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Qualitative Highly Divergent Nuclear Export Signals Can Regulate Export by the Competition for Transport Cofactors *in Vivo*

Peter Heger[1], Jens Lohmaier[1], Grit Schneider[1], Kristian Schweimer[2] and Roland H. Stauber[1,*]

[1]Institute for Clinical and Molecular Virology, University of Erlangen-Nürnberg, Schlossgarten 4, D-91054 Erlangen, Germany
[2]Lehrstuhl für Biopolymere, University of Bayreuth, D-95440 Bayreuth, Germany
**Corresponding author: Roland H. Stauber, rdstaube@viro.med.uni-erlangen.de*

Nucleo-cytoplasmic transport of proteins is mediated by nuclear export signals, identified in various proteins executing heterologous biological functions. However, the molecular mechanism underlying the orchestration of export is only poorly understood. Using microinjection of defined recombinant export substrates, we now demonstrate that leucine-rich nuclear export signals varied dramatically in determining the kinetics of export *in vivo*. Thus, nuclear export signals could be kinetically classified which correlated with their affinities for CRM1-containing export complexes *in vitro*. Strikingly, cotransfection experiments revealed that proteins containing a fast nuclear export signal inhibited export and the biological activity of proteins harboring a slower nuclear export signal *in vivo*. The affinity for export complexes seems therefore predominantly controlled by the nuclear export signal itself, even in the context of the complete protein *in vivo*. Overexpression of FG-rich repeats of nucleoporins affected a medium nuclear export signal containing protein to the same extent as a fast nuclear export signal containing protein, indicating that nucleoporins appear not to contribute significantly to nuclear export signal-specific export regulation. Our results imply a novel mode for controlling the biological activity of shuttle proteins already by the composition of the nuclear export signal itself.

Key words: CRM1, NES, nucleo-cytoplasmic transport, nucleoporins, HIV, Rev, PKI

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A hallmark of eukaryotic cells is their spatial and functional separation into the nucleus and the cytoplasm by the nuclear envelope. Although this separation introduces a potent and sophisticated level of regulation, it also requires a highly effective and selective transport machinery. All known transport between the nucleus and the cytoplasm occurs through the nuclear pore complex (NPC) (for reviews see (1–3). Theoretically, proteins with masses <40 kDa can enter and leave the nucleus by passive diffusion. However, even most of the smaller proteins and nucleic acids appear to be transported by a signal-mediated pathway, probably because signal-mediated trafficking is more efficient and more amenable to specific regulation than diffusion. Transport is largely performed by the superfamily of transport receptors that interact directly or indirectly with the NPC, share a RanGTP-binding motif and are more or less related to importin β (for recent reviews see (4–7)). Exportins bind their cargoes in the presence of Ran-GTP and the trimeric complex of cargo, exportin, and Ran-GTP is subsequently translocated to the cytoplasm. Ran-GTP hydrolysis triggers the dissociation of the complex which is induced by the cytoplasmic Ran-GTPase-activating protein and its accessory Ran-binding protein 1 (reviewed in (4,6,8)). The Ran-GTPase cycle appears to create a RanGTP-gradient which determines transport directionality by regulating the stability of the transport receptor–cargo complexes (9).

The most prevalent nuclear export signals (NESs) found consist of a short leucine-rich stretch of amino acids in which the leucine residues are critical for function (for review see (4,6) and references therein). Leucine-rich NESs have been identified in an increasing number of cellular and viral proteins executing quite heterologous biological functions. Those include RNA transport (10–12), cell cycle and transcriptional control (13–16), the regulation of kinase activity (17–19) or even the controlled localization of cytoskeletal proteins (20). The existing data indicate that CRM1 acts as a general export receptor for all leucine-rich NESs identified so far, although additional receptors have been suggested (21). Although CRM1 was also reported to interact with the FG-repeats of several nucleoporins (e.g. CAN/Nup214, RIP, Nup88, etc.) (22–26), the detailed functional role of these interactions remains to be clarified. Currently, distinct factors acting downstream or upstream of CRM1 are actively discussed which would allow the orchestration of export to independently regulate the transport of the various shuttle proteins and cargoes (25,27–30). However, this mode of regulation is currently poorly understood and could be achieved on different levels during transport.

Although various aspects of the Ran-GTPase system or the CRM1 dependence of export have been addressed in detail, the contribution of the individual NESs in the regulation of nuclear export *in vivo* has been neglected so far. Using a microinjection approach, we found that export varied dra-

Table 1: Qualitative classification of various NES sequences

NES-sequence	Protein	Nuclear export[a]
MTKK**F**GT**L**T**I**	Minute virus of mice NS2	fast (5–10 min)
LA**L**K**L**AG**L**D**I**G	Protein kinase inhibitor (PKI)	fast (5–10 min)
AA**L**K**A**AG**L**D**I**G	Protein kinase inhibitor (PKI) (mut)	inactive (>16 h)
LSAQ**L**YSS**L**S**L**D	HTLV-1 Rex	medium type 1(10–20 min)
LQ**L**PP**L**ER**L**T**L**	HIV-1 Rev	medium type 1 (10–20 min)
LYPE**L**RR**IL**T**I**	Adenovirus type 5 E1B-55K	slow (30–60 min)
MFRE**L**NEA**L**E**L**K	Tumor suppressor protein p53	very slow (>10 h)
MFRE**L**NA**L**E**L**K	Tumor suppressor protein p53 (mut)	very slow (>10 h)
LKK**L**TQ**L**ATKY**L**	HTDV/HERV-K cORF	inactive (>16 h)
LKK**L**TQ**L**ATLY**L**	HTDV/HERV-K cORF (mut)	inactive (>16 h)
MV**L**TREE**L**V**I**	Adenovirus type 5 E4orf6	inactive (>16 h)
LV**L**TREE**L**V**I**	Adenovirus type 5 E4orf6 (mut)	inactive (>16 h)
L-$x_{(2,3)}$-**FILVM**-$x_{(2,3)}$-**L**-x-**L/I**	Consensus I*	
ΩxxxΩxx**L**x**L/I**[$]	Consensus II**	

[a]Assayed by microinjection of GST-NES-GFP recombinant substrates into the nucleus.
Amino acids reported to be essential for function are marked in bold.
*As proposed by (33). **As proposed by (30). [$]$\Omega$ denotes amino acids M, V, I, L, F, or W.

matically in somatic mammalian or *Xenopus* cells, ranging from minutes up to hours, depending on the NES examined. Importantly, the kinetics of export could further be translated into the affinities of the NESs for export cofactors even in the context of the complete proteins, suggesting a novel mode of regulation.

Results

Minimal NESs determine the kinetics of nuclear export in vivo

To directly compare the export capabilities of different NESs, we tested them in the context of a defined *in vivo* system. Recombinant GST-GFP export substrates containing the various NESs (see Table 1) were expressed in bacteria and purified. SDS-polyacrylamide gel electrophoresis (PAGE) revealed a major single band of approximately 54 kDa. Equal amounts of recombinant substrates (2 mg/ml, as adjusted by PAGE) were microinjected into the nuclei of Vero cells and export monitored over time under identical experimental conditions. Autofluorescent substrates containing the protein kinase inhibitor (PKI)- or the parvovirus minute virus of mice NS2 protein-NES were efficiently exported after 10 min (Figure 1A–C and data not shown). As a control a mutated PKI-NES in which two essential leucine residues were changed into alanines (see Table 1) did not support export (data not shown). The export kinetics of other NESs ranged from 20 min (Figure 1D–F, for the Rev-NES), to 60 min for the E1B-NES (Figure 1G–I) and to about 16 h (Figure 1J–L, for the p53-NES). NESs were considered inactive if their corresponding export substrates were still predominantly nuclear 16 h post injection. Therefore, the biological significance of the previously proposed HERV-K cORF NES (31) and the E4orf6 NES (32, see Table 1), being clearly inactive in our assay, might need reevaluation. Similar results were obtained in at least three independent microinjection experiments.

Microinjection of different concentrations of the export substrates (4 mg/ml, 2 mg/ml) did not significantly influence the kinetics of export, indicating that the cellular export machinery was not saturated by the amounts of substrate used in our assay (data not shown). Thus, the tested NESs were categorized into fast, medium, slow, very slow and inactive export signals (see Table 1). Of note, the qualitative classification of the NESs was also observed in HeLa cells.

To control the possibility that the export potential of the NESs was influenced by their accessibility in the context of the glutathione S-transferase (GST)-NES-green fluorescent protein (GFP) hybrids, we fused the PKI- and the E1B-NES to the C-terminus of GST-GFP. We observed that GST-GFP-E1BNES and GST-GFP-PKINES were exported with similar kinetics as compared to GST-E1BNES-GFP and GST-PKINES-GFP, respectively (Figure 2A–C; and data not shown). The observed kinetics of export were therefore independent of the intramolecular position of the NESs.

In analogy to transcriptional regulation where the number of transcription factor binding sites correlates with promoter activity, we investigated whether a fast NES could be reconstituted by multiple slow NESs. Addition of a second E1B-NES (i.e. GST-E1BNES-GFP-E1BNES) did not significantly enhance export (Figure 2D–F), indicating that a fast NES appears not to be reconstitutable by multiple slow NESs in a cumulative way.

Although NES consensus sequences have been proposed (30,33), potential NESs still need to be verified experimentally. In order to identify a consensus sequence for fast NESs we attempted to convert a very slow NES or an inactive NES into a more functional NES by changing the composition of the NESs according to the proposed consensus sequences (see Table 1). However, optimizing the spacing of essential leucines in the p53-NES or the HTDV/HERV-K cORF-NES by

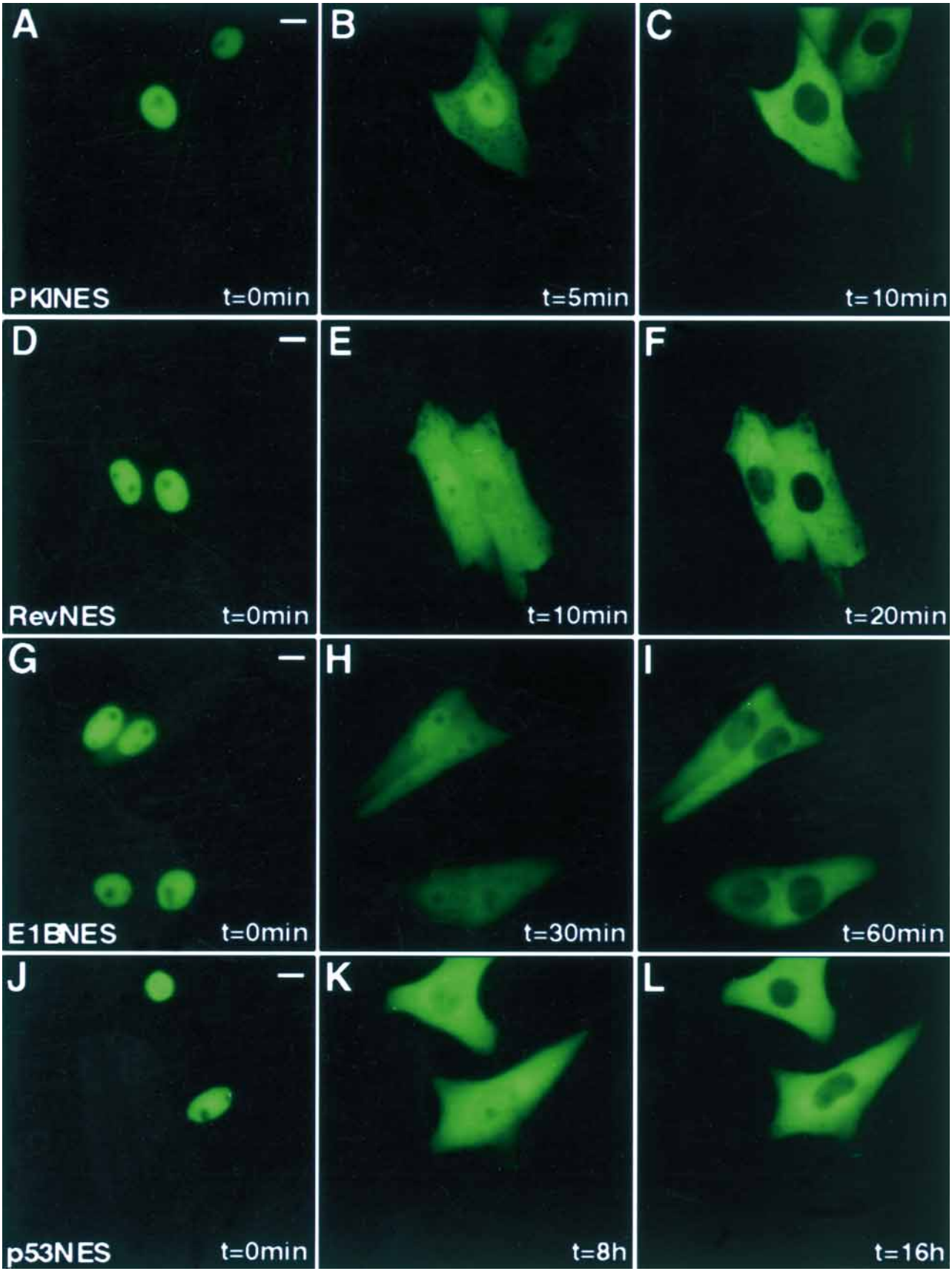
A
PKINES
t=0min
B
t=5min
C
t=10min
D
RevNES
t=0min
E
t=10min
F
t=20min
G
E1BNES
t=0min
H
t=30min
I
t=60min
J
p53NES
t=0min
K
t=8h
L
t=16h

Figure 1: Minimal NESs display different export kinetics *in vivo*. Equal amounts of recombinant GST-NES-GFP substrates (2 mg/ml) were microinjected into the nuclei of Vero cells under identical experimental conditions and export was monitored by GFP-fluorescence in living cells. Whereas the PKI-NES-directed nuclear export was completed after 10 min (A–C) the export kinetics of the other NESs tested ranged from 20 min for the Rev-NES (D–F), to 60 min for the E1B-NES (G–I) to about 16 h for the p53-NES (J–L). Bar, 20 μm.

the deletion of a glutamine or the substitution of a lysine by a leucine residue, respectively, did not improve export activities (data not shown).

Export correlates with the affinity of the NESs for CRM1-containing complexes in vitro

In order to investigate if the kinetics of export were mediated by different affinities for export complexes, we performed pulldown assays. *In vitro* translated labeled CRM1 was incubated with equal amounts of the different immobilized GST-GFP transport substrates (as controlled by PAGE) in the presence of GST-RanQ69L. Figure 3 indicates that the fast PKI-NES (lane 5) bound CRM1-containing complexes with higher affinity compared to slow NESs. To control for unspecific binding activity the SV40 nuclear import signal was included in the experiment (lane 1). Although the different kinetics of export were reflected by the affinities of the NESs for export complexes *in vitro*, the significant differences in export activity between the p53 and the E1B-NES (lanes 2 and 3) could no longer be adequately resolved by this *in vitro* assay.

Attempts to quantitate the affinities of recombinant His-tagged CRM1 to the different NESs by surface-plasmon-resonance failed due to the high background binding of recombinant CRM1 to GST (data not shown).

Proteins containing a fast NES inhibit the export and the biological activity of proteins containing a slower NES, but not vice versa

A critical question was if the different kinetics and affinities observed for the isolated NESs in the GST-GFP system were maintained also in the context of the complete proteins. Thus, it was crucial to investigate the NES classification in the context of the complete proteins *in vivo*. If the affinities for export factors were maintained, we reasoned that a shuttle protein containing a fast NES should be able to affect the trafficking of a shuttle protein harboring a slower NES *in trans* by the competition for export factors. As hypothesized, in cotransfection

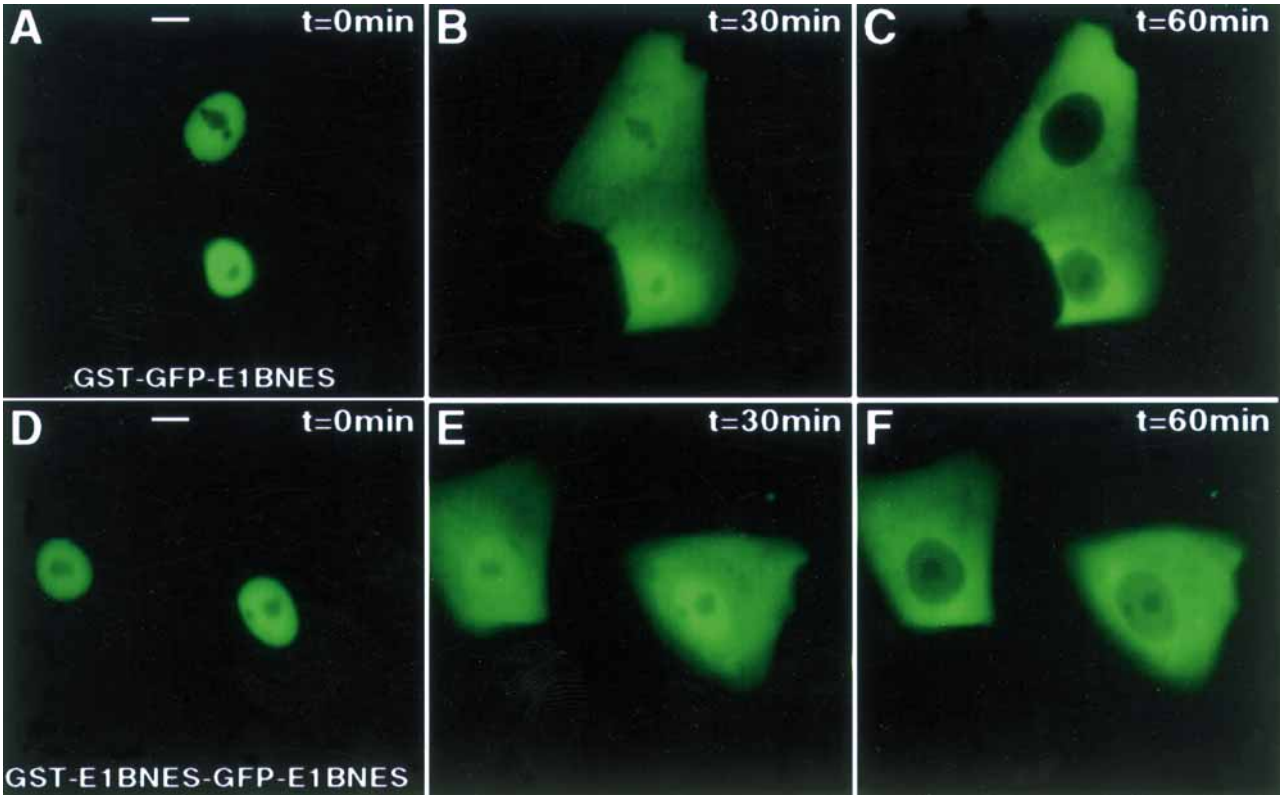

Figure 2: The intramolecular position or number of the E1B-NES does not affect export kinetics. Equal amounts of the indicated recombinant substrates (2 mg/ml) were microinjected into the nuclei of Vero cells and export monitored by fluorescence microscopy. Compared to the GST-E1BNES-GFP substrate (Figure 1 G–I), GST-GFP-E1BNES (A–C) or GST-E1BNES-GFP-E1BNES (D–F), respectively, were exported with similar kinetics. Bar, 20 μm.

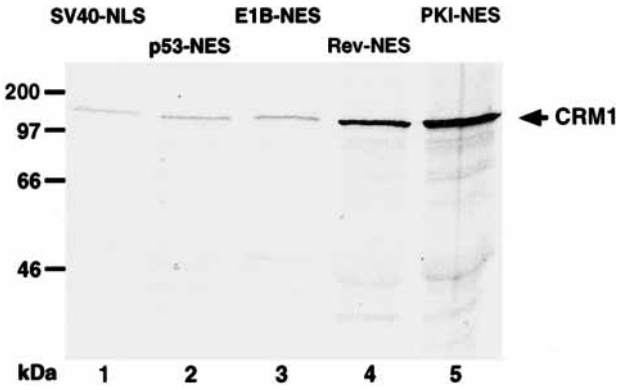

Figure 3: Kinetically different NESs display different affinities to CRM1. *In vitro* translated and ^{35}S-labeled CRM1 protein was incubated with equal amounts of the various immobilized GST-NES-GFP substrates or GST-SV40-NLS-GFP in the presence of GST-RanQ69L. Fast NESs bound with higher-affinity CRM1-containing complexes compared to slower NESs (PKI-NES > Rev-NES > E1B-NES > p53-NES). GST-SV40-NLS-GFP (lane 1) served to control for unspecific binding to GST-GFP.

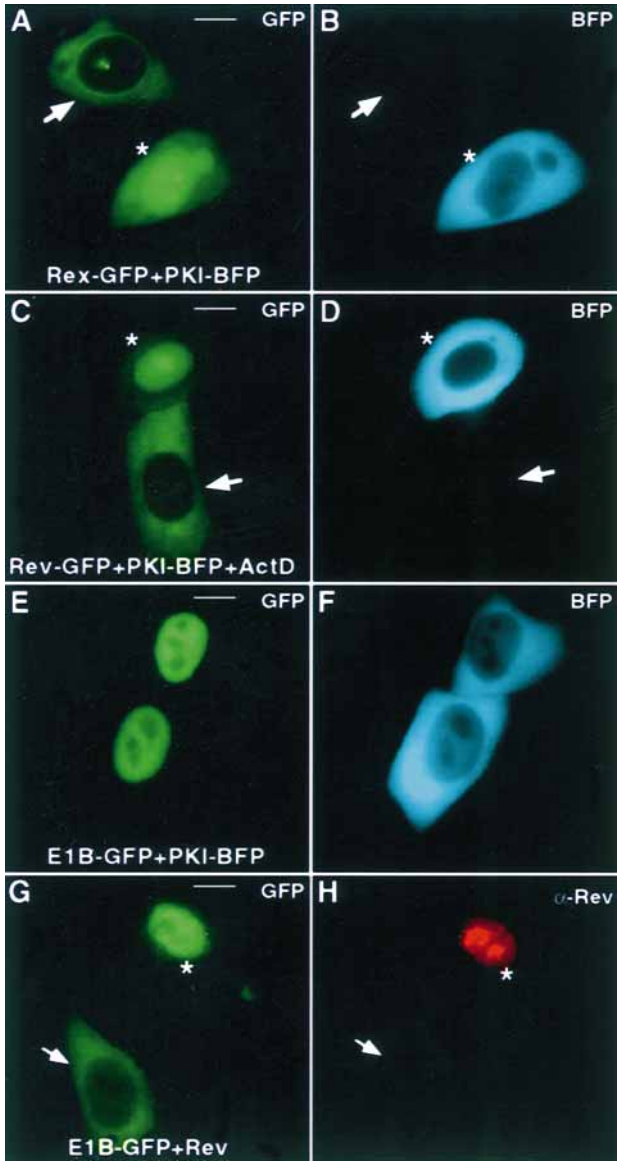

Figure 4: Overexpression of a protein containing a fast NES affects the nuclear export of proteins containing a slower NES. HeLa cells were transfected with the various expression plasmids and examined 16 h post transfection. Overexpression of PKI-BFP or the HIV-1 Rev protein (3 μg of plasmid-DNA) inhibited the nuclear export of HTLV-1 Rex-GFP (A/B), HIV-1 Rev-GFP (C/D) and the Ad5 E1B-55K-GFP protein (E/F; G/H) (1 μg of plasmid-DNA), resulting in nuclear accumulation. Asterisks indicate cells co-expressing both proteins, whereas the arrows mark cells expressing only one of the specified proteins. BFP and GFP signals were detected independently using the appropriate filters. Expression of HIV-1 Rev was verified by indirect immunofluorescence. Bar, 20 μm.

assays overexpression of PKI-blue fluorescent protein (BFP) (a fast NES containing shuttle protein) inhibited the nuclear export of a Human T-cell leukemia virus (HTLV)-1 Rex-GFP hybrid (Figure 4A,B) (a medium NES containing protein) and of the Ad5 E1B-55K-GFP protein (Figure 4E,F) (a slow NES containing protein). Rex-GFP and E1B-55K-GFP are active shuttle proteins with a predominant cytoplasmic steady-state localization (Figure 4A,G) and affecting nuclear export resulted in their nuclear accumulation resembling the treatment with the export inhibitor leptomycin B (LMB) (11,34). Likewise, overexpression of the HIV-1 Rev protein (a medium NES containing protein) caused nuclear accumulation of E1B-55K-GFP (Figure 4G,H). Since the interaction partners were localized in different intracellular compartments (Figure 4G,H; nuclear vs. nucleolar) inhibition was not mediated by direct protein interaction. To test for the inhibition of the HIV-1 Rev protein export, cells were transfected with plasmids expressing PKI-BFP together with Rev-GFP and treated with actinomycin D (Act D). As reported (35), Rev-GFP which is accumulating at the nucleoli (Figure 5A) redistributed into the cytoplasm following Act D treatment (Figure 4C) (cell marked with an arrow). However, the presence of PKI-BFP (Figure 4C,D) (cells marked with asterisks) inhibited Rev's nucleo-cytoplasmic transport, resulting in nuclear accumulation. In contrast, referring to Table 1, overexpression of Rev-GFP (Figure 5A,B) or p53-BFP (Figure 5C,D) did not affect the nuclear export of PKI-BFP or the HTLV-1 Rex protein, respectively. To further control for the NES-dependence of inhibition, cotransfection experiments were performed using PKI- and Rev-mutants lacking the NES. As illustrated in Figure 5(E–J), the cytoplasmic localization and thus nuclear export of Rex-GFP or E1B-55K-GFP remained unaffected by coexpression of PKIΔNES-BFP or RevΔNES-BFP, respectively.

Previously, it was suggested (36,37) that the assembly of Rev into multimers on its natural target RNA, the Rev response element (RRE), significantly increases Rev's affinity for export components. Thus, in the presence of an RRE containing RNA, Rev's export (i.e. biological activity) should no longer be inhibited by the presence of PKI. However, cotransfection

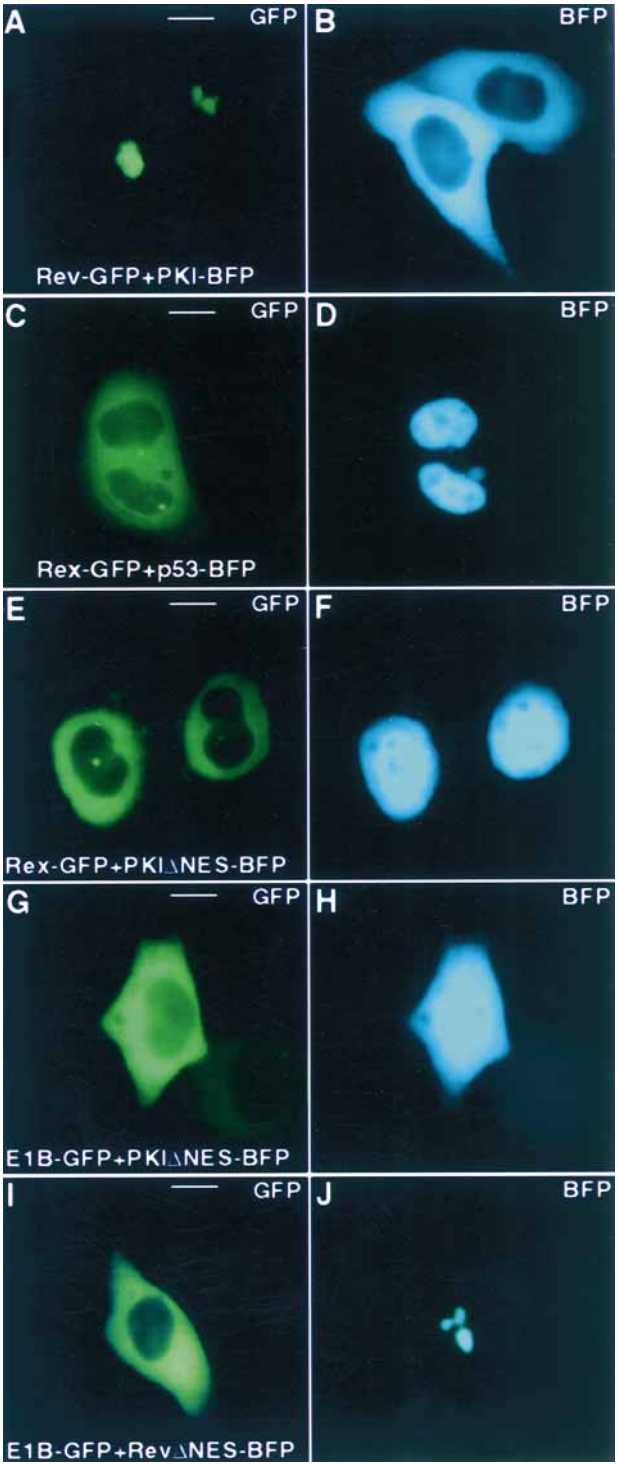

Figure 5: Proteins containing a slower NES do not inhibit nuclear export of proteins containing a fast NES. Overexpression of Rev-GFP (A/B) or p53-BFP (C/D) (3 μg of plasmid-DNA) did not affect the nuclear export of PKI or of the HTLV-1 Rex protein (1 μg of plasmid-DNA), respectively. Likewise, coexpression of a PKI (PKIΔNES-BFP) or a Rev protein mutant (RevΔNES-BFP) lacking the NES (3 μg of plasmid-DNA) did not inhibit nuclear export of Rex-GFP (E/F) or E1B-GFP (G/H; I/J) (1 μg of plasmid-DNA). Bar, 20 μm.

experiments revealed that Rev mediated mRNA export, which was reflected by the measured CAT-activity (Figure 6, lane 1), was still significantly impaired by the overexpression of PKI in a dose-dependent way (Figure 6, lanes 2–4). Similar results were obtained in HeLa cells and for the inhibition of Rex activity. Overexpression of a NES-deficient PKI-mutant (Figure 6, lane 5) or E1B-55K (Figure 6, lane 6) verified that the inhibitory effect on Rev activity was dependent on the presence of a high affinity NES. Thus, the multimerization on the RRE did not convert the medium-affinity Rev-NES into a high-affinity NES.

Interestingly, coexpression of CRM1 *in trans* could not restore Rev or Rex activity, suggesting that CRM1 alone is not the only rate-limiting factor, and additional components are necessary to reconstitute functional export complexes (data not shown).

Comparison between HIV-1 Rev proteins containing qualitative different NESs

In order to investigate if the localization and biological activity of a protein is influenced by the NES, we compared the HIV-1 Rev protein (containing a medium NES) with a Rev-PKI hybrid (containing a fast NES). Rev-PKI consists of the Rev amino acids 1–66 (containing the specific RNA-binding site but lacking the Rev NES) and the complete PKI coding sequence and was shown to be functional in *trans*-activation (38). Both proteins were expressed as GFP hybrids, and transient transfection revealed that the Rev/PKI-GFP hybrid localized predominantly to the cytoplasm (Figure 7A), in contrast to the preferential nucleolar localization of Rev-GFP (Figure 5A). To confirm the shuttling of Rev/PKI-GFP, cells were treated with LMB. Blocking nuclear export resulted in rapid nucleolar accumulation of Rev/PKI-GFP (Figure 7B), indicating that the steady-state localization of a protein can highly depend on the balance of nuclear retention vs. nuclear export signals.

In *trans*-activation assays, Rev-GFP and Rev/PKI-GFP displayed similar activity (Figure 8). Of note, untagged versions of Rev and Rev/PKI were equally active (data not shown).

Several groups suggested that nucleoporins participate in the regulation of export and also Rev activity (22–26). To examine the possibility that nucleoporins specifically regulate the export of individual NESs, we tested the effect of overexpressing FG-rich repeats of nucleoporins (NP). Coexpression of CANc or Nup98-NP inhibited RRE-dependent *trans*-activation by Rev as well as by Rev-PKI to a similar extent (Figure 8). Although reported to play a critical role in multiple types of export (39) Nup153-NP affected significantly neither Rev (as reported by (25)) nor Rev-PKI activity. Similar results were obtained using a GFP-Nup153-NP fusion protein which allowed verification of efficient expression of Nup153-NP (data not shown). Thus, the tested nucleoporins do not appear to interact NES-selectively with preassembled export complexes.

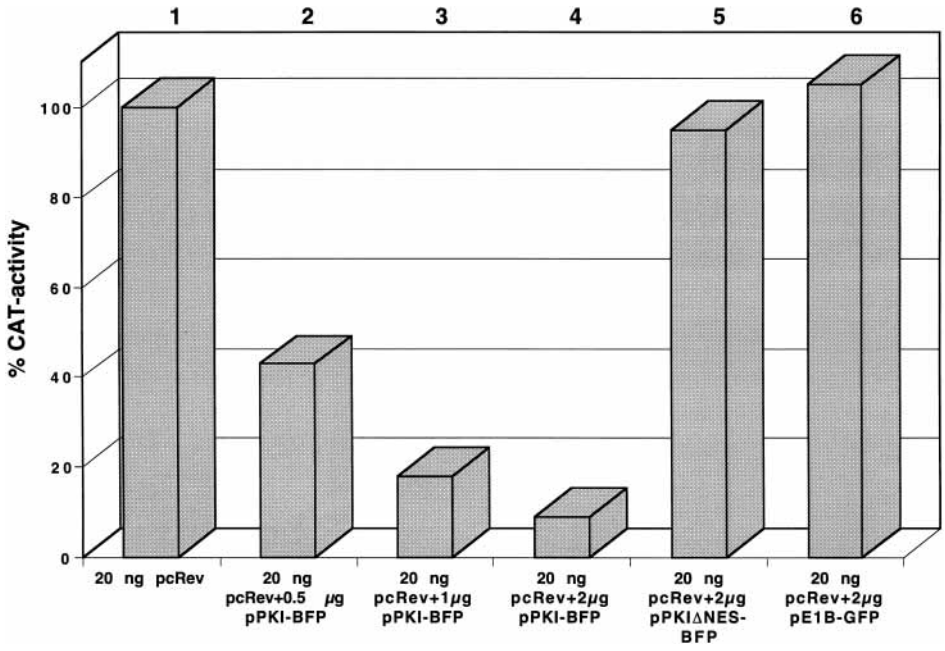

Figure 6: NES-dependent inhibition of HIV-1 Rev activity by PKI but not by E1B-55K. Rev-mediated *trans*-activation was determined by cotransfection of 293 cells with a Rev-responsive CAT-expression plasmid, the indicated expression constructs and a constitutive internal control vector encoding β-galactosidase. All CAT values were adjusted for transfection efficiency (β-galactosidase activity) and were corrected for background (mock) activity. Transfection of 20 ng Rev expression plasmid was arbitrarily set to 100% CAT-activity. Duplicate plates were used and the results were averaged. Similar results were obtained in three independent experiments.

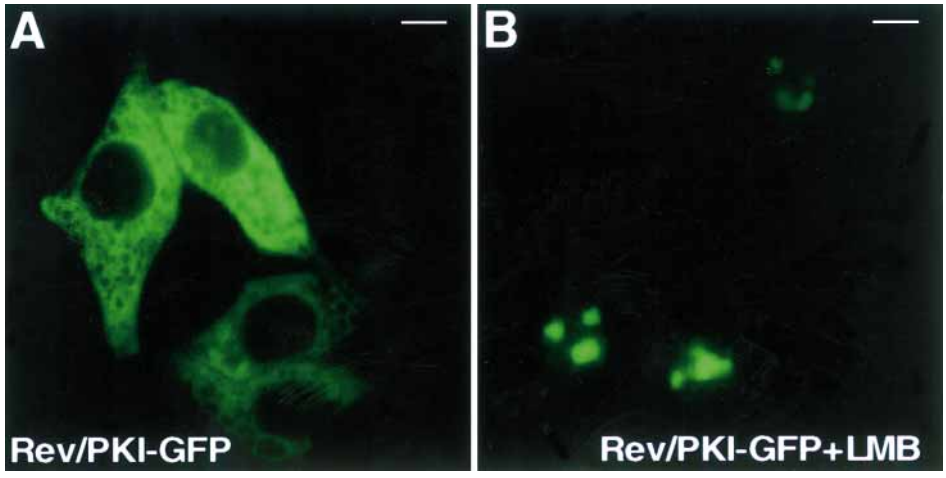

Figure 7: Localization of Rev/PKI-GFP in living human cells. HeLa cells were transfected with pcRev/PKI-GFP and observed by fluorescence microscopy 16 h post transfection. (A) Rev/PKI-GFP localized predominantly to the cytoplasm. (B) Treatment with LMB caused rapid nucleolar accumulation of Rev/PKI-GFP, demonstrating its CRM1-dependent shuttling activity. Bar, 20 µm.

Discussion

To date, NESs using the CRM1 export pathway have been identified in a growing list of regulatory viral and cellular proteins. In spite of that, the orchestration of export is still unclear. Regulation can be envisaged to occur at different levels during transport. First, the interaction of the CRM1-cargo complex with the NPC could be regulated by specific adaptors acting upstream of CRM1 (e.g. nucleoporins). Second, specific modifications or cofactors binding to the NESs and/or to other regulatory domains of the shuttle proteins could modulate the interaction with CRM1. Finally, the affinity

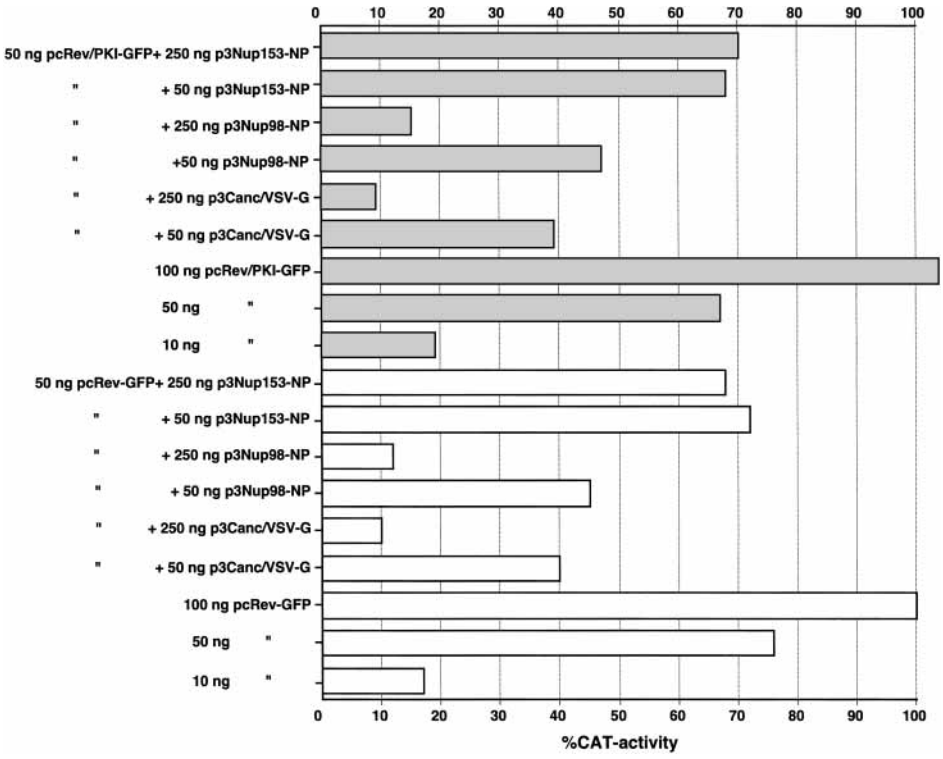

Figure 8: Rev and Rev/PKI are active in *trans*-activation and inhibited by the overexpression of nucleoporin FG-repeats. Rev-mediated *trans*-activation was determined as described in Figure 6. Transfection of different amounts of Rev-GFP or Rev/PKI-GFP expression plasmids, respectively, indicated that the two proteins displayed comparable activity. Coexpression of CANc or Nup98-NP inhibited *trans*-activation to a similar extent, whereas overexpression of Nup153-NP did not significantly affect Rev- or Rev/PKI-mediated CAT expression.

for a general CRM1-containing export complex could already be directly or indirectly predetermined by the composition of the NESs themselves.

Consequently, this study was undertaken to broaden the knowledge of the CRM1-dependent, NES-mediated nuclear export pathway by addressing the effects of individual NESs on transport. Comparison of leucine-rich NESs from various proteins in the defined GST-GFP system revealed that the kinetics of directed export varied dramatically in living mammalian cells. This resulted in a qualitative classification of the tested NESs which was independent of their intramolecular position. After completion of our studies, the results of Henderson and Eleftheriou (40), although obtained in a system involving treatment with transcription inhibitors, known to affect transport (41,42), also suggested qualitative differences for isolated NESs. Importantly, the activity of the tested NESs appears to correlate with their affinity for CRM1-containing export complexes *in vitro* (see Figure 3). Our observations are also supported by Askjaer and colleagues (30), who reported similar results for NES peptides, qualitatively classified in this study, using an indirect *in vitro* assay. However, the *in vitro* assays were unable to reflect exactly the different kinetic activities of the NESs as observed by microinjection in living cells (see Figure 1). Most likely the unspecific binding of CRM1-containing export complexes to GST-GFP masks the low-affinity interaction with slow NESs. Alternatively, cellular components mediating the interaction of slow NESs with CRM1-containing export complexes may not be sufficiently supplied by the addition of the reticulocyte lysates to the binding reactions.

However, to verify the *in vivo* significance of these findings it was crucial to confirm the classification of the isolated NESs in the context of the complete proteins *in vivo*. Importantly, cotransfection studies could demonstrate that proteins containing a fast NES affected the export and biological activity of proteins containing slower NESs. Since this effect was dependent on a functional NES (Figures 4–6) inhibition appears to be based on the NES-mediated competition for export factors. This suggests that the kinetics and affinities observed for the isolated NESs in the GST-GFP system can be translated into affinities for export components in the context of the complete proteins in a test system that takes all potential modifications into account, the living cell. Thus, at least for the proteins tested, the affinities for export components seem to be determined predominantly by the composition of the NES itself, and not significantly modulated by post-translational modifications or additional factors interacting with other domains of the shuttle proteins.

These results are interesting since it was speculated that processes like extensive multimerization or conformational

changes significantly influence the affinities for export factors (16,30,43). However, this conclusion was mainly based on the observation that in *Xenopus* oocytes a Rev-NES peptide could only function as an effective export competitor when conjugated in multiple copies to BSA (37), but has not been addressed in the context of the complete protein in living mammalian cells. We found that even supplying an RRE containing RNA *in trans*, being prerequisite for the biological relevant formation of Rev multimers on its target RNA (Figure 6), did not convert the medium-affinity Rev-NES into a high-affinity export factor binding site. Rev's multimerization on the RRE, which is critical for RNA export, might therefore not qualitatively change the Rev-NES but quantitatively increase the number of bound Rev molecules. Since Rev-dependent RNAs appear to be retained in the nucleus by their interaction with cellular factors (44,45), multiple Rev molecules are required to bind in order to overcome retention and to promote RNA export. The observation that a monomeric Rev-binding site (46) can only mediate export of a small RNA (47), but needs to be present in multiple copies to export a larger RNA (48), favors this model.

It is striking that signals as short as 10 amino acids are capable of dramatically influencing export. But how is this regulation mediated? Do fast NESs display a specific conformation that fits better into the binding site of CRM1, or do NESs function by the formation of mere hydrophobic interaction patches? Since a PKI-NES peptide was described to be active in inhibiting export and NESs were functionally independent from their intramolecular position (19,49, this report) it appears that complex tertiary structure is not necessary for function. Using nuclear magnetic resonance (NMR), Hauer and colleagues reported that the PKI-NES consists of an amphipathic helix in which the essential leucines form a hydrophobic face (50). However, the NES of p53 showed a similar distribution of large hydrophobic residues in an amphipathic α-helix (50) and yet was significantly less active in export (see Figure 1). Of note, these NMR structures were recorded at a low temperature (2 °C), and attempts to resolve the structure of a PKI-NES peptide at a higher, more physiological temperature (25 °C) did not reveal a specific conformation (Schweimer and Stauber, data not shown). In order to gain insight into an active NES structure it would thus be important to analyze an NES in the complex with its specific receptor, as achieved for a nuclear localization signal (NLS)-importin α complex (51). Resolving the three-dimensional active NES structure might also allow design of inhibitors by molecular modeling to specifically target the biological functions of individual shuttle proteins.

We and Askjaer and colleagues (30) reported different affinities of the NESs for CRM1-containing export complexes. This might suggest that the qualitative classification of the NESs *in vivo* reflects their affinities for CRM1, and the inhibitory effect of fast NESs would be due to the competition for CRM1 only. However, ectopic expression of CRM1 could relieve the inhibition neither by CRM1-binding nucleoporins nor by fast NES-containing proteins (25,34, this report), favoring the existence of still-unidentified export components besides CRM1, Ran-GTP and the NESs. Efficient Rev-NES/CRM1 complex formation was observed using CRM1 derived from reticulocyte lysates (22, this report), which could serve as a source for additional export cofactors. In contrast, when purified CRM1 was used, only weak binding of the Rev-NES to CRM1 but not to the PKI-NES was reported (22,30). Hofmann and colleagues (27) observed the efficient binding of the Rev-NES to recombinant CRM1 only in the presence of recombinant eIF-5A, underlining the idea of additional export cofactors. The possibility that the different affinities are mediated by individual adapter proteins recognizing specifically distinct NESs would, however, require a highly complex regulatory network. Although speculative, the composition of the NES most likely creates a structured interaction face contacting the CRM1 export complex containing still unidentified factors. For low-affinity NESs this interaction might need to be strengthened by additional bridging factors contacting both the NES and CRM1. Although nucleoporins are essential components of the nuclear transport machinery they do not seem to interact NES-selectively with export complexes. The inhibitory effect of overexpressing FG-rich repeats of certain nucleoporins is most likely based on the competition for CRM1-containing export complexes. Thus, as investigated so far, the orchestration of export appears to be mainly regulated downstream of CRM1 at the level of the NES.

Export signals might not only mark a protein for the export pathway, but already represent a mechanism to regulate the biological activity of proteins. Although the HIV-1 Rev and the Rev-PKI hybrid protein harbor qualitatively different NESs, both proteins were equally active in *trans*-activation assays. One could speculate that the Rev-NES is already optimized for the nuclear export of RRE-containing RNAs. Alternatively, the Rev-dependent expression of a RRE-CAT mRNA might not be the most adequate system to detect differences in Rev-activity. Therefore, we are currently probing the influence of the different NESs on HIV-1 replication in the context of the complete virus.

Our study underlines that qualitatively different NESs exist also in their natural context and are able to compete for export cofactors. This finding might have significant impact in understanding the regulation of viral and cellular systems. One will be faced with questions: why, for example, did complex retroviruses develop regulatory shuttle proteins containing a medium but not a fast NES? Does this make them vulnerable to competition by fast cellular shuttle proteins, especially during the initial phase of replication, or already represent a mechanism to maintain latency by inhibiting for example HIV-Rev function? Would a fast NES containing p53 be a more efficient 'guardian of the genome', and what are the reasons for the evolutionary conservation of the NESs? To understand the functional relevance of NES diversity will certainly be an important challenge for the future.

Materials and Methods

Plasmids

Vectors pc3-GFP and pc3-BFP, expressing the green and blue fluorescent protein, respectively, were constructed by PCR-amplifying the coding sequence of GFP or BFP, using appropriate primers containing *BamHI*- and *EcoRI*-restriction sites and the plasmids pCMV-GFPsg25 and pCMV-BFPsg50 (52) as templates. The PCR products were digested with *BamHI* and *EcoRI* and inserted into the vector pcDNA3 (Invitrogen).

To generate pc3-PKI-BFP, the coding sequence of the human protein kinase inhibitor (PKI) was amplified by PCR using appropriate primers containing *BamHI*- and *NheI*-restriction sites and cloned into pc3-BFP. Plasmid pc3-PKIΔNES-BFP was generated by PCR-mutagenesis using primers mutating the essential leucines in the PKI-NES at positions 37, 39 and 41 to alanines.

Plasmids pc3-p53-BFP encoding a BFP-tagged version of p53, and pE1B-GFP encoding a hybrid between the adenovirus type 5 E1B-55K protein and GFP have already been described (11). Plasmids pRex-GFP encoding GFP-tagged HTLV-1 Rex and the HIV-1 Rev and HTLV-1 Rex expression vectors pcRev, pcRevM9 and pcRex, respectively, have been described (34,38). To generate pcRevM9ΔNES-PKI, the coding sequence of PKI was amplified by PCR, using appropriate primers containing *BglII*- and *XhoI*-restriction sites and cloned into pcRevM9. pcRevM9ΔNES-PKI encodes a hybrid protein composed of the HIV-1 Rev protein aa 1–66 (lacking the NES) and the complete PKI protein. To generate pcRev/PKI-GFP, the coding sequence of the Rev-PKI hybrid was amplified by PCR using appropriate primers containing *NheI*-restriction sites and plasmid pcRevM9ΔNES-PKI as the template. The PCR product was cloned into the plasmid pcRev-GFP (53), replacing the Rev coding sequence. Plasmid pBrevM10BL-BFP encodes a BFP-tagged Rev mutant lacking the NES (RevΔNES-BFP) (53). pDM128/CMV/RRE or pDM128/CMV/RxRE is a Rev- or Rex-responsive reporter construct, respectively, encoding the bacterial chloramphenicol acetyltransferase (CAT) (34). pBC12/CMV/βGal encodes β-galactosidase (34). Plasmid p3-CRM1-VSV, encoding a VSV-tagged version of human CRM1 and p3CANc/VSV-G, encoding the carboxy terminus of CAN/Nup214, have already been described (54). The coding regions of Nup98 (aa 2–494) and Nup153 (aa 894–1475) were PCR amplified, using specific oligonucleotides containing *BamHI*-/*EcoRI*- or *HindIII*-/*XhoI*-restriction sites, respectively, and plasmids Nup98-NP and Nup153-NP as templates (25). Subsequently, the PCR products were cloned into the vector pcDNA3, resulting in the construct p3Nup98-NP and p3Nup153-NP. Plasmid GFP-Nup153 encodes a GFP-tagged version of the Nup153-NP (25).

Potential nuclear export signals were cloned into the bacterial expression vector pGEX-GFP (55), using complementary oligonucleotides containing *BamHI*-/*NheI*-restriction sites. The resulting plasmids (see Table 1) encode fusions of glutathione S-transferase (GST) and the nuclear export signals linked to GFP. Alternatively, the export sequences were inserted into the *KpnI*-/*EcoRI*-digested vector pGEX-MCS, using complementary oligonucleotides, encoding a GST-GFP-NES fusion protein. To express a GST-GFP fusion protein containing two E1B-NESs, pGEX-E1BNES-GFP was digested with *BamHI*/*EcoRI* and the small DNA-fragment replaced by the corresponding fragment from the vector pGEXMCS-E1BNES. The resulting plasmid pGEX-E1BNES-GFP-E1BNES encodes a GST-E1BNES-GFP-E1BNES hybrid. Plasmid pGEX-SV40NLS-GFP encodes as GST-GFP fusion containing the SV40 large T-antigen nuclear import signal (41).

To generate the bacterial expression vector pGEX-RanQ69L, the coding region of RanQ69L was amplified by PCR from the plasmid pET3dRanQ69L (56), using appropriate oligonucleotides containing *BamHI*- or *EcoRI*-restriction sites, respectively. Subsequently, the PCR product was digested with *BamHI*/*EcoRI* and cloned into the bacterial expression vector pGEX-3X (Pharmacia, Peapack, NJ, USA). The coding regions of all constructs were confirmed by sequence analysis.

Purification of recombinant GST-fusion proteins

GST-GFP hybrid proteins or GST-RanQ69L, respectively, were expressed and affinity purified under nondenaturing conditions as described (55). Protein concentrations were in the range of 2–4 mg/ml. His-tagged CRM1 was prepared as described (27).

CRM1 pull-down assays and in vitro translation

Coupled transcription/translation was performed using the TNT reticulocyte lysate system (Promega, Madison, WI, USA), supplemented with [^{35}S]methionine (Amersham, Princeton, NJ, USA) and plasmid p3CRM1-VSV as the template. One hundred micrograms of the recombinant GST-NES-GFP substrates or GST-SV40-NLS-GFP were bound to 50 μl of glutathione-sepharose 4B (Pharmacia) in PBS for 30 min at 4 °C, washed extensively with PBS to remove unbound protein, and washed with binding buffer as described (57). Subsequently, 15 μl of the loaded beads were incubated with 4 μl of the CRM1-TNT product and 5 μg GST-RanQ69L. Binding was performed in binding buffer for 4 h at 4 °C and the beads were subsequently washed three times with 1 ml binding buffer. Proteins were analyzed by SDS-PAGE and fluorography.

Cells, microscopy and microinjection

Vero, 293 or HeLa cells were prepared for microinjection or transfected as described (34,55). Following injection, cells were immediately observed or fixed and analyzed using the appropriate fluorescence filters as described (34,55). 1Twelve-bit images were captured using a cooled MicroMax CCD camera (Princeton Instruments, Stanford, CA, USA). Image analysis, quantitation of fluorescence and presentation were performed using the IPLab Spectrum and Adobe Photoshop software.

Rev or Rex *trans*-activation assays were performed in 293 or HeLa cells as described (34). Briefly, cells were transfected with 300 ng of pDM128/CMV/RRE or pDM128/CMV/RxRE DNA, respectively, 100 ng of pBC12/CMV/βGal DNA, together with indicated amounts of different expression plasmids. pDM128/CMV/RRE or pDM128/CMV/RxRE express the CAT gene in a Rev- or Rex-dependent manner, and CAT expression was quantitated 48 h after transfection.

Treatment with leptomycin B was performed as described (34).

Indirect immunofluorescence

Cells were fixed, permeabilized and incubated with a mouse monoclonal anti-Rev antibody (34).

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