Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 334 (2005) 284–293

VIROLOGY

www.elsevier.com/locate/yviro

Nucleocytoplasmic shuttling of the rabies virus P protein requires a nuclear localization signal and a CRM1-dependent nuclear export signal

David Pasdeloup[a,1], Nicolas Poisson[a,1], Hélène Raux[a], Yves Gaudin[a], Rob W.H. Ruigrok[b], Danielle Blondel[a,*]

[a]*Unité Mixte de Virologie Moléculaire et Structurale UMR2472 CNRS, UMR1157 INRA, 91198 Gif sur Yvette Cedex, France*
[b]*Laboratoire de Virologie Moléculaire et Structurale, FRE 2854 CNRS-UJF, c/o EMBL, BP 181, 38042 Grenoble Cedex 9, France*

Received 22 December 2004; returned to author for revision 31 January 2005; accepted 7 February 2005

Abstract

Rabies virus P protein is a co-factor of the viral RNA polymerase. It has been shown previously that P mRNA directs the synthesis of four N-terminally truncated P products P2, P3, P4, and P5 due to translational initiation by a leaky scanning mechanism at internal Met codons. Whereas P and P2 are located in the cytoplasm, P3, P4, and P5 are found in the nucleus. Here, we have analyzed the molecular basis of the subcellular localization of these proteins. Using deletion mutants fused to GFP protein, we show the presence of a nuclear localization signal (NLS) in the C-terminal part of P (172–297). This domain contains a short lysine-rich stretch (^{211}**KKYK**^{214}) located in close proximity with arginine 260 as revealed by the crystal structure of P. We demonstrate the critical role of lysine 214 and arginine 260 in NLS activity. In the presence of Leptomycin B, P is retained in the nucleus indicating that it contains a CRM1-dependent nuclear export signal (NES). The subcellular distribution of P deletion mutants indicates that the domain responsible for export is the amino-terminal part of the protein. The use of fusion proteins that have amino terminal fragments of P fused to β-galactosidase containing the NLS of SV40 T antigen allows us to identify a NES between residues 49 and 58. The localization of NLS and NES determines the cellular distribution of the P gene products.

Keywords: Rabies virus; P protein; Nuclear import; Nuclear export

Introduction

Eucaryotic cells possess a double nuclear membrane, containing multiple nuclear pores that regulate bi-directional transport of macromolecules that is critically required for the maintenance of normal cell physiology.

Transport proceeds through the nuclear pore complex (NPC), a 125 MDa macromolecular assembly of 50–100 polypeptides that are frequently termed nucleoporins. The NPC spans the nuclear membrane and creates an aqueous channel allowing passive diffusion of globular proteins of up to approximately 50 kDa. Translocation across the NPC into the nucleoplasm or into the cytoplasm is mediated by importins and exportins, respectively, which interact with cargo molecules, the Ran GTPase, and proteins of the NPC. Cargo molecules have localization signals that allow their interaction with importins and exportins. The classical nuclear localization signal (NLS) comprises one or two short stretches of basic amino acids. Examples are the simian virus 40 (SV40) T antigen NLS (PKKKRKV) (Kalderon et al., 1984) or the cellular nucleoplasmin protein NLS (KRPAATKKAGQAKKK) (Robbins et al., 1991). These sequences are recognized in the cytoplasm by importin α which forms a stable heterodimeric complex with the importin β (Kohler et al., 1999). Importin β mediates the interaction of the importin α–β complex with the NPC and Ran GTPase. The complex translocates into the nucleus where importin α and β receptors dissociate from the cargo and shuttle back into the cytoplasm. The cycle is controlled by a Ran GTP gradient across the nuclear

* Corresponding author. Fax: +33 1 69 82 43 08.
E-mail address: danielle.blondel@vms.cnrs-gif.fr (D. Blondel).
[1] These authors contributed equally to this work.

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.02.005

envelope. Low levels of Ran GTP in the cytoplasm allow the binding of importins to their cargoes, whereas high levels of Ran GTP in the nucleus induce their dissociation (Gorlich and Mattaj, 1996; Gorlich et al., 1995; Nigg, 1997). More recently, many other NLSs have been identified that do not conform to the classical NLS consensus motif with respect to size and/or highly basic character. Some of them are non-conventional importin α-interacting domains. This is the case of the influenza virus nucleoprotein (Wang et al., 1997), and the Borna disease virus p10 protein (Wolff et al., 2002).

The nuclear export of proteins and RNA is also a signal-dependent process mediated by soluble receptors called exportins. The best-characterized nuclear export signal (NESs) was first described in the HIV-Rev protein and then identified in an increasing number of proteins (Fischer et al., 1995). These leucine-rich NESs are recognized by the CRM1 nuclear export receptor. Leptomycin B (LMB) binds to the central domain of CRM1 to disrupt its interaction with the NES and provides a useful reagent for studying CRM1-mediated nuclear export (Fornerod et al., 1997; Nishi et al., 1994).

Rabies virus replicates in the host cell cytoplasm. It has a linear, non-segmented, single-strand RNA genome of negative polarity. The ribonucleoprotein (RNP) contains the RNA genome tightly encapsidated by the viral nucleoprotein (N) and the RNA polymerase complex which consists of the large protein (L) and its cofactor, the phosphoprotein (P). Both L and P are involved in transcription and replication. A positive-stranded leader RNA and five mRNAs are synthesized during transcription. The replication process yields nucleocapsids containing full-length antisense genome RNA, which in turn serves as a template for the synthesis of sense genome RNA.

Like the VSV P protein, the rabies virus P protein is a non-catalytic cofactor and a regulatory protein: it associates with the L protein in the polymerase complex and interacts with both soluble and genome-associated N proteins. The P protein contains two N protein-binding sites: one located in the amino-terminal part and the other in the carboxy terminal region (Chenik et al., 1994; Fu et al., 1994; Jacob et al., 2001). The major L-binding site resides within the first 19 residues of P (Chenik et al., 1998). The rabies virus P protein is phosphorylated by two kinases: the unique cellular protein kinase, RVPK (rabies virus protein kinase), and protein kinase C (Gupta et al., 2000). Both kinases phosphorylate specific sites on the P protein, leading to the formation of different phosphorylated forms of the P protein with different motilities in SDS–PAGE (Gupta et al., 2000). In addition, four other amino-terminally truncated products (P2, P3, P4, and P5) translated from P mRNA have been found in the purified virus, in infected cells and in cells transfected with a plasmid encoding the complete P protein. These shorter proteins are translated from internal in-frame AUG initiation codons by a leaky scanning mechanism (Chenik et al., 1995). Whereas P and P2 are located in the cytoplasm, P3–P5 are found mostly in the nucleus. The nuclear products of the P gene have been shown to interact with PML nuclear bodies that could be involved in a cellular defense mechanism against viral infection (Blondel et al., 2002).

In this study, we have analyzed the nuclear shuttling of the products of the rabies virus P gene. We show that the complete P has one NLS and one CRM1-dependent NES. The presence of these signals determines the intracellular localization of P and its products (P2–P5).

Results

The short P3 protein is conserved throughout the Lyssavirus genus

We have previously shown that the rabies CVS strain P mRNA directs the synthesis of four N-terminally truncated P products P2, P3, P4, and P5 due to translational initiation by a leaky scanning mechanism at internal Met codons 20, 53, 69, and 83, respectively (Chenik et al., 1995). Whereas P and P2 are located in the cytoplasm, P3, P4, and P5 are found mostly in the nucleus. Since Met 53 is conserved in the P protein of the most divergent lyssaviruses genotype, GT3 (Badrane et al., 2001), we analyzed first whether P3 was synthesized from Mokola P mRNA and then looked at its subcellular localization. For this, we constructed a plasmid containing the P gene of Mokola with the nucleotide sequence context of the first initiation codon of P mRNA.

Cells were infected with VTF7-3 recombinant vaccinia virus and were transfected with the plasmid encoding the P protein of Mokola (P_{Mok}) under the control of the T7 RNA polymerase promoter. Proteins present in cell extract were analyzed by immunoblotting with a mouse polyclonal anti-P antibody. As shown in Fig. 1A, the $P3_{Mok}$ protein that comigrated with the truncated $P\Delta N52_{Mok}$ protein initiated from the third AUG codons was also expressed from the Mokola P gene. In order to examine the intracellular localization of $P3_{Mok}$ in intact cells, the plasmid $pP\Delta N52_{Mok}$-GFP (encoding P3 fused to GFP) was transfected as described previously. Analysis by confocal microscopy shows that $P3_{Mok}$-GFP is found mainly in the nucleus and showed a distinct speckled nuclear localization similar to that of P3-GFP of the CVS strain (Figs. 1B and 3). This observation provides support for the conservation of the P3 protein and for its nuclear localization throughout the genus.

The difference in cellular localization of P and P3 suggests that there must be targeting information located either within P or P3 to account for their distinctive distributions. A conformational change resulting from truncation of the 52 first residues of P protein might reveal new targeting information within P3. Alternatively the lack of these 52 residues might remove a nuclear export signal

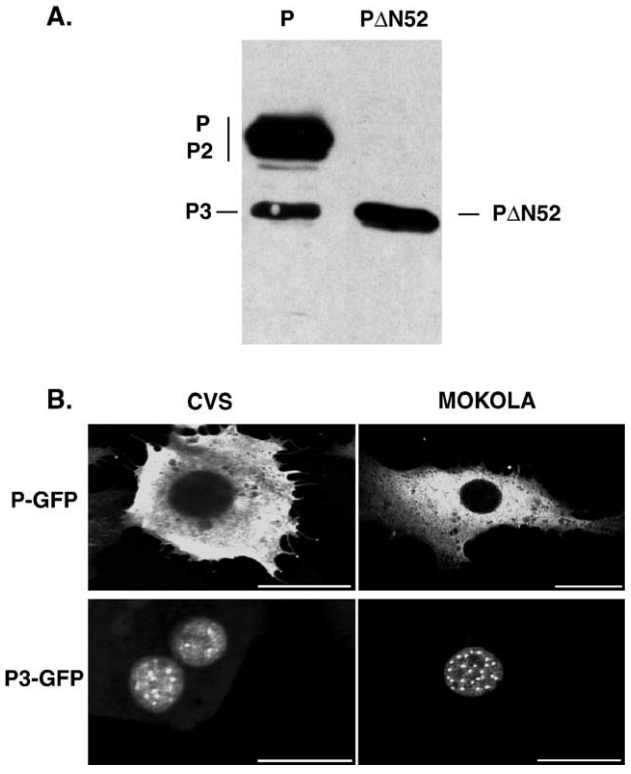

Fig. 1. P3 is expressed from P mRNA of Mokola and is nuclear. (A) BSR cells were infected with VTF7-3 and transfected with plasmids encoding P Mokola for 24 h. Cell extracts were analyzed by Western blotting. The blot was immunostained with the polyclonal anti-P antibody and visualized by the ECL system. (B) Immunostaining of P and P3 in transfected cells. Cells were transfected with plasmids encoding P-GFP or P3-GFP from Mokola or from CVS. After fixation, cells were analyzed for GFP expression by confocal fluorescence microscopy. The scale bars correspond to 20 μm.

that is dominant over nuclear targeting in the context of the complete P protein.

Nuclear localization of P3 requires the presence of a nuclear targeting sequence located between the residues 173–297

The localization of P3 within the nucleus suggested the presence of an NLS but a sequence resembling a classical motif could not be identified. To identify sequence(s) within the P protein that could function as an NLS, N-terminal and C-terminal truncations of P were fused to GFP (Fig. 2A). In contrast to the previous constructs encoding P_{Mok}, sequences upstream the AUG initiation codon have been converted to a Kozak consensus site to further increase the translation efficiency preventing downstream scanning. These truncated P proteins were expressed efficiently in BSR cells with the vaccinia T7 system and migrated as proteins of the expected molecular sizes (data not shown). Protein localization was determined by confocal microscopy (Fig. 2B). In contrast to GFP alone, which was distributed diffusely in both the cytoplasm and the nucleus, the proteins P3-GFP accumulated in the nucleus as P3. This result indicated that the nuclear targeting activity of P is located between residues 52–297. More extended deletions of 83, 138, and 172 amino acids from the amino-terminal part of P had a weak effect upon nuclear localization and these truncated proteins were predominantly nuclear. In contrast a C-terminal truncation of 125 residues abrogated the nuclear localization of the GFP fusion protein.

These findings indicate that the *carboxyl*-terminal part of P from 173 to 297 contains an active NLS.

Identification of the P residues required for nuclear localization

As mentioned above, P protein does not possess an obvious polybasic NLS sequence. However, inspection of the carboxy terminal part of P reveals a motif with a high proportion of basic residues (^{211}**KK**Y**K**^{214}) that is conserved in the seven different genotypes of lyssavirus. Moreover, the crystal structure of the C-terminal domain (186–297) of the P protein reveals that this lysine-rich motif lies on a surface exposed loop in close proximity to arginine 260, another basic residue (Mavrakis et al., 2004).

Mutational analysis of P3-GFP was performed in order to evaluate the role of this motif on nuclear import activity (Fig. 3). Upon transfection, mutated protein P3(^{211}**AA**YK^{214}-R^{260})-GFP exhibited a diffuse staining pattern in the nucleus indicating that this protein was efficiently imported into the nucleus. In contrast, cells expressing the protein P3(^{211}KKY**A**^{214}-R^{260})-GFP presented different staining patterns: an even fluorescence distribution between the nucleus and the cytoplasm or a cytoplasmic staining with few dots in the cytoplasm. The presence of cytoplasmic dots observed with the alanine substitution of lysine 214 could be the result of misfolding or altered stability of P3. However, when a negative aspartic acid residue was substituted to lysine 214, the localization of the protein P3(^{211}KKY**D**^{214}-R^{260})-GFP appeared diffuse in the cytoplasm without any granular staining. This finding indicates that the cytoplasmic localization of the mutants is not an artefact due to protein aggregation. Thus, a substitution of lysine residue at position 214 was sufficient to reduce the nuclear localization of P3-GFP whereas substitutions of the lysine residues at position 211 and 212 have little effect upon nuclear localization. In order to investigate the contribution of the arginine residue 260 in the nuclear import, we constructed the plasmid encoding P3(^{211}KKYK^{214}-**A**^{260})-GFP. This mutant protein exhibited a predominantly cytoplasmic and diffuse distribution indicating that the arginine residue also contributed efficiently to nuclear import activity. When both substitutions of lysine at position 214 and arginine in position 260 by alanine were introduced in the P3 sequence, the corresponding protein P3(^{211}KKY**A**^{214}-**A**^{260})-GFP was completely excluded from the nucleus. As expected, an aspartic acid substitution of lysine 214 associated with the alanine substitution on the arginine 260 also abolished nuclear import. These results

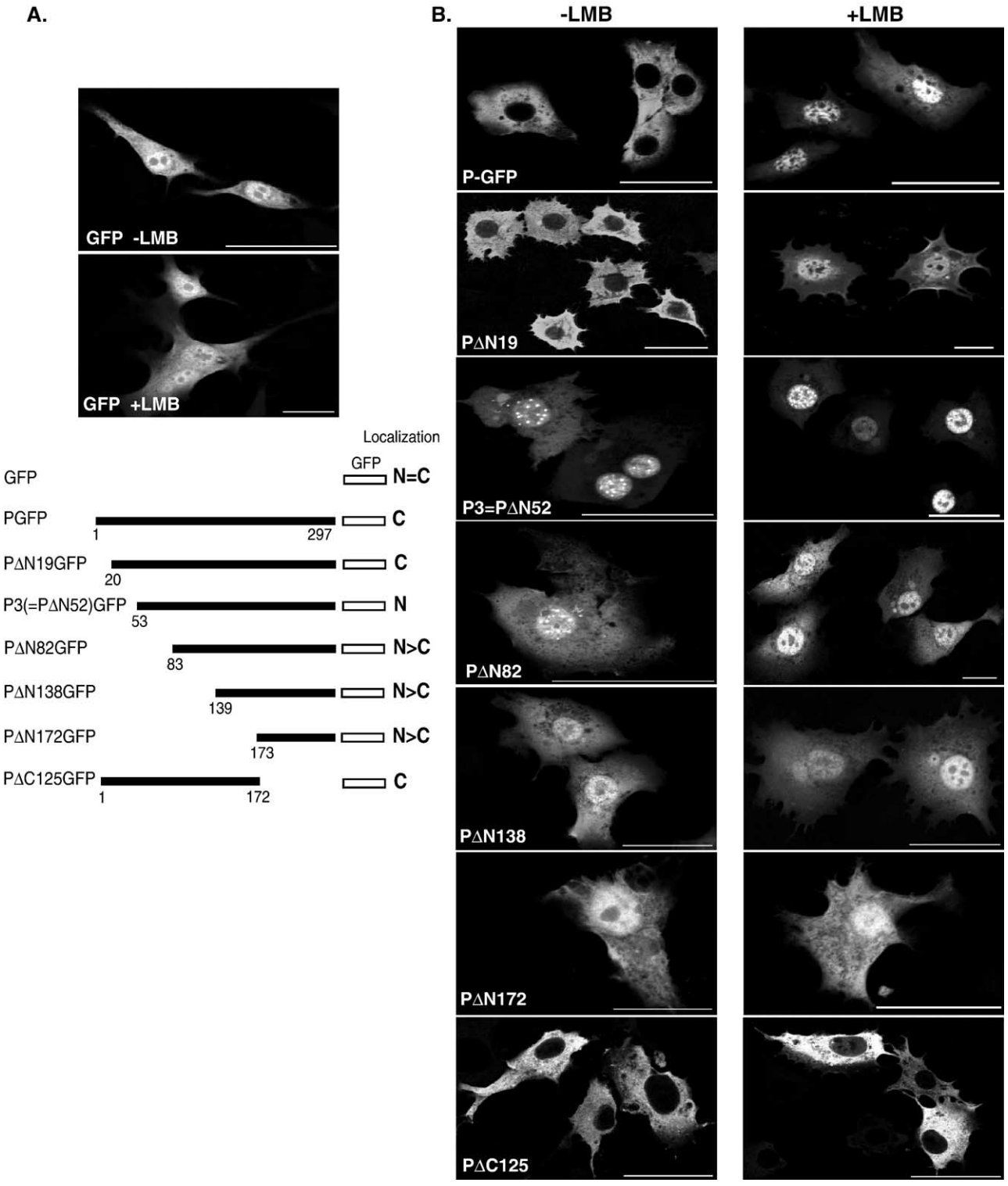

Fig. 2. Identification of an NLS in the P protein. (A) Construction of plasmids encoding truncated P protein fused to GFP. In this schematic representation, dark bars represent the protein product of each deleted gene with amino acid positions indicated. These mutants fused to GFP were expressed in BSR cells with the vaccinia T7 system as described in Material and methods. An immunofluorescence analysis was performed and the intracellular localization of these mutants in the cytoplasm (C) or in the nucleus (N) is summarized. (B) Cells were transfected with plasmids encoding truncated P proteins and incubated in the absence (−LMB) or presence of leptomycin B (+ LMB) at a final concentration of 20 nM during the time of transfection (6 h). After fixation, cells were analyzed for GFP expression by confocal fluorescence microscopy. GFP protein localization was also shown as control in the upper left panel. Representative results for a large number of cells examined in independent experiments are shown. The scale bars correspond to 40 μm.

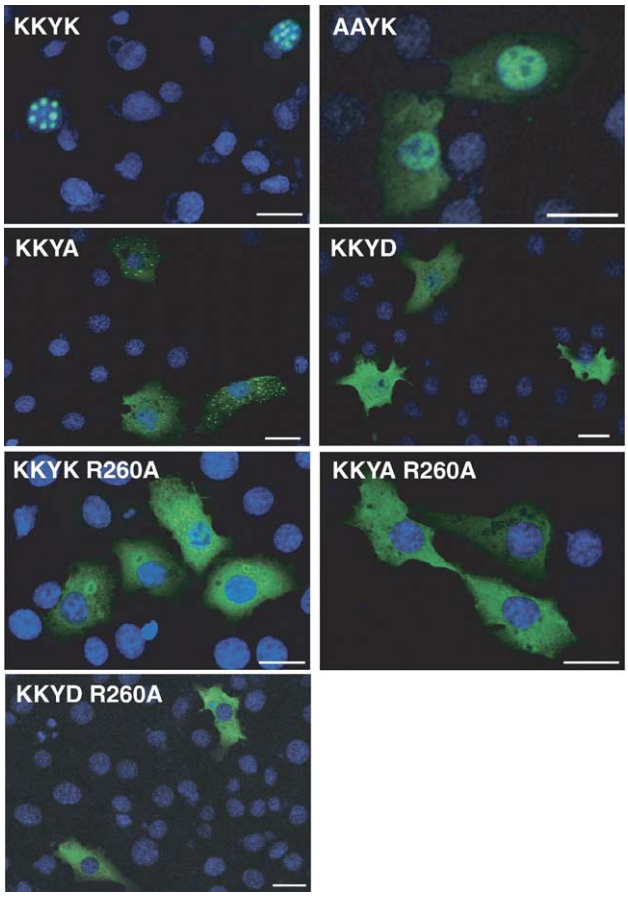

Fig. 3. Delineation of the residues involved in the NLS activity. Site-directed mutagenesis was performed on the motif ^{211}KKYK^{214}R^{260} of the plasmid encoding P3 protein fused to GFP as described in Material and methods. Cells were transfected with plasmids encoding P3(^{211}KKYK^{214}R^{260}), P3(^{211}**AA**YK^{214}R^{260}), P3(^{211}KKY**A**^{214}R^{260}), P3(^{211}KKY**D**^{214}R^{260}), P3(^{211}KKYK^{214}**A**^{260}), P3(^{211}KKY**A**^{214}**A**^{260}), and P3(^{211}KKY**D**^{214}**A**^{260}). Six hours after transfection, cells were fixed, stained with DAPI, and analyzed for GFP expression by confocal fluorescence microscopy. The scale bars correspond to 20 μm.

indicate that K214 and R260 are essential in the P3 nuclear activity and part of a conformational NLS.

Identification of a CRM1-dependent nuclear export signal (NES) within the P sequence

As the complete P protein contains the NLS motif but is cytoplasmic, its nuclear exclusion could be explained by the presence of a nuclear export activity. To determine whether there is a CRM1-dependent NES in P, cells expressing P-GFP were treated with LMB (20 nM), a specific inhibitor of nuclear export. Although P-GFP was cytoplasmic in untreated cells, its distribution was sensitive to LMB with retention of GFP fluorescence in the nucleus indicating that part of P protein was trapped in the nucleus (Figs. 2B and 4). LMB had no effect on the localization of GFP (Fig. 2A). Besides demonstrating the presence of CRM1-dependent NES within the P protein, this finding also indicates the nuclear entry of the complete P protein. To map the putative export signal, we analyzed the subcellular distribution of P deletion mutants fused to GFP and expressed in cells in the presence or absence of LMB (Figs. 2B and 4).

The localization of the amino-terminal deleted PΔN19-GFP protein was sensitive to the addition of LMB which resulted in a significant increase in the nuclear retention of the protein (Fig. 2B). In contrast, the localizations of others P constructs were all insensitive to LMB but for different reasons. As expected the nuclear localization of PΔN52 (P3-GFP) was unchanged in the presence of LMB. For shorter amino-terminal truncated P proteins (PΔN82, PΔN138, PΔN172) which are located in both cytoplasm and nucleus, LMB has no effect indicating the absence of a NES between residues 83–297 (Fig. 4). The fact that the carboxy terminal truncated P protein PΔC125 remained cytoplasmic in the presence of LMB confirms the absence of an NLS between residues 1–172. All these results show that a NES is located in the first 83 residues of P.

CRM1-dependent NESs are characterized by leucine-rich sequences (**L**XXX**L**XX**L**X**L**) although large hydrophobic amino acids (V, M, I) may be substituted to leucine. The examination of the first 83 residues of P revealed two stretches of amino acid residues similar to the characterized hydrophobic rich NES-like sequences (Fig. 5A). They were located between residues 11 and 20, 49, and 58.

In order to address whether the N-terminal domain confers nuclear export to a protein normally restricted to the nucleus, a chimerical protein with residues 6–60 of P fused to β-galactosidase carrying the SV40 T antigen NLS motif was expressed in transfected cells in the presence or absence of LMB (Figs. 5B and C). As expected NLS-β gal (used as a control) was exclusively nuclear. The fusion protein (NLS $P_{6–60}$-β gal) was excluded from the nucleus in the absence of LMB and its localization was sensitive to LMB (Fig. 5B). This result confirms that the domain 6–60 confers nuclear export ability to the fusion protein and contains at least one NES.

To determine more closely which motif is important for the nuclear export, we transferred two distinct fragments of the amino-terminal part of P ($P_{6–44}$, $P_{45–60}$) to the β-galactosidase carrying the SV40 T antigen NLS motif (Figs. 5B and C). The cells expressing the fusion protein NLS-$P_{6–44}$-βgal displayed a predominantly nuclear localization, which was unchanged in LMB treated cells. In contrast, the chimerical protein NLS-$P_{45–60}$-βgal was predominantly localized in the cytoplasm of untreated cells and the LMB treatment induced its accumulation in the nucleus. These findings are consistent with the presence of one functional NES between residues 49–58.

Since the first methionine of P3 (PΔN52) is located inside the NES sequence, P3 does not contain the intact NES. This probably explains the nuclear localization of P3. We then constructed the fusion protein PΔN44-GFP containing 9 more residues than P3-GFP in order to reconstitute the NES (Fig. 6A). In contrast to the original nuclear profile of P3, the completion of the NES to P3-GFP

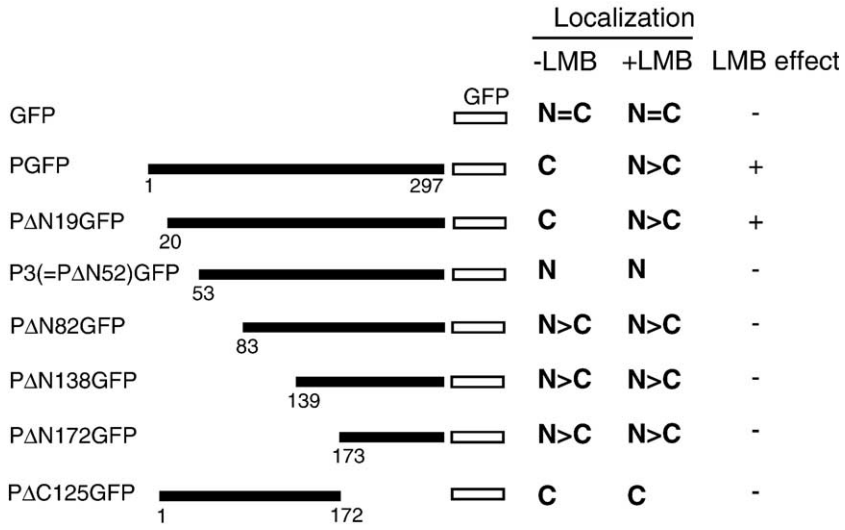

Fig. 4. Detection of the nuclear export signals in the N-terminal part of P. The localization of the truncated P proteins shown in Figs. 2A and B was compared in the absence (−LMB) or presence (+ LMB). The proteins were predominantly cytoplasmic (C), nuclear (N), or evenly distributed in cytoplasm and nucleus (N = C). From these results, the effect of LMB on the intracellular distribution of these truncated P proteins was summarized.

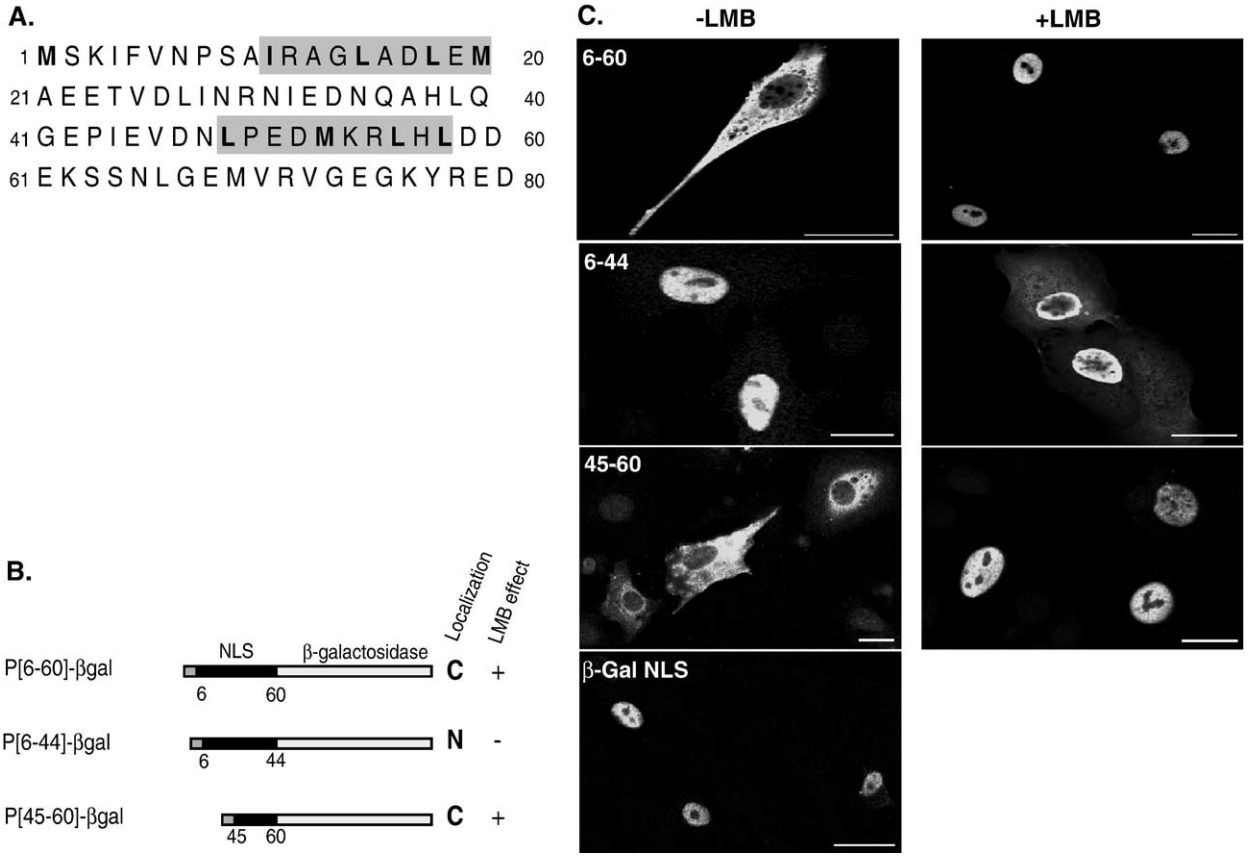

Fig. 5. Identification of a functional NES in the N-terminal part of P. (A) Examination of the N-terminal part of the sequence of P (1–83) revealed two stretches of amino acids residues similar to the characterized leucine-rich sequences NES sequence (**L**XXX**L**XX**L**X**L**), V, M, I may replaced leucine. These sequences were located between amino acids 11 and 20, 49, and 58 (gray boxes). (B) Different domains of the N-terminal part of P containing the putative NES (6–60, 6–44, 45–60, dark bars) were fused to the β-galactosidase (white bars) carrying the SV40 T antigen NLS motif (gray bars). The intracellular localization of these mutants in the cytoplasm (C) or in the nucleus (N) and the effect of LMB are summarized. (C) The plasmids described above were transfected into BSR cells. After 24 h, cells were incubated in the absence or presence of LMB at a final concentration of 20 nM for 2 h. After fixation, cells were permeabilized and were immunostained using the mouse monoclonal anti-β galactosidase followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG. The scale bars correspond to 20 μm.

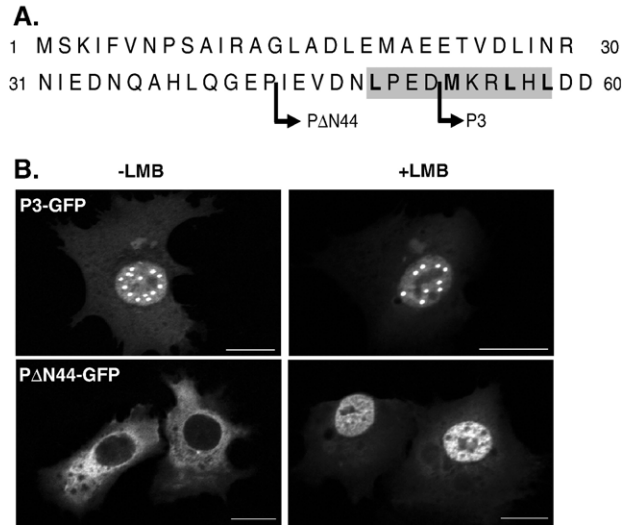

Fig. 6. NES is missing in the P3 protein. (A) The first methionine (bold) of P3 is located inside the NES (gray box). A fusion protein PΔN44-GFP containing 9 more residues than P3-GFP was constructed in order to reconstitute the NES. (B) PΔN44-GFP was expressed in BSR cells treated (right) or not (left) with LMB (as described in Fig. 2). After fixation, the cells were analyzed for GFP expression by confocal fluorescence microscopy. The scale bars correspond to 16 μm.

resulted in the redistribution of the protein to the cytoplasm (Fig. 6B). As expected, the protein PΔN44-GFP was sensitive to the LMB treatment. This result shows that the nuclear localization of P3 is due to the absence of the NES.

Discussion

In this report, we describe that rabies virus P protein is a nucleocytoplasmic shuttling protein that contains a nuclear localization signal (NLS) located in the C-terminal part and a nuclear export signals (NES) located in the N-terminal part of the protein. Although we have not demonstrated a direct interaction of the P protein with a karyopherin, the motif that mediates nuclear import (^{211}KKY**K**^{214} and **R**^{260}) is a cluster of basic residues exposed on the surface as shown from the recently solved crystal structure of the carboxy terminal domain of the P protein (Mavrakis et al., 2004). Therefore, this basic patch probably constitutes a conformational NLS which is conserved in the seven different genotypes of lyssavirus. The same situation exists for M1 protein of Influenza virus that has a positively charged patch on the surface of its N-terminal domain that functions as NLS (Arzt et al., 2001; Ye et al., 1995).

The nuclear export signal of rabies P protein at positions 49–58 is canonical with a high leucine content and is absolutely conserved throughout the lyssavirus genus.

In the light of these results, both P and the small P products P2–P5 contain the NLS but P and P2 are excluded from the nucleus because they contain the NES. It appears that NES counteracts the nuclear import of P to remove P from the nucleus. However, the inhibition of CRM1-dependent export by LMB does not result in a complete nuclear retention of P in the nucleus. Therefore, it is also possible that only a fraction of P goes in the nucleus.

Our data suggest that the intracellular localization of the products of the P gene is tightly regulated. The fact that we have also identified P3 from Mokola virus, the most divergent lyssaviruses genotype, and that its localization is also nuclear, suggests that this regulation is probably conserved throughout the lyssavirus genus. However, the interplay between NLS and NES sequences may only be part of the complexity of the cellular localization of P. Other control mechanisms such as phosphorylation, oligomerization, or interaction with viral or cellular partners may influence the localization of the P products.

It has been shown that phosphorylation or dephosphorylation in the vicinity of an NLS or NES may play a role in the intracellular distribution of proteins (Jans and Hubner, 1996). Rabies virus P proteins are phosphorylated (Gupta et al., 2000; Tuffereau et al., 1985) and the position of one of the phosphorylation sites (S210) is right next to the NLS on the surface of the protein (Mavrakis et al., 2004). Therefore, phosphorylation of P proteins could have an effect on nuclear import as it has been shown for nuclear import of the T-antigen (Hubner et al., 1997). Alternatively, oligomerization of P products could also play a role in their localization. Indeed, P and small P products contain the oligomerization domain which is most probably located between the residues 93 and 130 (Gigant et al., 2000; Jacob et al., 2001) and form oligomers although it is not clear whether these are trimers or tetramers (Gigant et al., 2000; Mavrakis et al., 2003).

In infected cells, the interaction of P with viral partners such as the polymerase L and the N protein may also contribute to the regulation of nucleocytoplasmic shuttling of the P protein. Interestingly the motif ^{211}KKYK^{214}, which is part of the NLS motif, is also required for the interaction with the N-RNA (Jacob et al., 2001; Mavrakis et al., 2004). Therefore, the nuclear import activity of P may be blocked by the direct binding of N to P. Finally, interaction with cellular partners may contribute to the localization of the P gene products. P protein has been shown to interact with the dynein light chain LC8, a cellular factor implicated in retrograde axonal transport that mediates the RNP transport along the neuronal axons (Jacob et al., 2000; Raux et al., 2000). In addition, the nuclear P3 protein has been shown to interact with PML nuclear bodies (Blondel et al., 2002). This could explain why P3 is the only small P protein that shows punctuate staining in nuclei of transfected cells. However, formation of nuclear dots does not require PML since the same localization exists in PML−/− cells (Blondel et al., 2002). The nature of these nuclear structures is still unknown but could be due to specific interaction of P3 (but not P4 and P5) with nuclear proteins, which may retain P3 in such structures. Such an interaction could involve the first residues of P3 corresponding to amino acids 53–67 of P and explain that P3 is more nuclear than shorter P products.

In regards to the role of the nuclear products of the P gene, it has been proposed that P3 via its interaction with PML may overcome the antiviral response of the infected cells (Blondel et al., 2002). Interaction of viral proteins with nuclear proteins is not restricted to viruses that replicate in the nucleus. Many RNA viruses whose replication strictly takes place in the cytoplasm use the nucleus or nuclear components in order to facilitate replication and/or alter host cell function and inhibit antiviral responses (Hiscox, 2003).

The presence of nuclear import and export signals on P protein insures representation of complete or truncated P proteins in the two cellular compartments. This contributes to an increase in the number of various P forms already observed as oligomeric or phosphorylated forms and which perform diversified functions in the virus life cycle.

Materials and methods

Cell cultures, antibodies, and leptomycin B (LMB) treatment

BSR cells, cloned from BHK 21 (baby hamster kidney), were grown at 37 °C in DMEM supplemented with 10% fetal calf serum (FCS). The mouse polyclonal anti-P antibody has been described previously (Raux et al., 1997). The monoclonal anti-β galactosidase antibody was bought from Promega and used as described by the manufacturer. LMB (Sigma) was added to culture medium to a final concentration of 20 nM.

Plasmid constructions

pMC.P encoding the wild-type P of the CVS has been described previously (Chenik et al., 1994). The constructs pP-GFP, pP△N19-GFP, pP△N52-GFP, pP△N82-GFP, pP△N138-GFP, and pP△N172-GFP have been described previously (Blondel et al., 2002). The plasmid pP△C125-GFP was generated by PCR using reverse primers to delete the last 375 nucleotides of P. Amplified cDNA was cut with *AgE*1 and *Eco*R1 and used to replace the P gene *AgE*1/*Eco*R1—excised from the plasmid pCDNA1 encoding P-GFP.

The plasmid pCMV-NLS.βgal containing the SV40 T antigen NLS into plasmid pCMVβ has been described elsewhere (Hiriart et al., 2003) and was a generous gift from Dr. E. Manet (from the Unité de Virologie Humaine, U412 INSERM-ENS Lyon). Different fragments of P (6–60, 6–44, and 45–60) were amplified by PCR using, respectively, the sets of oligonucleotides (5′CCGAGATCTGTTAATAGTGCA3′, 5′CCGAGATCTATCGTCCAGGTG3′; 5′CCGAGATCTGTTAATAGTGCA3′, 5′CCGAGATCTTTCTATGGGTTC3′; 5′CCGAGATCTGAAGTGGACAAC3′, 5′CCGAGATCTATCGTCCAGGTG3′). The amplified double-stranded cDNAs were digested with *bgl*II and inserted downstream of the SV40 T antigen NLS into a *bgl*II site of pCMVNLS βgal.

Site-directed mutagenesis

A two-step PCR-based site-directed mutagenesis approach was used to generate amino acid substitutions in pP-GFP. In a first step, two overlapping PCR fragments were amplified from pP-GFP with primer combinations specific of the mutation (1 and 2) as described in Table 1. In a second step, aliquots of the two PCR products were mixed, annealed, and used as template for a second round of amplification with P- or P3-specific forward primers (5′GCCGAATTCATGAGCAAGATCTTT3′, 5′GCCGAATTCATGAAGCGACTTCAC3′) and GFP-specific reverse primer (5′GCCTCTAGATTACTTGTACAGCTC 3′) in order to amplify the whole mutation containing gene. The products of this second reaction were digested with *Eco*RI/*Xba*I and used to replace the *Eco*RI/*Xba*I excised P-GFP. By using this strategy, we obtained the constructs encoding mutated P or P3 in fusion with GFP with one or two amino acid substitutions (in boldface) in the motif ^{211}KKYK^{214} associated or not with one amino acid R^{260} substitution: pP or pP3(^{211}KKY**A**^{214}-R^{260}), pP or pP3(^{211}KKY**D**^{214}-R^{260}), pP or pP3(^{211}**AA**YK^{214}-R^{260}), pP or pP3(^{211}KKYK^{214}-

Table1
Primers used for the first step of the two-step mutagenesis PCR products

Construction	Sense (1) and antisense (2) primer for the first PCR step	Template
pP(^{211}KKY**A**^{214}-R^{260})	(1) 5′ CCAAGAAGTAC**GCG**TTTCCCTCCCGA 3′	
pP3(^{211}KKY**A**^{214}-R^{260})	(2) 5′ TCGGAGGGAAA**CGC**GTACTTCTTGG 3′	pP-GFP
pP(^{211}KKY**D**^{214}-R^{260})	(1) 5′ CCAAGAAGTAC**GAC**TTTCCCTCCCGA 3′	
pP3(^{211}KKY**D**^{214}-R^{260})	(2) 5′ TCGGGAGGGAAA**GTC**GTACTTCTTGG 3′	pP-GFP
pP(^{211}**AA**YK^{214}-R^{260})	(1) 5′ GAAAGCTTTTCC**GCGGCG**TACAAGTTTCCCTCCCGA 3′	
pP3(^{211}**AA**YK^{214}-R^{260})	(2) 5′ TCGGGAGGGAAACTTGTA**CGCCGC**GGAAAAGCTTTC 3′	pP-GFP
pP(^{211}KKYK^{214}-**A**^{260})		pP-GFP
pP3(^{211}KKYK^{214}-**A**^{260})		pP-GFP
pP(^{211}KKY**A**^{214}-**A**^{260})	(1) 5′ AAAATCCCCCT**GGC**GTGCGTACTGGGA 3′	pP (^{211}KKY**A**^{214}-R^{260})
pP3(^{211}KKY**A**^{214}-**A**^{260})	(2) 5′ TCCCAGTACGCAC**GCC**AGGGGGATTTT 3′	pP3 (^{211}KKY**A**^{214}-R^{260})
pP(^{211}KKY**D**^{214}-**A**^{260})		pP (^{211}KKY**D**^{214}-R^{260})
pP3(^{211}KKY**D**^{214}-**A**^{260})		pP3 (^{211}KKY**D**^{214}-R^{260})

$\mathbf{A}^{260}$, pP or pP3(^{211}KKYK$\mathbf{A}^{214}$-$\mathbf{A}^{260}$), and pP or pP3(^{211}KKYK$\mathbf{D}^{214}$-$\mathbf{A}^{260}$).

Plasmid transfections

BSR cells were grown on sterile glass cover slips in 6 well plates and were transfected (Parker and Stark, 1979). After 24 h at 37 °C cells were prepared for immunofluorescence staining.

In some experiments, proteins were transiently expressed by using the vaccinia T7 expression system according to the method of Fuerst et al. (1986). Briefly, BSR cells were infected with VTF7-3 at a multiplicity of infection of 5 PFU per cell. After 1 h of adsorption the cells were transfected with 2 μg of plasmid and were incubated for 6 h at 37 °C.

Immunofluorescence staining and confocal microscopy

Cells were fixed in 4% paraformaldehyde for 15 min at 4 °C and permeabilized for 5 min with 0.1% Triton X-100 in PBS. GFP distribution was analyzed directly after fixation. The intracellular distribution of β-galactosidase constructions was analyzed by immunostaining using the anti-β-galactosidase antibody at a dilution of 1/1000 and the corresponding anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Molecular Probes). The cells were mounted in mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) to stain nuclei.

Confocal laser microscopy was performed on a Leica SP2 microscope (40× oil-immersion objective) using ultraviolet excitation at 351 nm (DAPI) and/or blue laser excitation at 488 nm (Alexa 488, GFP) in simultaneous recording mode.

Western blot analysis

Cells were washed and re-suspended in PBS, lysed in hot Laemmli sample buffer, and boiled for 5 min. About 20 μg of protein was analyzed on a 10% SDS–PAGE gel and transferred onto a nitrocellulose membrane. The proteins were blocked on the membranes with 10% skimmed milk in TBS for 2 h and incubated overnight at 4 °C with the rabbit polyclonal anti-P antibody. The blots were then washed extensively in TBS–Tween and incubated for 1 h with the peroxidase-coupled secondary antibodies (Amersham). The blots were revealed by chemoluminescence (ECL, Amersham).

Acknowledgments

We acknowledge Dr. E. Manet (from the Unité de Virologie Humaine, U412 INSERM-ENS Lyon) for the plasmid pCMV-NLS.βgal. We would like to thank C. Maheu for technical assistance.

References

Arzt, S., Baudin, F., Barge, A., Timmins, P., Burmeister, W., Ruigrok, R.W.H., 2001. Combined results from solution studies on intact influenza virus M1 protein and from a new crystal form of its N-terminal domain show that M1 is an elongated monomer. Virology 279, 439–446.

Badrane, H., Bahloul, C., Perrin, P., Tordo, N., 2001. Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. J. Virol. 75, 3268–3276.

Blondel, D., Regad, T., Poisson, N., Pavie, B., Harper, F., Pandolfi, P.P., De The, H., Chelbi-Alix, M.K., 2002. Rabies virus P and small P products interact directly with PML and reorganize PML nuclear bodies. Oncogene 21, 7957–7970.

Chenik, M., Chebli, K., Gaudin, Y., Blondel, D., 1994. In vivo interaction of Rabies virus phosphoprotein (P) and nucleoprotein (N): existence of two N-binding sites on P protein. J. Gen. Virol. 75, 2889–2896.

Chenik, M., Chebli, K., Blondel, D., 1995. Translation initiation at alternate in-frame AUG codons in the rabies virus phosphoprotein mRNA is mediated by a ribosomal leaky scanning mechanism. J. Virol. 69, 707–712.

Chenik, M., Schnell, M., Conzelmann, K.K., Blondel, D., 1998. Mapping the interacting domains between the rabies virus polymerase and phosphoprotein. J. Virol. 72, 1925–1930.

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

Fornerod, M., Ohno, M., Yoshida, M., Mattaj, I.W., 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell 90, 1051–1060.

Fu, Z.F., Zheng, Y., Wunner, W.H., Koprowski, H., Dietzschold, B., 1994. Both the N- and the C-terminal domains of the nominal phosphoprotein of Rabies virus are involved in binding to the nucleoprotein. Virology 200, 590–597.

Fuerst, R.T., Niles, E.G., Studier, F.W., Moss, B., 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 83, 8122–8126.

Gigant, B., Iseni, F., Gaudin, Y., Knossow, M., Blondel, D., 2000. Neither phosphorylation nor the amino-terminal part of Rabies virus phosphoprotein is required for its oligomerization. J. Gen. Virol. 81, 1757–1761.

Gorlich, D., Mattaj, I.W., 1996. Nucleocytoplasmic transport. Science 271, 1513–1518.

Gorlich, D., Kostka, S., Kraft, R., Dingwall, C., Laskey, R.A., Hartmann, E., Prehn, S., 1995. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. Curr. Biol. 5, 383–392.

Gupta, AK., Blondel, D., Ghoudhary, S., Banerjee, A.K., 2000. The phosphoprotein of Rabies virus is phosphorylated by a unique cellular protein kinase and specific isomers of protein kinase C. J. Virol. 74, 91–98.

Hiriart, E., Farjot, G., Gruffat, H., Nguyen, M.V., Sergeant, A., Manet, E., 2003. A novel nuclear export signal and a REF interaction domain both promote mRNA export by the Epstein–Barr virus EB2 protein. J. Biol. Chem. 278, 335–342.

Hiscox, J.A., 2003. The interaction of animal cytoplasmic RNA viruses with the nucleus to facilitate replication. Virus Res. 95, 13–22.

Hubner, S., Xiao, C.Y., Jans, D.A., 1997. The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. J. Biol. Chem. 272, 17191–17195.

Jacob, Y., Badrane, H., Ceccaldi, P.E., Tordo, N., 2000. Cytoplasmic dynein LC8 interacts with lyssavirus phosphoprotein. J. Virol. 74, 10217–10222.

Jacob, Y., Real, E., Tordo, N., 2001. Functional interaction map of lyssavirus phosphoprotein: identification of the minimal transcription domains. J. Virol. 75, 9613–9622.

Jans, D.A., Hubner, S., 1996. Regulation of protein transport to the nucleus: central role of phosphorylation. Physiol. Rev. 76, 651–685.

Kalderon, D., Richardson, W.D., Markham, A.F., Smith, A.E., 1984. Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature 311, 33–38.

Kohler, M., Speck, C., Christiansen, M., Bischoff, F.R., Prehn, S., Haller, H., Gorlich, D., Hartmann, E., 1999. Evidence for distinct substrate specificities of importin alpha family members in nuclear protein import. Mol. Cell. Biol. 19, 7782–7791.

Mavrakis, M., Iseni, F., Mazza, C., Schoehn, G., Ebel, C., Gentzel, M., Franz, T., Ruigrok, R.W.H., 2003. Isolation and characterisation of the rabies virus N°-P complex produced in insect cells. Virology 305, 406–414.

Mavrakis, M., McCarthy, A., Roche, S., Blondel, D., Ruigrok, R.W., 2004. Structure and function of the C-terminal domain of the polymerase cofactor of rabies virus. J. Mol. Biol. 343, 819–831.

Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S., Beppu, T., 1994. Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. J. Biol. Chem. 269, 6320–6324.

Nigg, E.A., 1997. Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature 386, 779–787.

Parker, B.A., Stark, G.R., 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. 31, 360–369.

Raux, H., Iseni, F., Lafay, F., Blondel, D., 1997. Mapping of monoclonal antibody epitopes of the Rabies virus P protein. J. Gen. Virol. 78, 119–124.

Raux, H., Flamand, A., Blondel, D., 2000. Interaction of the rabies virus P protein with the LC8 dynein light chain. J. Virol. 74, 10212–10216.

Robbins, J., Dilworth, S.M., Laskey, R.A., Dingwall, C., 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell 64, 615–623.

Tuffereau, C., Fisher, S., Flamand, A., 1985. Phosphorylation of the N and M1 proteins of rabies virus. J. Gen. Virol. 66, 2285–2289.

Wang, P., Palese, P., O'Neill, R.E., 1997. The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal. J. Virol. 71, 1850–1856.

Wolff, T., Unterstab, G., Heins, G., Richt, J.A., Kann, M., 2002. Characterization of an unusual importin alpha binding motif in the Borna disease virus p10 protein that directs nuclear import. J. Biol. Chem. 277, 12151–12157.

Ye, Z., Robinson, D., Wagner, R.R., 1995. Nucleus-targeting domain of the matrix protein (M1) of influenza virus. J. Virol. 69, 1964–1970.