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Exportin-5 Mediates Nuclear Export of Minihelix-containing RNAs*

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The adenovirus VA1 RNA (VA1), a 160-nucleotide (nt)-long RNA transcribed by RNA polymerase III, is efficiently exported from the nucleus to the cytoplasm of infected cells, where it antagonizes the interferon-induced antiviral defense system. We recently reported that nuclear export of VA1 is mediated by a cis-acting RNA export motif, called minihelix, that comprises a double-stranded stem (>14 nt) with a base-paired 5' end and a 3–8-nt protruding 3' end. RNA export mediated by the minihelix motif is Ran-dependent, which indicates the involvement of a karyopherin-related factor (exportin) that remained to be determined. Here we show using microinjection in *Xenopus laevis* oocytes that VA1 is transported to the cytoplasm by exportin-5, a nuclear transport factor for double-stranded RNA binding proteins. Gel retardation assays revealed that exportin-5 directly interacts with VA1 RNA in a RanGTP-dependent manner. More generally, *in vivo* and *in vitro* competition experiments using various VA1-derived, but also artificial and cellular, RNAs lead to the conclusion that exportin-5 preferentially recognizes and transports minihelix motif-containing RNAs.

Nucleo-cytoplasmic transport of most RNAs and proteins is dependent on soluble receptors called karyopherins that can dock at and translocate through the nuclear pore complex. Interaction between cargo and karyopherin β is governed by the GTPase Ran. The asymmetric distribution of the Ran regulatory proteins provides a steep gradient of RanGDP (cytoplasmic)/RanGTP (nuclear) across the nuclear envelope that ensures the directionality of nuclear transport (1, 2). Nuclear

import receptors unload their cargo upon binding to RanGTP in the nucleus, whereas RanGTP is used to assemble export complexes which are in turn destabilized by dissociation of RanGTP in the cytoplasm (3, 4).

Our understanding of the nuclear export of RNAs has been greatly facilitated by the study of viral RNAs. For this reason, we focused our attention on the adenovirus VA1 RNA (VA1), a 160-nt¹-long RNA transcribed by RNA polymerase III that massively accumulates in the cytoplasm of infected cells. It serves to antagonize the interferon-induced cellular antiviral defense system. Indeed, VA1 binds and inhibits the double-stranded RNA-dependent protein kinase R (PKR), which otherwise phosphorylates eIF2 α and leads to the inhibition of protein synthesis (5, 6). Adenovirus VA1 RNA contains a new cis-acting RNA export motif that comprises a double-stranded stem (>14 nt) with a base-paired 5' end and a 3–8-nt protruding 3' end and that can tolerate some mismatches and bends (7). This export signal, called minihelix, is present not only in VA1 but in a large family of small viral and cellular RNAs transcribed by polymerase III. RNA export mediated by the minihelix motif is Ran-dependent, which indicates the involvement of a karyopherin-related factor (exportin). This exportin is distinct from Crm1 and exportin-t (7, 8). Therefore, we sought to identify cellular factors that bind to and mediate the export of minihelix-containing RNAs.

EXPERIMENTAL PROCEDURES

RNA Mutants—VA Δ IV, Mut10 mutants, and artificial stems have been described previously (7). VARdm and Mut9 mutants are derived from VA Δ IV. VARdm contains a 3'-terminal oligouridine stretch replaced by a UAG sequence. Mut9 presents a 6-base mispairing in the 5' end. These mutants have been generated by PCR and confirmed by sequencing. *In vitro* transcription was performed as described previously (7).

t-RNA^{Met} with additional GGG at the 5' end and a mature 3' end presents a pseudo-minihelix structure but is exported to the cytoplasm only after the processing of the 5' end (9, 10).

Electrophoretic Mobility Shift Assay—EMSA was performed in binding buffer (20 mM Hepes, pH 7.9, 50 mM KCl, 5 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol) containing 250 nM Mut10 to reduce unspecific binding, in a final sample volume of 20 μ l. Recombinant proteins were preincubated with unlabeled competitors for 10 min at room temperature and incubated with the radiolabeled probe for 25 additional minutes. Then, 1 μ l of loading DTT-dithiothreitol buffer (0.6 mg ml⁻¹ heparin, 1 mg ml⁻¹ bromphenol blue) was added, and incubation was pursued for another 5 min. Half of each sample was loaded on a 5% non-denaturing polyacrylamide gel, and electrophoresis was carried out at a constant voltage of 13.3 V cm⁻¹ at 4 °C in 0.5 \times TBE (45 mM Tris borate, 1 mM EDTA).

***Xenopus laevis* Oocyte Microinjections**—Oocytes injections and analysis of microinjected RNA by denaturing gel electrophoresis and autoradiography were performed as described previously (7). Stability of competitor VA RNA has been verified previously (7).

RESULTS AND DISCUSSION

VA1 consists of three functional domains, an apical stem-loop required for PKR binding, a central domain responsible for PKR inhibition, and a terminal stem, which brings together the 5' and 3' ends of the RNA. The 3' end consists in an unpaired oligouridine stretch characteristic of polymerase III transcripts that not only acts as a transcription termination signal but also as a primary binding site for the La protein. To analyze the role

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¹ The abbreviations used are: nt, nucleotide; PKR, protein kinase R; EMSA, electrophoretic mobility shift assay; dsRBD, double-stranded RNA binding domains.

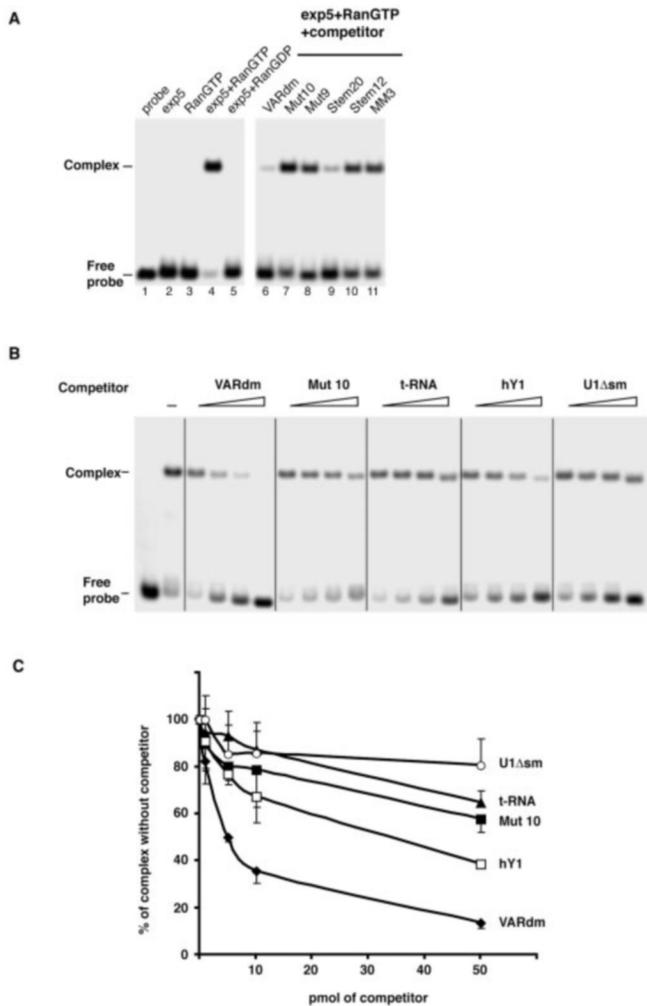


FIG. 2. Exportin-5 directly interacts with minihelix-containing RNAs in a RanGTP-dependent manner. *A*, EMSA was performed on radiolabeled VARdm probe using recombinant exportin-5 (*exp5*, 0.4 μ M), Ran GDP (1 μ M), or RanQ69L-GTP (1 μ M) as mentioned in the absence or in the presence of 5 pmol of indicated competitor RNA. *B*, EMSA was performed on radiolabeled VARdm probe using recombinant exportin-5 (*exp5*, 0.4 μ M) and RanQ69L-GTP (1 μ M) in the presence of 1, 5, 10, or 50 pmol of the indicated competitor RNA. *C*, data from three independent competition experiments performed as shown in *B* were quantified using ImageQuant 5.1 after scanning gels on a Storm 840 PhosphorImager (Amersham Biosciences).

various cellular RNAs on the exportin-5-VARdm complex. Addition of 1 pmol of unlabelled VARdm already affected the formation of the radiolabeled probe-exportin-5-RanGTP complex that was completely prevented with 50 pmol of VARdm (Fig. 2, *B* and *C*). No significant competition could be observed using U1 Δ sm that do not contain the minihelix motif, and a poor competition was observed using Mut10. hY1 RNA presents a 18-nt-long double-stranded stem with mismatches, a base-paired 5' end, and a protruding 3' end, thus corresponding to a minihelix structure less optimal than the VA1. Consistently, 5-fold more hY1 RNA than VARdm were required to compete out the formation of the radiolabeled probe-exportin-5-RanGTP complex. Finally tRNA^{Phe} that contains a highly degenerated minihelix motif displayed a very weak competition effect (Fig. 2, *B* and *C*). Similar results were obtained with purified *Escherichia coli* tRNA than *in vitro* transcribed human tRNA^{Phe}. Together, these data indicate that exportin-5 preferentially recognizes minihelix-containing RNAs with affinity varying as a function of the minihelix structure.

To determine whether the ability of exportin-5 to interact

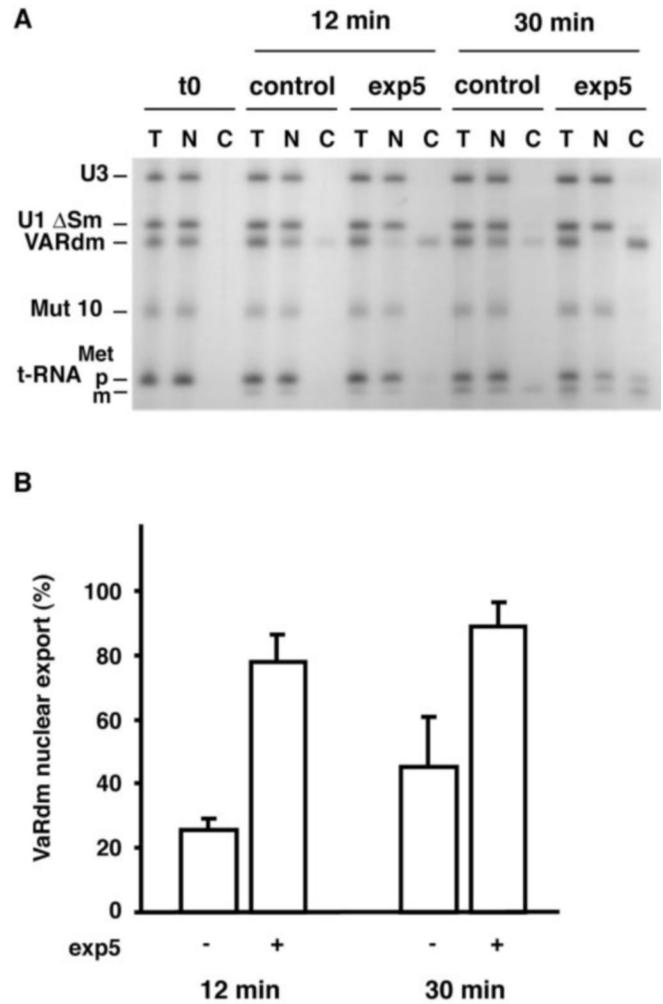


FIG. 3. Exportin-5 facilitates nuclear export of VARdm from *Xenopus oocyte* nuclei. *A*, a mixture of ³²P-labeled VARdm, Mut10, U1 Δ Sm, U3, and tRNA^{Met} was injected into oocyte nuclei preinjected with either buffer (*control*) or exportin-5 (*exp5*, 300 fmol per oocyte). After 12 or 30 min at 19 °C, total (*T*), nuclear (*N*), and cytoplasmic (*C*) RNAs were analyzed as in Fig. 1. *p*, unprocessed tRNA; *m*, mature. *B*, results from three independent experiments performed as in *A* were quantified using the Bioprint acquisition system and Bioprofil program (Vilbert Lourmat, Marne La Vallee, France) and expressed as the percent of VaRdm RNA located in the cytoplasmic fraction at the indicated time.

with minihelix structure in a RanGTP-dependent manner correlates with its ability to mediate the nuclear export of minihelix-containing RNAs, we examined the effect of microinjection of recombinant exportin-5 on the transport of various RNA in *Xenopus oocytes* (Fig. 3). For this purpose, a mixture of labeled VARdm, U3, U1 Δ Sm, Mut10, and tRNA^{Met} RNAs were injected into oocyte nuclei following preinjection of control buffer or recombinant exportin-5, and their nucleocytoplasmic distribution was analyzed after short incubation periods (12–30 min). It should be noted that tRNA^{Met} used in this experiment contained an additional GGG at the 5' end allowing the formation of a pseudo-minihelix structure 2 nt longer than mature tRNA. This tRNA is normally exported to the cytoplasm only after the processing of the GGG 5' end (9, 10). As shown on Fig. 3, *A* and *B*, preinjection of exportin-5 dramatically accelerated VARdm export and weakly stimulates the export of unprocessed tRNA. Importantly, the exportin-5 did not significantly affect the export of other RNAs.

Altogether, our results demonstrate that exportin-5 promotes the nuclear export of the viral VA1 RNA. More generally,

this exportin appears to specifically interact with the minihelix RNA motif in a RanGTP-dependent manner and is likely responsible for the nuclear transport of viral or cellular RNAs containing such a motif. Based on our results, an optimal exportin-5-interacting minihelix presents a 20-nt-long double-stranded stem with a base-paired 5' end and a protruding 3' end. It has been reported recently that exportin-5 is able to stimulate nuclear export of tRNA in microinjected *Xenopus* oocytes nuclei when transport of endogenous tRNA was artificially saturated (14). However, in normal experimental conditions, we found that exportin-5 does not affect tRNA transport. In addition, although exportin-5 can bind tRNA, our results show that its affinity for the optimal minihelix structure is much higher. This strongly suggest that tRNAs, which contain a degenerate minihelix, do not represent a preferential cargo for exportin-5 but can eventually use this transport receptor when their own transport pathway using exportin-t is deficient. Interestingly, the predicted secondary structures of intermediates of the recently discovered class of micro-RNAs and siRNAs resemble the minihelix motif and might mediate their nuclear export by the exportin-5 pathway. In addition, exportin-5 acts as a nuclear export receptor for proteins containing dsRBD. This could occur through an indirect interaction mediated by dsRNA similarly to exportin-5/EF1A interaction via tRNA (15), but one can certainly not exclude that certain dsRBD-

containing proteins might participate in the nuclear export of minihelix RNAs.

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