolvable complex with HAV Ia (an 18 bp helix confined by a tetraloop) under the conditions used. To expand our studies, we tested vigilin's binding properties to yeast $tRNA^{Asp}$, human $tRNA^{Lys}$, human initiator $tRNA^{Met}$ and an export-deficient mutant thereof ($tRNA^{Met}$ G57-U) that is retained in the nucleus [32]. All these tRNA species were able to form gel-resolvable complexes with recombinant vigilin (Figure 1B). A titration experiment, with trace amounts of radiolabelled $tRNA^{Lys}$ and increasing amounts of vigilin, revealed the formation of two distinct complexes (I and II; Figure 1C). From the binding curve shown in Figure 1(D) an overall K_d (app) of 20 nM was derived. These results provide first direct experimental evidence that vigilin itself has a high affinity for tRNA. To ascertain that RNA retardation was not due to an contaminant from *E. coli* in the recombinant protein preparation, anti-vigilin immunostaining of the shifted complexes in each retardation assay was performed. These control experiments confirmed that tRNA probes in complexes I and II (Figure 1C) co-migrated exactly with immunostained vigilin bands (results not shown). From previous studies and as also observed for our recombinant protein preparation (results not shown), vigilin seems to be able to bind a broader spectrum of RNA species. However, an excess of tRNA apparently did not compete for vigilin's binding with

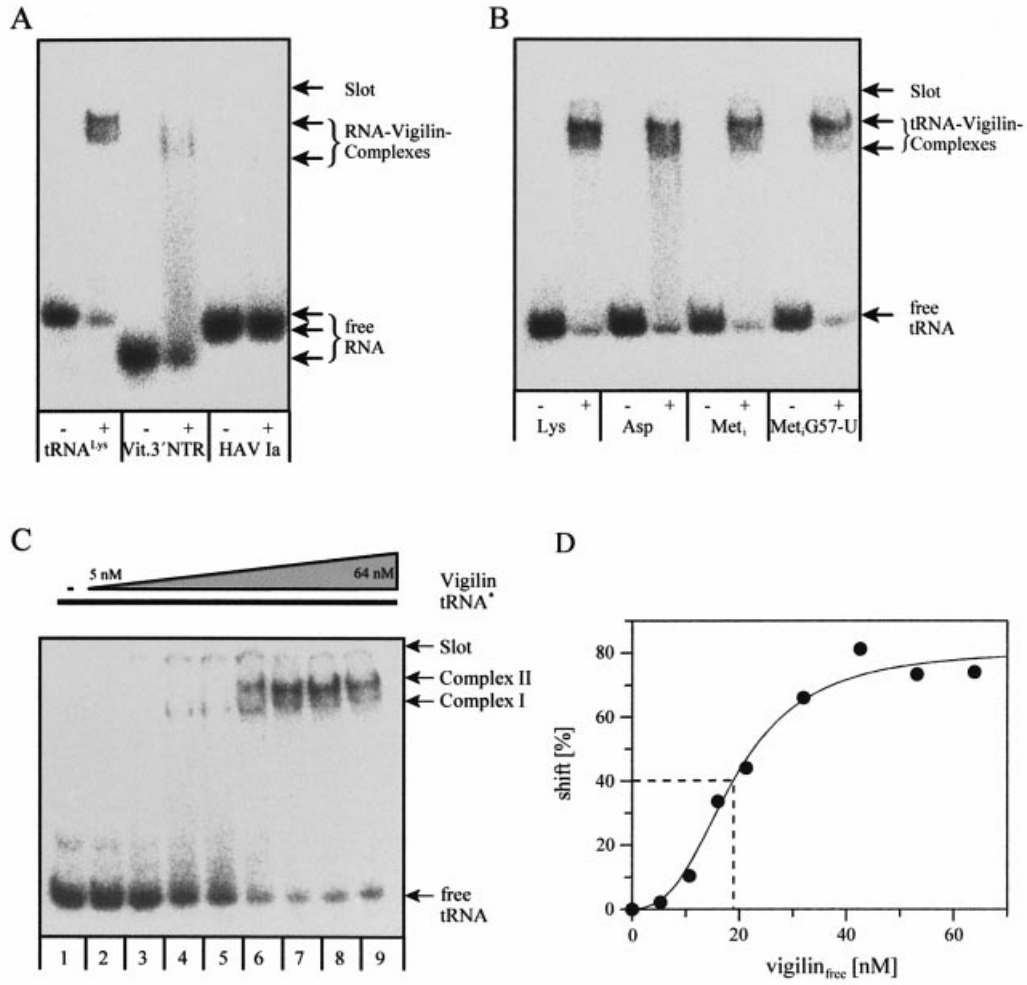


Figure 1 Gel retardation of vigilin and RNA

(**A**) Gel retardation experiment with recombinant vigilin purified from *E. coli* and different species of RNA. ^{32}P-labelled RNA (50 fmol) was incubated without (−) or with (+) 35 ng (approx. 0.2 pmol) of recombinant vigilin. Lane labels: tRNA^{Lys}, human tRNA^{Lys} (UUU); Vit. 3′ NTR, a 27 nt fragment of the *Xenopus* vitellogenin 3′ NTR identified as a high-affinity binding RNA for vigilin; HAV Ia, stem–loop structure (18 bp stem confined by a tetraloop) of the hepatitis A 5′ NTR. (**B**) Comparative gel retardation experiment documenting the binding of different tRNA species to recombinant vigilin. Equal quantities (50 fmol) of the respective tRNA species were incubated without (−) or with (+) 30 ng (approx. 0.2 pmol) of vigilin. Lane labels: Lys, human tRNA^{Lys} (UUU); Asp, yeast tRNA^{Asp} (GUC); Met$_i$, human initiator tRNA; Met$_i$G57-U, derivative of human initiator tRNA carrying a G^{57} → U point mutation. (**C**) Trace amounts of ^{32}P-labelled tRNA^{Lys} (2.6 nM) were incubated with increasing amounts of recombinant vigilin (0–64 nM, lanes 1–9) and analysed by gel retardation. (**D**) Binding curve derived from (**C**) after quantification of free and bound tRNA. The vigilin concentration at which half of the binding-competent fraction of the probe (approx. 80%) was complexed with the protein (indicated by broken lines) defines the overall apparent equilibrium dissociation constant [K_d (app) = 20 nM, verified in two additional independent experiments].

the 3′ NTR of *Xenopus* vitellogenin mRNA [22], suggesting that vigilin might bind distinct structural classes of RNA with different binding modes.

tRNA-depleted VCC$_N$ and vigilin alone stimulate nuclear tRNA export

The presence of tRNA in the vigilin-containing multi-protein complex isolated from nuclei by anti-vigilin antibody affinity chromatography prompted us to study the effect of vigilin on nuclear tRNA export in human HEp-2 cells. For this purpose, different fluorescently labelled RNA species were injected into the nuclei of living human cells (HEp-2) in the presence or absence of recombinant vigilin or tRNA-depleted VCC$_N$. One set of tRNA probes (a bacterial tRNA^{Gly}, human initiator tRNA^{Met}, tRNA^{Met}G57-U, *Xenopus* U1ΔSm and U6Δss RNA species [5]) were 3′ end-labelled with fluorescein. *E. coli* tRNA^{Phe} was internally labelled with proflavin in the D loop, its label therefore being less prone to potential intranuclear removal than the 3′-terminal fluorescein tag exposed to potential 3′-exonucleases. Figures 2(A) and 2(B) illustrate two representative examples (U1ΔSm and tRNA^{Met} co-injected with the tRNA-depleted VCC$_N$) of time-dependent changes in fluorescence intensity and localization at various time points after injection into human HEp-2 cells. Only minor decreases in nuclear staining intensity and no measurable cytoplasmic staining were observed with the control U1ΔSm RNA probe 10 min after injection (Figure 2A), whereas the tRNA^{Met} probe was equally distributed between nucleus and cytoplasm at the same time point (Figure 2B). Injection experiments performed with the various RNA probes are summarized in Figure 2(C). The time-dependent decrease in nuclear staining intensity was lowest for control RNA species

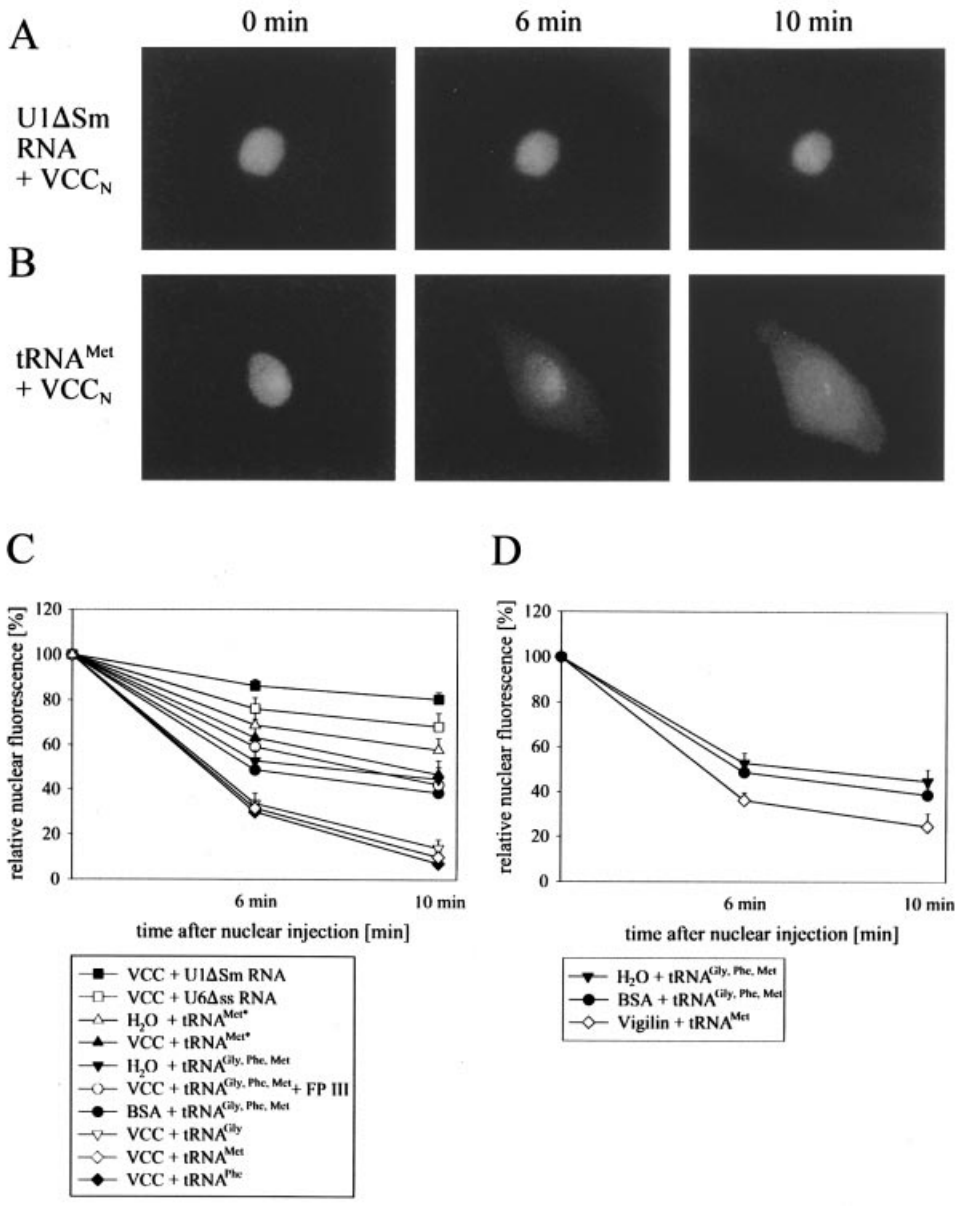


Figure 2 Effect of the VCC$_N$ and recombinant vigilin on the nuclear export of fluorescence-labelled RNA species co-injected into HEp-2 cell nuclei

(**A**, **B**) Time-dependent distribution of cellular fluorescence after co-injection of the VCC$_N$ and fluorescein-labelled U1ΔSm RNA (**A**) or tRNA^{Met} (**B**) into the nuclei of human HEp-2 cells. Fluorescence-labelled RNA species were incubated for 15 min at room temperature with the tRNA-depleted VCC$_N$ and then injected into the nuclei of HEp-2 cells. Cells were analysed by fluorescence microscopy immediately after injection (0 min) as well as 6 and 10 min afterwards. (**C**, **D**) Time-dependent changes in nuclear fluorescence as a function of co-injected VCC$_N$ (**C**) or vigilin alone (**D**). Nuclei injected with fluorescently labelled RNA were bordered and intensities of fluorescence were measured immediately after injection (0 min) as well as 6 and 10 min later. The nuclear fluorescence intensity at 0 min was defined as 100%. Results are means ± S.E.M. from several independent experiments; fluctuations seen in individual experiments are indicated by error bars. Note that in all experiments a fading, bleaching and quenching effect of approx. 25% must be taken into account. Results obtained with tRNA^{Gly}, tRNA^{Phe} or tRNA^{Met} in water, co-injected with BSA or with VCC plus FP III were essentially identical, and have therefore been combined as tRNA^{Gly,Phe,Met} for the sake of clarity. Preincubation and co-injection of tRNAs (tRNA^{Gly}, tRNA^{Phe} or tRNA^{Met}) with the purified VCC$_N$ led to a 90% decrease in nuclear staining intensity within 10 min (C). The loss was decreased to 55% when the VCC$_N$ was omitted (water control) or replaced with BSA. The additional presence of affinity-purified anti-vigilin antibodies (FP III) prevented the stimulation of tRNA export, decreasing the loss of nuclear staining intensity to the background level of 55% after 10 min. A proportion (40%) of tRNA^{Met*} (tRNA^{Met}G57-U) left the nucleus after 10 min in the absence of VCC$_N$; this increased slightly to 55% after 10 min in the presence of VCC$_N$. Control RNA species U1ΔSm and U6Δss were injected in concentrations equal to those of the tRNA probes. Symbols in (**C**): ■, VCC + U1ΔSm RNA; □, VCC + U6Δss RNA; △, water + tRNA^{Met*}; ▲, VCC + tRNA^{Met*}; ▼, water + tRNA^{Gly,Phe,Met}; ○, VCC + tRNA^{Gly,Phe,Met} + FP III; ●, BSA + tRNA^{Gly,Phe,Met}; ▽, VCC + tRNA^{Gly}; ◇, VCC + tRNA^{Met}; ◆, VCC + tRNA^{Phe}. Symbols in (**D**): ▼, water + tRNA^{Gly,Phe,Met}; ●, BSA + tRNA^{Gly,Phe,Met}; ◇, vigilin + tRNA^{Met}.

U1ΔSm and U6Δss co-injected with the VCC$_N$, and most rapid for tRNA^{Gly}, tRNA^{Phe} and tRNA^{Met} co-injected with the VCC$_N$. Stimulation of nuclear tRNA export was not observed when tRNA probes were preincubated and co-injected with BSA. Co-injection of tRNA with the VCC$_N$ and an affinity-purified monospecific anti-vigilin antibody (FP III) [15] abolished export stimulation, resulting in a time course very similar to that of the control (water + tRNA). In contrast, co-injection of the tRNA

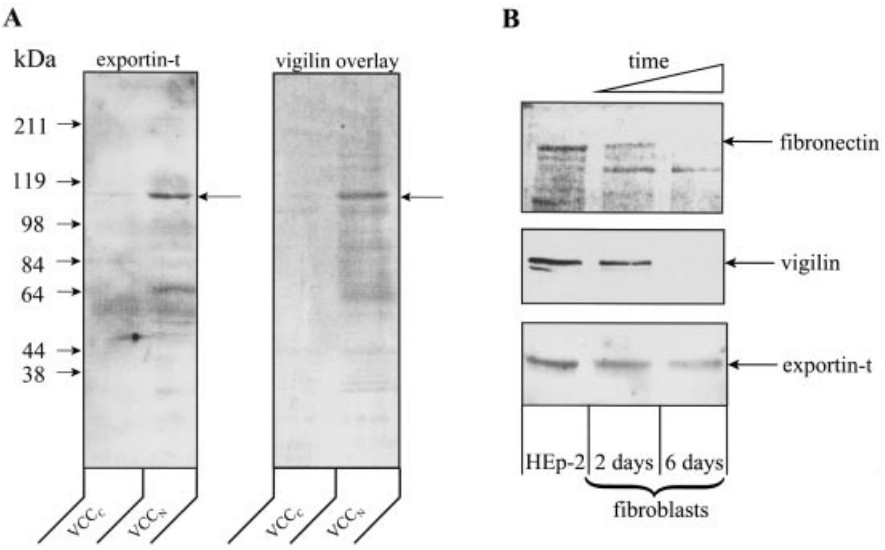


Figure 3 Exportin-t is part of the VCC_N, and evidence for direct binding of a 110 kDa protein to vigilin

(**A**) VCC_N and VCC_C were analysed for the presence of exportin-t by immunoblotting with an anti-(exportin-t) antiserum (left panel) and for polypeptides interacting with radioactively labelled vigilin (overlay assay, right panel). The overlay assay, which was preceded by renaturing of blotted proteins, identified a prominent 110 kDa polypeptide in the VCC_N, which interacts with the labelled vigilin. The positions and sizes of co-electrophoresed molecular marker proteins are indicated at the left. (**B**) High cellular levels of vigilin are associated with high translational activity. Primary human fibroblasts grown under culture conditions produced higher levels of extracellular matrix proteins, such as fibronectin, 2 days after seeding than at 6 days after seeding, when cells had reached confluence. Significant levels of vigilin were detectable by immunostaining 2 days after seeding. After 6 days, vigilin was decreased to undetectable levels. In contrast, no significant changes in the level of exportin-t were detected by immunostaining 2 and 6 days after seeding, suggesting the constitutive expression of exportin-t in this cellular system.

probe, the VCC_N and an anti-(mouse IgG) antibody did not affect VCC_N-mediated export stimulation (results not shown), indicating specificity of the inhibition caused by antibody FP III. Relatively slow decreases in nuclear fluorescence intensity were observed for mutant $tRNA^{Met}$G57-U when injected alone, with a minor increase in export velocity when co-injected with the VCC_N. This is in line with the previous findings that mutant $tRNA^{Met}$G57-U is actively retained in the nucleus [4,32]. Further controls included microinjection of fluorescein-labelled dextran (150 kDa), resulting in time-dependent losses in nuclear staining intensity very similar to those observed for U1ΔSm and U6Δss co-injected with the VCC_N. Fluorescein-5-thiosemicarbazide dye alone was found to leave the nucleus within 2 min, whereas proflavin was entirely retained within the nucleus for the first 20 min after injection, exceeding the time span of our experiments (results not shown). Therefore, if significant release of fluorescein or proflavin from the labelled RNA species had occurred owing to intranuclear RNA degradation or maturation, one would have expected different results for the fluorescein-labelled and proflavin-labelled tRNA probes; however, such differences were not observed. Additionally, RNA integrity in the injected samples was demonstrated by analysing an aliquot of each injection mix on a polyacrylamide gel after 2 h of incubation at room temperature.

The tRNA-depleted VCC_N has a complex protein composition [16], which makes it impossible at present to assign unambiguously its stimulatory effect on tRNA export to individual protein components. We therefore analysed the role of isolated recombinant vigilin on tRNA export (Figure 2D). Injection of vigilin alone stimulated the nuclear export of wild-type $tRNA^{Met}$ significantly in comparison with controls ($tRNA^{Gly,Phe,Met}$ with water or BSA). These results indicate that vigilin itself is a limiting tRNA export factor in our experimental system, i.e. under conditions of relatively high nuclear tRNA concentrations. However, on co-injection of equal total amounts of protein, $tRNA^{Met}$ export was accelerated more with the VCC_N than with vigilin alone, as inferred from decreases in nuclear staining intensity of more than 90% (VCC_N, Figure 2C) compared with less than 80% (vigilin alone, Figure 2D) after 10 min. In view of the relatively small amount of vigilin in the complex, this indicates that other components besides vigilin contribute to the efficacy of tRNA export. In addition, at present we cannot exclude the possibility that recombinant vigilin is functionally less competent than authentic vigilin.

Exportin-t is part of the VCC_N, preliminary evidence for binding of exportin-t to vigilin, and differential expression of exportin-t and vigilin

The human 110 kDa protein exportin-t has recently been identified as a tRNA-specific nuclear export receptor [12,13]. We therefore examined whether exportin-t is a constituent of VCC complexes. Protein components of the VCC_N and the VCC_C were separated by electrophoresis in the presence of SDS, blotted to membranes and renatured. When the blot was developed with anti-(exportin-t) antibodies (Figure 3A, left panel), exportin-t was readily detectable in the VCC_N lane, whereas the corresponding band was very weak for the VCC_C. In an overlay assay, radioactively labelled vigilin bound preferentially to one polypeptide band at the same position (approx. 110 kDa in size) in the VCC_N lane; again only a very faint signal was observed for the VCC_C (Figure 3A, right panel). These results demonstrate that exportin-t is part of the VCC_N and further indicate that a 110 kDa protein, probably exportin-t, is the major nuclear factor interacting with vigilin. At present we cannot discern whether low levels of exportin-t in the VCC_C reflect a genuine feature of the cytoplasmic complex or whether they are due to some preparational cross-contamination of nuclear and cytoplasmic complexes.

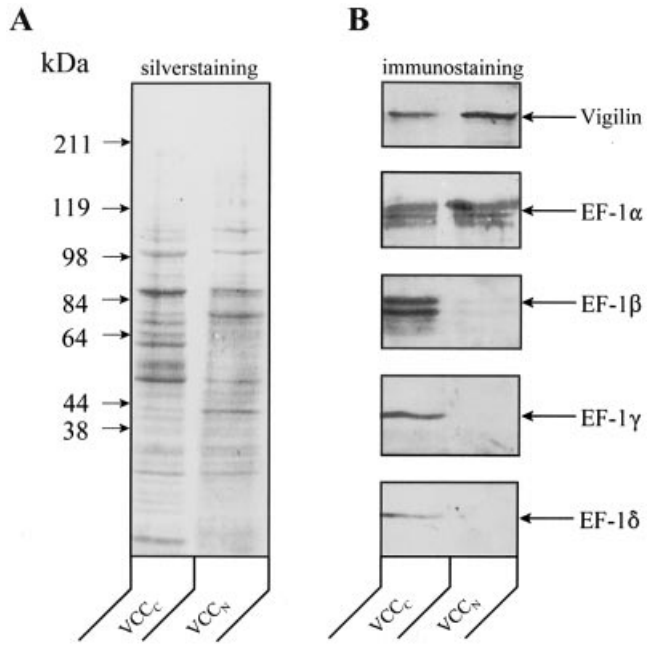


Figure 4 EF-1 is part of VCC complexes

Protein compositions of VCC_N and VCC_C were analysed by SDS/PAGE followed by staining with silver (**A**) and by immunoblotting with anti-vigilin and antibodies against EF-1α, EF-1β, EF-1γ and EF-1δ (**B**). EF-1α is enriched in the VCC_C and in the VCC_N. EF-1β, EF-1γ and EF-1δ are essentially confined to the VCC_C. The positions and sizes of co-electrophoresed molecular marker proteins are indicated at the left.

Because vigilin is most abundant in cells with intensive protein synthesis [16,17,33], we analysed the levels of exportin-t and vigilin as a function of culturing time *in vitro* (Figure 3B). Primary human fibroblasts, producing large amounts of extracellular matrix proteins [28], were analysed 2 and 6 days after seeding. At day 6, cells reached confluence and protein synthesis was significantly decreased, as indicated by the disappearance of fibronectin. Vigilin also disappeared, whereas levels of exportin-t remained essentially constant (Figure 3B). Thus, in consistency with previous observations, vigilin is most abundant in cells containing high protein synthesis activity [17], whereas exportin-t seems to be expressed constitutively.

Compositional differences between the nuclear and cytoplasmic tRNA-binding protein complexes (tRNP)

Vigilin is located in the nucleus and cytoplasm of human cells [15]. However, isolated VCC_C and VCC_N display similar yet distinct protein patterns (Figure 4A). Although the enrichment of elongation factor 1α (EF-1α) is a feature of both complexes [16] (Figure 4B), immunostaining with antibodies directed against various subunits of EF-1 shows that the presence of the β, γ and δ subunits is essentially restricted to the VCC_C (Figure 4B).

DISCUSSION

So far, relatively few investigations have focused on nuclear tRNA export. One mechanism is the export of tRNA by the dimeric complex exportin-t/RanGTP [12,13,34,35]. Additional mechanisms might exist and processes such as tRNA maturation and nuclear aminoacylation [34–36] have been reported to affect the efficiency of tRNA export.

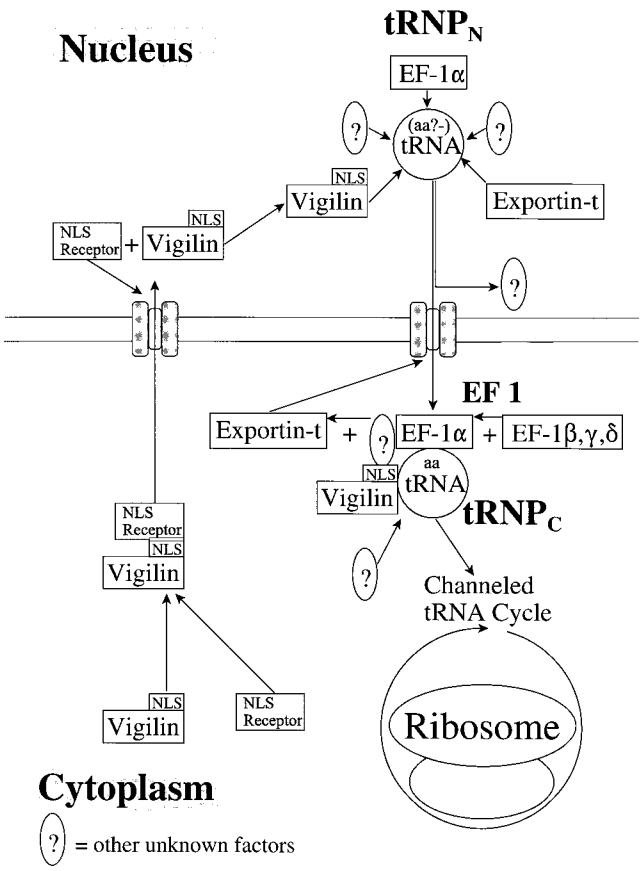
Vigilin, a protein with 15 reiterative putative RNA-binding domains of the KH type [37], has been shown to be capable of binding RNA [16,38,39]. The observation that vigilin is part of a nuclear as well as a cytoplasmic multi-protein tRNP [16,17] suggested that one cellular function of the protein might be associated with tRNA binding and/or export. This idea was supported by the finding that cellular levels of vigilin are always highest under conditions of increased protein synthesis [17,33,40,41], which imply a high demand for tRNA molecules.

We have demonstrated that nuclear microinjection of the VCC_N or purified vigilin itself accelerates tRNA export from the nucleus of human cells. Previous investigations in the *Xenopus* oocyte system have shown that tRNA export by exportin-t/RanGTP is more efficient for tRNA substrates with mature 3′ and 5′ ends [34,35]. In our study, $tRNA^{Phe}$ internally labelled with proflavin had mature 3′ and 5′ ends, whereas $tRNA^{Gly}$ 3′ end-labelled with fluorescein carried a three-nucleotide extension beyond the CCA sequence at the 3′ end. The VCC_N stimulated the nuclear export of $tRNA^{Phe}$ and $tRNA^{Gly}$ to very similar extents (Figure 2C), which apparently contradicts the results obtained in the *Xenopus* oocyte system [34,35]. However, *in vitro*-transcribed tRNA with an extra 10 nt at the 3′ end was bound by exportin-t/RanGTP with only an approx. 2.5-fold decreased affinity in comparison with mature tRNA transcribed *in vitro* [35], indicating that complete tRNA maturation is not an absolute prerequisite for tRNA export. Such minor differences in the affinity of $tRNA^{Phe}$ and $tRNA^{Gly}$ for human nuclear export factors might have been too small to affect the results of the experiments shown in Figure 2, particularly considering that we measured tRNA export in the presence of nuclear tRNA concentrations that exceeded the concentration of endogenous nuclear tRNA. These intranuclear conditions might have allowed immature tRNA to escape partly from functional proofreading processes controlling tRNA export [36].

Co-injection of the VCC_N or vigilin stimulated the nuclear export of human mutant $tRNA^{Met}$G57-U to a smaller extent than the export of wild-type $tRNA^{Met}$ (Figure 2C). A decreased efficiency of $tRNA^{Met}$G57-U export was also observed in the *Xenopus* oocyte system [32]. The authors proposed that tRNA export factors compete with nuclear retention factors for tRNA binding and attributed decreased $tRNA^{Met}$G57-U export to a relatively high affinity of the mutant tRNA for nuclear retention factors. The consistency of our results (Figure 2D) with those of Boelens et al. [32] suggests that similar export phenomena were analysed in both studies.

Although the isolated vigilin complexes are composed of a large number of polypeptides [16], they nevertheless satisfy several criteria of specificity. First, they have been isolated from soluble nuclear and cytoplasmic protein fractions by using affinity-purified anti-vigilin antibodies, yielding highly reproducible protein patterns with different immunoaffinity matrix preparations and different batches of soluble protein fractions from HEp-2 cells (Figure 4A) [16]. Secondly, such complexes resist treatment with high salt concentrations (0.8–1.0 M NaCl), which releases the bound tRNA. Thirdly, tRNA-depleted complexes can be recharged with tRNA [16,17]; lastly, RNA retained within complexes is strongly protected against nucleases [17].

Nuclear tRNA export is more efficiently stimulated by co-injection of the tRNA-depleted VCC_N than by vigilin alone (Figures 2C and 2D). This indicates that other factors from the complex are involved in the export process and prompted us to investigate the VCC composition. After immunological investigation, several components of the complexes have been identified. One complex constituent that might have contributed to the stimulation of tRNA export by the VCC_N is the 110 kDa protein



Scheme 1 Working model for the cellular function of vigilin

After the translation of vigilin mRNA on polysomes, the nuclear localization sequence of vigilin is thought to associate with importin α and β, which mediate import into the nucleus via nuclear pore complexes. Vigilin is then assumed to interact with other proteins (EF-1α, exportin-t) and immature and/or mature tRNA species, forming a nuclear tRNP particle (tRNP$_N$) that is exported from the nucleus through nuclear pore complexes with concomitant changes in complex composition, resulting in the formation of a cytoplasmic tRNP particle (tRNP$_C$). According to the model, the cytoplasmic vigilin tRNP, which is assumed to include components of the translation machinery such as aminoacyl-tRNA synthetases and translation factors (EF-1α, EF-1β, EF-1γ and EF-1δ), associates with ribosomes, thereby delivering aminoacyl-tRNA species to the ribosome for repeated rounds of polypeptide synthesis without transient release of deacylated tRNA into the cytoplasm, termed the channelled tRNA cycle [42]. Abbreviation: aa, amino acid. Other unknown factors are indicated by question marks.

exportin-t. We have observed that radioactively labelled vigilin interacts primarily with a 110 kDa protein of the VCC$_N$, probably exportin-t as inferred from immunostaining with an antiserum specific for exportin-t (Figure 3A). This suggests that vigilin might be able to interact directly with exportin-t on the tRNA export pathway. Additionally, the interaction of vigilin with the export receptor exportin-t would provide an explanation for vigilin export from the nucleus despite its lack of any known nuclear export signal. The precise role of vigilin in tRNA export and the putative conjunction with exportin-t remain to be characterized.

Analyses of primary human fibrobasts 2 and 6 days after seeding (Figure 3B) revealed a nearly complete disappearance of cellular vigilin after longer cultivation, together with decreasing translational activity of extracellular matrix proteins, while levels of exportin-t remained virtually unchanged. This result suggests that tRNA export processes might not be identical under different cellular conditions. In cells containing elevated protein synthesis activity and a high demand for tRNA molecules, a vigilin-involving export process might be operational, whereas the exportin-t/RanGTP/tRNA trimeric complex might be sufficient for basal levels of tRNA export under conditions of lower protein synthesis activity. Although our results point to a functional link between vigilin and exportin-t in human cells, genetic data obtained in yeast suggest that tRNA export can be maintained in the absence of the putative exportin-t analogue LOS 1p, because an inactivation of the gene encoding LOS 1p had no influence on the viability of yeast cells [14]. In contrast, a deletion mutation of the vigilin-analogous protein Scp 160p in yeast resulted in cells of decreased viability [18], indicating that the vigilin analogue is essential in yeast metabolism. One can speculate that, at least in yeast, a vigilin-dependent but exportin-t-independent export mechanism might be sufficient to maintain tRNA nuclear export in such mutant cells.

Taking together the available experimental evidence, the following, partly hypothetical, model for the role of the vigilin-tRNP in tRNA nuclear export seems conceivable (Scheme 1). Vigilin is imported into the nucleus by a classical nuclear localization sequence [15], presumably through interaction with the import receptors importin α and β (Scheme 1). In the nucleus, a tRNP is formed that contains, among other factors, vigilin, tRNA species, EF-1α [16] and the export receptor exportin-t. After the tRNP has passed the nuclear envelope, the export receptor is displaced from the complex and further components, such as EF-1β, EF-1γ and EF-1δ, associate with the complex. The further fate of the cytoplasmic tRNP is not yet clear. However, both the translation elongation factors identified in the complex and the immunohistochemical localization of vigilin at the outer surface of the rough endoplasmic reticulum [15] strongly point to an association of the cytoplasmic tRNP with translation. The complex might therefore serve as a direct shuttle for tRNA from the nucleus to the ribosome. This model accounts for the fact that tRNA has never been found free in the cytoplasm and provides a plausible explanation for how tRNA enters the channelled tRNA cycle for delivery to the tranlsational machinery [42,43].

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REFERENCES

1 Pennisi, E. (1998) Science **279**, 1129–1131
2 Izaurralde, E., Kutay, U., von Kobbe, C., Mattaj, I. W. and Görlich, D. (1997) EMBO J. **16**, 6535–6547
3 Ohno, M., Fornerod, M. and Mattaj, I. W. (1998) Cell **92**, 327–336
4 Zasloff, M. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6436–6440
5 Jarmolowski, A., Boelens, W. C., Izaurralde, E. and Mattaj, I. W. (1994) J. Cell Biol. **124**, 627–635
6 Görlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R. A., Mattaj, I. W. and Izaurralde, E. (1996) Cell **87**, 21–32
7 Dahlberg, J. E. and Lund, E. (1998) Curr. Opin. Cell Biol. **10**, 400–408
8 Görlich, D. (1998) EMBO J. **17**, 2721–2727
9 Nakielny, S. and Dreyfuss, G. (1998) Curr. Biol. **8**, 89–95
10 Weis, K. (1998) Trends Biochem. Sci. **23**, 185–189
11 Cheng, Y., Dahlberg, J. E. and Lund, E. (1995) Science **267**, 1807–1810
12 Kutay, U., Lipowsky, G., Izaurralde, E., Bischoff, F. R., Schwarzmaier, P., Hartmann, E. and Görlich, D. (1998) Mol. Cell **1**, 359–369

13 Arts, G. J., Fornerod, M. and Mattaj, I. W. (1998) Curr. Biol. **8**, 305–314
14 Hurt, D. J., Wang, S. S., Lin, Y. H. and Hopper, A. K. (1987) Mol. Cell. Biol. **7**, 1208–1216
15 Kügler, S., Grünweller, A., Probst, C., Müller, P. K. and Kruse, C. (1996) FEBS Lett. **382**, 330–334
16 Kruse, C., Grünweller, A., Willkomm, D. K., Pfeiffer, T., Hartmann, R. K. and Müller, P. K. (1998) Biochem. J. **329**, 615–621
17 Kruse, C., Grünweller, A., Notbohm, H., Kügler, S., Purschke, W. G. and Müller, P. K. (1996) Biochem. J. **320**, 247–252
18 Wintersberger, U., Kühne, C. and Karwan, A. (1995) Yeast **11**, 929–944
19 Raleigh, E. A. and Wilson, G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 9070–9074
20 Perret, V., Garcia, A., Puglisi, J., Grosjean, H., Ebel, J. P., Florentz, C. and Giegé, R. (1990) Biochimie **72**, 735–744
21 Harmon, S. A., Richards, O., Summers, D. F. and Ehrenfeld, E. (1991) J. Virol. **65**, 2757–2760
22 Dodson, R. E. and Shapiro, D. J. (1994) Mol. Cell. Biol. **14**, 3130–3138
23 Weiss, S., König, B., Müller, H.-J., Seidel, H. and Goody, R. S. (1992) Gene **111**, 183–197
24 Hamm, J., Kazmaier, M. and Mattaj, I. W. (1987) EMBO J. **6**, 3479–3485
25 Hamm, J. and Mattaj, I. W. (1989) EMBO J. **8**, 4179–4187
26 Schlegl, J., Fürste, J. P., Bald, R., Erdmann, V. A. and Hartmann, R. K. (1992) Nucleic Acids Res. **20**, 5963–5970
27 Cameron, V. and Uhlenbeck, O. C. (1977) Biochemistry **16**, 5120–5126
28 Aumailley, M., Krieg, T., Razaka, G., Müller, P. K. and Bricaud, H. (1982) Biochem. J. **206**, 505–510
29 Dignam, J. D., Lebowitz, R. M. and Roeder, R. G. (1983) Nucleic Acids Res. **11**, 1475–1489
30 Hatzfeld, M., Maier, G. and Franke, W. W. (1987) J. Mol. Biol. **197**, 237–255
31 Dodson, R. E. and Shapiro, D. J. (1997) J. Biol. Chem. **272**, 12249–12252
32 Boelens, W. C., Palacios, I. and Mattaj, I. W. (1995) RNA **1**, 273–283
33 Kruse, C., Emmrich, J., Rumpel, E., Klinger, M. H. F., Grünweller, A., Krammer, H.-J., Kühnel, W. and Müller, P. K. (1998) Exp. Cell Res. **239**, 111–118
34 Arts, G. J., Kuersten, S., Romby, P., Ehresmann, B. and Mattaj, I. W. (1998) EMBO J. **17**, 7430–7441
35 Lipowsky, G., Bischoff, F. R., Izaurralde, E., Kutay, U., Schäfer, S., Gross, H. J., Beier, H. and Görlich, D. (1999) RNA **5**, 539–549
36 Lund, E. and Dahlberg, J. E. (1998) Science **282**, 2082–2085
37 Musco, G., Stier, G., Joseph, C., Castiglione Morelli, M. A., Nilges, M., Gibson, T. J. and Pastore, A. (1996) Cell **85**, 237–245
38 Dejgaard, K. and Leffers, H. (1996) Eur. J. Biochem. **241**, 425–431
39 Kanamori, H., Dodson, R. E. and Shapiro, D. J. (1998) Mol. Cell Biol. **18**, 3991–4003
40 Chiu, D. S., Oram, J. F., LeBoeuf, R. C., Alpers, C. E. and O'Brien, K. D. (1997) Arterioscler. Thromb. Vasc. Biol. **17**, 2350–2358
41 Rumpel, E., Kruse, C., Müller, P. K. and Kühnel, W. (1996) Ann. Anat. **178**, 337–344
42 Stapulionis, R. and Deutscher, M. P. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7158–7161
43 Negrutski, B. N. and Deutscher, M. P. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 4991–4995

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