The HIV-1 Rev is a shuttling protein required for the nuclear export of unspliced and partially spliced viral mRNA. In this study, we have identified a new Rev-interacting protein, that specifically interacts with the Rev nuclear export signal both in yeast and mammalian cells. This protein has features found in nucleoporins including many phenylalanine-glycine repeats, a very high serine content, a putative zinc finger, and a coiled-coil domain; we thus called it NLP-1 (nucleoporin-like protein 1). In addition, gene expression analysis and wheat germ agglutinin chromatography experiments suggested that NLP-1 is an ubiquitous O-glycosylated nuclear protein. Recently, a cellular factor called CRM-1 has been shown to be an essential nuclear export factor interacting directly with nuclear export signals including the Rev nuclear export signal in a RanGTP-dependent manner. We show here that NLP-1, like the previously described Rev-interacting protein hRIP/Rab and several nucleoporins, also interacts with CRM-1 both in yeast and mammalian cells.

The genome of lentiviruses has evolved from simple retroviruses encoding the classical gag, pol, and env structural genes toward complexity, mainly by increasing the number of additional genes. Lentiviruses are limited to a single transcription unit, and controlled protein expression is mainly achieved by a very large usage of alternative and facultative splicing allowing the production of multiple mRNA with different coding specificities. Consequently, most mRNA are either unspliced or partially spliced and would normally be retained in the nucleus until fully spliced. This apparent problem is circumvented in lentivirus such as HIV-1 by the use of a particular transacting factor called Rev, which bypasses the cellular machinery by specifically inducing the nuclear export of these incompletely spliced viral mRNA.

HIV-1 Rev is a small nuclear/nucleolar protein that binds specifically to a highly structured 350-nucleotide-long RNA sequence called the Rev response element (RRE) present in all incompletely spliced viral RNA (1–3). The Rev/RRE interaction is an ordered process. Rev binds initially to a single high affinity site located at the apex of the longest stem of the RRE (4–7). This nucleation event allows additional Rev molecules to multimerize on the RRE through co-operative protein/protein and protein/RNA interactions (3, 4, 8, 9). Rev binding to the RRE is mediated by a highly basic N-terminal domain, which also specifies nuclear/nucleolar localization. In addition to this RNA recognition domain, Rev also carries a short C-terminal leucine-rich motif now called the nuclear export signal (NES) that authorizes Rev to shuttle between the nucleus and the cytoplasm (10, 11). Structurally similar NES have now been found in many other viral and cellular proteins (for review see Ref. 12). The NES is transferable to heterologous proteins, and mutations in this domain impair both Rev shuttling and Rev-dependent export of RNA. Furthermore, when injected into Xenopus oocyte nuclei, a Rev NES peptide is able to competitively inhibit the Rev-dependent export of RNA as well as the export of 5 S rRNA and U1 small nuclear RNA (13). When Rev multimerizes on the RRE, NES motifs are exposed to the outside of the RNA-protein complex and remain accessible for protein contact (14). Altogether, these observations have suggested that nuclear export of viral mRNA is mediated by a direct interaction between the Rev NES and cellular co-factors.

The first attempts to isolate Rev co-factors for export were performed using the two-hybrid interaction trap in yeast. A human nucleoporin-like protein called hRIP/Rab in human and a yeast nuclear pore-associated protein, Rip-1p, were shown to interact specifically with functional NES, which was consistent with a role of Rev in nuclear export of RNA (15–17). Subsequent experiments performed in yeast have demonstrated that many well characterized yeast or mammalian nucleoporins (Nup) including Nup214/can, yNup159, yNup100, Nup153, Nup98, Pom121, yNup49p, yNup57p, yNup100p, yNup116p, yNup145p, rNup98 also contact the Rev NES with a similar specificity (17–19). However, direct binding using recombinant proteins has been very difficult to demonstrate, suggesting that these interactions are indirect and involve a molecular bridge conserved between yeast and mammals.

Recently the identification of CRM-1 as an essential nuclear export factor has clarified the problem (20–24). CRM-1 was initially described in Schizosaccharomyces pombe as an essential protein involved in the control of higher order of chromosome structure and gene expression (25). Leptomycin B, an antifungal and antitumor antibiotic with cell cycle arresting activity, was shown to target specifically CRM-1 (26). Recently, the report of Wolff et al. (27) showing that leptomycin B was a potent inhibitor of HIV-1 Rev-dependent nuclear export has suggested the possible implication of CRM-1 in this process. Simultaneously, Fornerod et al. (28) have found CRM-1 associated with Nup214 and Nup88 in a dynamic subcomplex localized at the nuclear membrane. They have also shown that CRM-1 shares significant homology within its N-terminal domain with a family of Ran-binding proteins including importin β, a cellular protein involved in nuclear import. Definite demonstration of the implication of CRM-1 in nuclear protein ex-
port was shown by different approaches in yeast and human cells. Taking advantage of a temperature-sensitive CRM-1 mutant strain of Saccharomyces cerevisiae, two groups reported that nuclear export of NES-containing proteins is blocked at a nonpermissive temperature (21, 23). Furthermore, CRM-1 has been shown to interact with different NES motifs in vitro and in vivo in a complex that also includes the Ran GTPase in its GTP-bound form (20, 22, 23). In addition, binding of leptomycin B to CRM-1 inhibits the formation of the NES-CRM-1 complex (20).

Although the involvement of CRM-1 in nuclear export as a receptor for NES-bearing proteins is now well documented, the question of the translocation of the NES-CRM-1 trimolecular complex through the nuclear pore complex (NPC) has still to be addressed. For example, we do not know the exact significance of the association of CRM-1 with FG-rich proteins including those that have been reported to interact with Rev in the two-hybrid system (29). Furthermore, a significant fraction of mammalian NPC-associated proteins, which are possibly involved in nucleo-cytoplasmic transport, have not been characterized yet.

Here we report the identification of a new FG-rich NLP-1 (nucleoporin-like protein 1) interacting with functional NES sequences including the Rev NES. Similar to the previously identified Rev-interacting factor hRIP/Rab, this protein is nuclear and associate with yeast and human CRM-1. The possible involvement of NLP-1 as well as hRIP/Rab in the first steps of protein nuclear export through the recruitment of the NES-CRM-1 complex to the NPC is discussed.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides**—The oligonucleotides used are: 1, 5'-CGATCCTCGACTTGCTATCTGAAATTCGGCGTACTCACCAGTCGCCGCCCCT-3'; 2, 5'-TGACCTGGCTCCCAAGGNGNCCCTCCCNNGAGGCTNNACTNNAGTCCGAACAGGAAGTTGGGAACCTTGCGAGCGGTTGTGGGATCAGGGGACCCGTCCCTCACTCCATCCGGGATTTCA-3'; 3, 5'-CTGATTTGAAGGCTTCGTTCCACAACCACCTCTGGCCCTGACAGACTTGCCTTCTGTCGCCAATTGAACTGCGTCG-3'; 4, 5'-CTGATTTGAAGGCTTCGTTCCACAACCACCTCTGGCCCTGACAGACTTGCCTTCTGTCGCCAATTGAACTGCGTCG-3'.

**Yeast Interaction Trap**—The yeast two-hybrid interaction trap was carried out using the LexA system (30). Yeast cells were transformed with these plasmids expressing fusion proteins linking the LexA DNA-binding domain to NES mutated Rev proteins as described in Materials and methods. The plasmids expressing fusion proteins linking the LexA DNA-binding domain to NES mutated Rev proteins were constructed as described previously. Oligonucleotides 3 and 4 in which the sequence encoding for leucines 75, 78, 81, and 83 of the Rev NES have been randomized were phosphorylated, hybridized, and cloned into SpeI-BglI-cut plasmid pLexM10. XL1 bacterial strain was transformed with the ligated DNA, and the resulting plasmid library consisting of approximately 2 × 10^10 independent clones was expressed in yeast cells. The expression of LexA fused to NES containing plasmids was selected by growing colonies on plates containing 200 μg/ml G418. The LexA-Fus was visualized by indirect immunofluorescence (see below) and by classical Western blot. The clones expressing the LexA-Fus fusion protein were isolated by limited proteolysis of the fusion proteins. The resulting fusion proteins were precipitated by trypsin and analyzed by non-denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The positions of the LexA-Fus proteins were determined by alkaline phosphatase labeling of the fusion proteins and were identified by immunoblotting with an antibody specific for LexA.

**NLP-1 Expression Analysis**—Multiple tissue Northern blot (CLONTECH) was hybridized with a uniformly 32P-labeled NLP-1 cDNA probe generated by random priming (Amersham). Oligo-cytosylation of NLP-1 was also performed in vivo or in vitro expressed proteins with wheat germ agglutinin (WGA)-Sepharose (Sigma) for 30 min at 4 °C. After washing in 0.1% Nonidet P-40, proteins were separated on acrylamide gels and visualized either by direct autoradiography or by classical Western blot. NLP-1 subcellular localization was performed by Western blot with total, nuclear, or cytoplasmic extracts. HeLa cells were washed and scraped in ice-cold phosphate-buffered saline (PBS). Total cellular extract was obtained by cell lysis in RIPA buffer for 30 min at 4 °C. The nuclei were pelleted by centrifugation at 14,000 × g for 20 s at 4 °C, and the cytoplasmic fraction-containing supernatant was recovered. The nuclear fraction was obtained by incubating nuclei in 500 μl of high salt buffer (RIPA buffer, 500 mM NaCl, 1 mM Pefabloc, 2 mM leupeptin) for 40 min at 4 °C followed by a 15-min centrifugation at 12,000 × g.

**Indirect Immunofluorescence**—A GST-NLP-1Δ fusion protein was expressed in XL-1 bacteria and purified from inclusion bodies as described in Materials and methods. A specific antiserum against GST-NLP-1 (Covalab) and affinity-purified on a glutathione-agarose GST-NLP-1Δ column according to Ref. 34. Indirect immunofluorescence was done on HeLa cells transiently expressing NLP-1 as described before (35). 48 h after transfection, cells grown on coverglasses were washed three times with PBS and fixed in 4% paraformaldehyde for 15 min. They were washed with PBS, permeabilized in PBS with 0.5% Triton X-100 for 5 min, and incubated in PBS with 2% bovine serum albumin for another 10 min. Incubation with affinity-purified anti-NLP-1Δ antiserum was performed for 30 min at room temperature followed by three washes in PBS. Finally, cells were incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma), washed, and mounted in 1,4-diazabicyclo[2.2.2]octane.

**Degenerate NES Library Construction and Screening**—A library of plasmids expressing fusion proteins linking the LexA DNA-binding domain to NES mutated Rev proteins was constructed as described above. Oligonucleotides 3 and 4 in which the sequence encoding for leucines 75, 78, 79, 81, and 83 of the Rev NES have been randomized were phosphorylated, hybridized, and cloned into SpeI-BglII-cut plasmid pLexM10. XL1 bacterial strain was transformed with the ligated DNA, and the resulting plasmid library consisting of approximately 2 × 10^10 independent clones was expressed in yeast cells. The expression of LexA fused to NES containing plasmids was selected by growing colonies on plates containing 200 μg/ml G418. The LexA-Fus was visualized by indirect immunofluorescence (see below) and by classical Western blot. The clones expressing the LexA-Fus fusion protein were isolated by limited proteolysis of the fusion proteins.

**Rev Reporter Construct and Expression Plasmids**—Rev mutants interacting with NLP-1 were tested for their ability to substitute for Rev and to promote export of a RRE-containing unspliced mRNA. For this purpose, a new Rev reporter plasmid derived from pAAC plasmid (36) was constructed by assembling PCR-amplified fragments derived from pNL4.3 HIV-1 provirus (see Fig. 4A). The DNA fragment coding for the first tat 5′ splice site was generated by hybridizing phosphorylated oligonucleotides 7 and 6. The DNA sequence coding for the first tat 3′ splice site was amplified with oligonucleotides 9 and 8. Plasmid pAAC-tat5 was constructed by cloning tat 5′ splice site sequence into XbaI-EcoRI-digested pAAC. Sequences coding for Gag pl17 protein and for the 264-nucleotide-long RRE were amplified with oligonucleotides 9 and 10 and oligonucleotides 11 and 12. The PCR fragments were then digested with EcoRI and XhoI, NcoI and BglII, and BglII and HindIII, respectively, for the Gag pl17, the tat 3′ splice, site, and the RRE and were subsequently ligated into the EcoRI- and HindIII-cut pAACTat5 construct to generate pVR51. DNA sequences coding for wild type, M10, M4 as well as for selected Rev mutants were subcloned into pSG5Flag expression plasmid (37). 500 ng of CsCl-purified pVRS1 reporter plasmid and 20 or 100 ng of each Rev expressing plasmid were transfected into HeLa cells by the calcium phosphate method using 10 μg of pUC19 vector as a carrier DNA. Cells were harvested 48 h after transfection, and RNA was isolated from total cellular extracts. The RNA isolation was subject to RNase protection assay as described before (38). The RNA probe was prepared using HindIII-cut pmTX91 as template (a gift from Mark Churche) and T3 RNA polymerase. The probe hybridize to 93 nucleotides of the HIV-1 Gag pl17 coding sequence (nucleotides 926–1018 of the pNL4.3 sequence, GenBank™ accession number M19921).
Mammalian Two-hybrid System—To monitor interaction between Rev and its cellular partners in mammalian cells, we have set up a two-hybrid system homologous to what we have used in yeast. Plasmid pSG5LexA was constructed by cloning the LexA DNA-binding domain coding sequence into the pSG5 expression vector (Amersham Pharma- cia Biotech) and was used to generate the “bait” plasmids. The “prey” constructs derived from pFNV plasmid encoding the VP16 transcriptional activation domain (amino acids 413–490) (37). To monitor the interaction between the two proteins, we have used the reporter plasmid, pSEAP LEXA5X constructed by inserting five LexA-binding sites into the \textit{XhoI} site of pSEAP promoter plasmid (CLONTECH). Transfections were done in HeLa cells plated in 35-mm Petri dishes. 120 ng of pSEAP LEXA5X and various amount of the LexA or VP16 hybrids expressing plasmids were co-transfected by the calcium phosphate method with 5 mg of pUC19 as carrier. 48 h after transfection, secreted alkaline phosphatase was titrated by chemiluminescence in 10 ml of cells supernatant using the SEAP Reporter Gene Assay (Roche Molecular Biochemicals).

RESULTS

Two-hybrid Screen for Rev NES-interacting Proteins—In an attempt to clone Rev co-factors for nucleo-cytoplasmic export, we performed a two-hybrid interaction screen in yeast using the Rev protein as a bait. The L40-pLexRev yeast strain was transformed with a human B-cell line cDNA library cloned in pACT and spread on L/W/H amino acid-depleted medium. More than 2.5 million clones were plated, leading to the isolation of 200 clones positive for histidine and \( \beta \)-galactosidase expression. These clones were subsequently tested with pLexM10, encoding a fusion between the DNA-binding domain of LexA and M10, a Rev mutant impaired in its capacity of stimulating export of HIV-1 mRNAs (Leu\textsuperscript{78},Glu\textsuperscript{79} \rightarrow Asp-Leu) (11). Sixteen clones interacting with wild type Rev but not with M10 were selected. Sequence analysis revealed that two of these clones correspond to the C terminus part of nucleoporin Nup214/can (clone Nup214c), which has already been shown to interact with nuclear export signal in the yeast two-hybrid system (18, 19). However, a 1.1-kb cDNA clone corresponding to an unknown gene was also identified by this extensive screening. The sequence of a gene called \textit{CG1} (candidate gene 1) (39), corresponding exactly to this clone was subsequently deposited in the GenBank\textsuperscript{TM} data base (accession number U97198). The cDNA fragment we obtained, which extends from codons 50 to 408 of the 423-amino acid-long \textit{CG1} putative open reading frame, was called NLP-1\textit{D}. To evaluate the extent of the interaction, we have assayed \( \beta \)-galactosidase expression in yeast cells co-transformed by pLex, pLexM10, or pLexRev and plasmids encoding the two identified preys. As shown in Fig. 1 \( \textit{A} \), \( \beta \)-galactosidase expression was low with the control plasmids or when the LexM10 hybrid was used as a bait. However, a dramatic increase of \( \beta \)-galactosidase expression (up to 30 times), was obtained upon co-transfection of pLexRev with pACTNLP-1\textit{D} or pACTNup214c.

Interaction was further analyzed by testing the preys against the wild type NES motif or its mutated counterpart fused to LexA. Results are reported in Fig. 1 \( \textit{B} \). They clearly demonstrated that the NES domain alone could account for the Rev/NLP-1\textit{D} interaction, suggesting that no additional Rev sequences were necessary for this contact. Furthermore, we...
challenged the interaction by swapping the bait by the preys and vice versa. Plasmids expressing Lex-Nup214c and Lex-NLP-1 fusion proteins were co-transfected with plasmids expressing fusion proteins between Rev or M10 and the transcriptional activation domain of Gal4 (Gal4AD). As shown in Fig. 1C, the Rev/Nup214c and the Rev/NLP-1 interactions are preserved when Nup214c and NLP-1 are tethered to Lex and Rev to Gal4AD.

Sequence Analysis of the NLP-1 Protein—Having shown that the Rev/NLP-1 interaction is specific of a functional NES, we decided to analyze in more detail the gene corresponding to the NLP-1 cDNA and its associated product. The NLP-1 cDNA fragment was completely sequenced, and a 1.6-kb cDNA fragment encoding the full-length 1269-base pair open reading frame was cloned by 5' and 3' rapid amplification of cDNA ends PCR. The 423-amino acid sequence deduced from the cDNA is reported in Fig. 2A and is similar to what has been published for the CG1 gene (39). Statistical analysis of the sequence reveal that the protein is very rich in serine residues (one-sixth of the polypeptide is serine) and contains 12 FG repeats, four repeats being of the PAFG type. The FG repeats are concentrated at the C terminus part of the protein (amino acids 215–375), which is very reminiscent of the FG motifs found in most nucleoporins. At the N-terminal side of the protein a putative CCCH type zinc finger motif is also found. This sequence is homologous to zinc fingers motifs found in other proteins like U2AF35, U2AF1, U2AF23, or yLee1. In addition, a putative coiled-coil region predicted by the COILS program at ISREC (40) is found between amino acids 171 and 197 and separates the FG-rich domain from the N terminus. Despite no obvious homologies with any other proteins in the data bases, BLAST search at National Center for Biotechnology Information with the FG-rich domain reveal partial homology with various nucleoporins including Nup1, pom1, Nup159, Nup214, Nup62, Nup42, etc. Furthermore, we have been able to show that when translated in a rabbit reticulocyte lysate or when expressed in human cells, the protein binds to WGA-Sepharose, indicating that it is O-glycosylated in vitro (Fig. 2B) as well as in vivo (Fig. 2C). In accordance with previous observations and in agreement with protein expression, we have decided to name this protein nucleoporin-like protein 1 or NLP-1.

NLP-1 Expression and Localization—NLP-1 mRNA expression was analyzed by Northern blot using different mRNA sources. A major band corresponding to a 2-kb mRNA, consistent with the expected size, is found in all cell lines and tissues tested, indicating that NLP-1 mRNA is ubiquitously expressed (Fig. 3A). A minor band is also visible at around 4.4 kb, which may represent either an mRNA isoform or a cross-hybridization with nucleoporin mRNA.

Subcellular localization of NLP-1 protein was then addressed both by cell fractionation and by indirect immunofluorescence on HeLa cells transiently transfected with pSG5-NLP-1 or mock transfected. A protein of the expected 45-kDa molecular size was detected by Western blot using an immunopurified polyclonal antibody (Fig. 3B). NLP-1 is found solely in total and nuclear protein extract in both mock and pSG5-NLP-1 transfected HeLa cells. Indirect immunofluorescence on HeLa transfected cells indicates that NLP-1 protein expression is restricted to the nucleus and excluded from the nucleolus (Fig. 3C).

Specificity of the NES/NLP-1 Interaction—To further confirm that interactions revealed by the two-hybrid assay were specific of a functional nuclear export signal, we have set up a genetic screen in yeast to select for Rev NES mutants that have maintained the ability to interact specifically with NLP-1. We first constructed a small library of plasmids derived from pLexM10 by randomly mutating the sequence coding for the four essential leucines located in the Rev nuclear export signal (Fig. 4A). This library (2 × 10^5 independent clones) was used to...
transform the L40-pACTNLP-1Δ yeast strain expressing the Gal4 activation domain fused to the NLP-1Δ polypeptide. To eliminate self-activating construct that could result from the generation of a functional activation domain in yeast, clones positive for both the histidine and β-galactosidase reporter genes were isolated and reintroduced into the L40-pACT strain expressing the Gal4 activation domain alone. Fourteen clones that appeared to be positive with pACTNLP-1Δ and negative with pACT in co-transformation experiments were finally sequenced (Fig. 4A). Among these clones, five were identical to the wild type, indicating that leucines in the Rev NES are probably the optimal amino acids required for Rev/NLP-1 interaction in yeast. Three were essentially wild type except for an leucine to isoleucine mutation, which is already known not to interfere dramatically with NES activity (41). Finally, four clones had two leucines of four changed to other hydrophobic residues, isoleucine and phenyalanine, or to tyrosine. It should be noted that very few of the selected clones have the same DNA sequence, suggesting that most of them if not all are in fact independent ones (data not shown). Furthermore, as shown in Fig. 4A, all clones were also positive when assayed with pACTNLP-1Δ and negative with pACT in co-transformation experiments were finally sequenced (Fig. 4A). Among these clones, five were identical to the wild type, indicating that leucines in the Rev NES are probably the optimal amino acids required for Rev/NLP-1 interaction in yeast. Three were essentially wild type except for an leucine to isoleucine mutation, which is already known not to interfere dramatically with NES activity (41). Finally, four clones had two leucines of four changed to other hydrophobic residues, isoleucine and phenyalanine, or to tyrosine. It should be noted that very few of the selected clones have the same DNA sequence, suggesting that most of them if not all are in fact independent ones (data not shown). Furthermore, as shown in Fig. 4A, all clones were also positive when assayed with pACTNup214c, indicating that NLP-1Δ and Nup214 exhibit the same specificity of interaction with the Rev NES.

To definitely correlate the ability of these mutated NES to interact with NLP-1Δ with their activity as a nuclear export signal, we decided to challenge the ability of these sequences to substitute for the wild type NES in a functional assay. For this purpose, a new Rev synthetic reporter construct was assembled that drives the expression of a Rev responsive RNA under the control of the CMV enhancer/promoter region and the SV40 small t polyadenylation signal. The premessenger RNA expressed from this reporter gene is composed of sequences coding for the HIV-1 Gag p17 inhibitory sequence region flanked by the first tat 5′ and 3′ splice sites and fused to a 264-nucleotide-long version of the HIV-1 RRE (Ref. 41 and Fig. 4B). When Rev is absent, this RNA undergoes splicing at tat sites. However, Rev expression leads to cytoplasmic accumulation of the unspliced version of the RNA (data not shown). As shown in Fig. 4C, RNase protection analysis of transcripts expressed from our reporter construct revealed that very little Gag containing RNA is found in the cytoplasm of transfected HeLa cells and that most of the precursor RNA undergoes complete splicing. However, cytoplasmic accumulation of full-length Gag p17 RNA is dramatically induced upon expression of Rev but not M10 or M4, a Rev mutant impaired in its capacity to bind RNA (10). Having shown that expression of our test RNA was dependent on a functional Rev protein (and also dependent on the RRE, data not shown), we recloned the four selected Rev proteins into the mammalian expression vector pSG5Flag to evaluate their activity in this new system. As shown in Fig. 4D, the four mutants can substitute for wild type Rev in inducing the nuclear export of the unspliced Gag RNA (activity reaching 71–95% of wild type). The former observation indicates that our screen for NLP-1-interacting Rev mutants has led to the identification of functional Rev proteins harboring efficient NES and strengthens the idea that the Rev/NLP-1 interaction identified in yeast is the result of a functional relationship between the two proteins.

**Rev Interacts with NLP-1 in Mammalian Cells**—Interaction between the NLP-1 protein and functional NES domains has
In this study we report the characterization of a cellular protein, encoded by a previously identified gene of unknown function called CG1 (39) that interacts specifically with the Rev nuclear export signal in the yeast two-hybrid system. Because it has strong homologies with nucleoporins including multiple FG repeats and a high serine content and because it is glycosylated in vitro and in vivo, this protein was named NLP-1. In addition, a putative CCCH type zinc finger and a coiled-coil region are suggested by a thorough analysis of the NLP-1 primary sequence. Thus, NLP-1 closely resembles the previously identified Rev-interacting protein hRIP/Rab, which also harbors many FG repeats, a high serine content, and a zinc finger of the CCC type (15, 16). In both cases, the Rev NES region are suggested by a thorough analysis of the NLP-1 primary sequence. Thus, NLP-1 closely resembles the previously identified Rev-interacting protein hRIP/Rab, which also harbors many FG repeats, a high serine content, and a zinc finger of the CCC type (15, 16). In both cases, the Rev NES region are suggested by a thorough analysis of the NLP-1 primary sequence. Thus, NLP-1 closely resembles the previously identified Rev-interacting protein hRIP/Rab, which also harbors many FG repeats, a high serine content, and a zinc finger of the CCC type (15, 16). In both cases, the Rev NES region are suggested by a thorough analysis of the NLP-1 primary sequence. Thus, NLP-1 closely resembles the previously identified Rev-interacting protein hRIP/Rab, which also harbors many FG repeats, a high serine content, and a zinc finger of the CCC type (15, 16). In both cases, the Rev NES region.

**DISCUSSION**

In the mammalian cell, CRM-1 associates with NLP-1 in a CRM-1-dependent manner. In this study, we report the characterization of a cellular protein, encoded by a previously identified gene called 

- **Function called CG1 (39)** interacts specifically with the Rev nuclear export signal in the yeast two-hybrid system. Because it has strong homologies with nucleoporins including multiple FG repeats and a high serine content and because it is glycosylated in vitro and in vivo, this protein was named NLP-1. In addition, a putative CCCH type zinc finger and a coiled-coil region are suggested by a thorough analysis of the NLP-1 primary sequence. Thus, NLP-1 closely resembles the previously identified Rev-interacting protein hRIP/Rab, which also harbors many FG repeats, a high serine content, and a zinc finger of the CCC type (15, 16). In both cases, the Rev NES region are suggested by a thorough analysis of the NLP-1 primary sequence. Thus, NLP-1 closely resembles the previously identified Rev-interacting protein hRIP/Rab, which also harbors many FG repeats, a high serine content, and a zinc finger of the CCC type (15, 16).

We have shown that NLP-1 and Nup214 interact specifically with a functional Rev NES in yeast. To confirm the functional significance of this contact, we asked whether motifs able to bind to NLP-1 can specify nuclear export. To do so, we randomized the four essential leucines in Rev NES and selected for NLP-1-interacting mutants. A similar approach based on the HTLV-1 Rev/hRIP/Rab interaction was reported previously and led to the same conclusion, i.e. the ability to bind FG-rich
domains of hRIP/Rab or NLP-1 is predictive of NES function (41). Taken altogether, the selected sequences also indicate that although leucines 1, 2, and 4 can be replaced by isoleucine or other hydrophobic amino acids, the leucine in position 3 is critical (Fig. 4). However, we still have no clear idea about the molecular interactions that sustain binding of the NES to its receptor. The consensus sequence for a functional NES proposed by Bogerd et al. (41) suggests that similar to nuclear localization signals, these targeting signals are not highly structured. In contrast, the nuclear export receptor CRM-1 interacts with NES in a RanGTP-dependent manner, and binding of RanGTP and the NES to CRM-1 is highly co-operative (20). This suggests that a RanGTP-dependent conformational change of CRM-1 is probably required to stabilize NES binding.

It has been shown previously that the FG-rich domain found in many nucleoporins of the GLFG and the XXFG but not the EXFG type are capable of interacting with the Rev NES in both yeast and higher eucaryotic cells (17–19). More recently, CRM-1, which is highly conserved from yeast to mammals, was shown to be a major export receptor for leucine-rich NES (20–24). Because CRM-1 was found associated with Nup88 and Nup214 in a dynamic complex (28), it was conceivable to postulate that CRM-1 was the necessary bridge allowing interaction of NES to nucleoporins in yeast. This hypothesis was indeed clearly demonstrated for yRip1p (29). In the present work, we show that NLP-1 also interacts with CRM-1, and we believe that the NES-NLP-1 interaction observed in yeast and HeLa cells is mediated by CRM-1 as well. However, we cannot rule out the possibility that Rev binds directly to NLP-1 as well as CRM-1. Considering the size of the NES motif, if these contacts exist, they might be mutually exclusive.

CRM-1 can bind FG-rich domains both in yeast and human cells (Refs. 28 and 29 and this work). However, we have no information about the molecular status of CRM-1 within this complex. In particular, we do not know whether RanGTP and the NES-containing proteins participate to this complex and whether they have to associate with CRM-1 before it can bind to nucleoporins. CRM-1 belongs to a family of import/export receptors that bind to the small GTPase Ran, an essential constituent of the bidirectional nucleocytoplasmic transport of proteins and RNA (42). Binding of GTP-bound Ran to import receptors induced dissociation of the cargo/transporter complex (43, 44). Conversely, binding of RanGTP to export receptors like Cas, Exportin t, or CRM-1 is believed to be necessary both for complex assembly with the export substrate and for the translocation process (20, 45–47). In the case of Cas and Exportin t, the complex is disassembled in the cytoplasm by the combined action of RanBP1 and RanGAP1, resulting in GTP hydrolysis (45, 48). For CRM-1-mediated export, NES binding requires RanGTP (20). Therefore, it is conceivable that binding to the FG domain of NPC-associated proteins only occurs when CRM-1 is complexed to both RanGTP and to the NES-containing cargo. Hydrolysis of GTP by RanGAP1 and RanBP1 at the cytoplasmic face of the NPC would completely dissociate the complex in a way similar to what happens following RanGTP binding for the import process.

**FIG. 5.** The Rev-NLP-1 interaction is reproduced in mammalian cells. **A,** schematic view of the reporter construct used for two-hybrid experiments in HeLa cells. OP stands for LexA operator binding sites, and SEAP stands for secreted alkaline phosphatase. PSV40e and SV40 PA correspond, respectively, to promoter/enhancer and polyadenylation sequences of SV40. **B,** schematic representation of the Lex and VP16 fusion proteins expressed from the indicated plasmids used in panel C and Fig. 6B. The VP16 hybrids proteins are tagged with the SV40 large T nuclear localization signal (nls) and the Flag peptide (Eastman Kodak). C, two-hybrid experiments performed in HeLa cells, pSEAP LEX5X reporter plasmid was co-transfected with constructs expressing the Lex or VP16 hybrid proteins as indicated. SEAP activity in cell culture medium was assayed by chemiluminescence.
indicated plasmids and spread on leucine, tryptophan, and histidine amino acids-depleted agar plates. Growth on selective plates is indicated by chemiluminescence. The values are given as multiples of the number of relative light units obtained for cells transfected with pLexNLP-1 alone.

The NPC is thought to be composed of 50–100 different nucleoporins, but only a fraction of these have been identified so far (for review see Refs. 49–51). Nucleoporins can be separated into two classes: integral membrane proteins, which make up the core of the NPC, and the dipeptide FG-rich nucleoporins, which are mostly located at the NPC periphery. It is noteworthy that although not necessary for proper nucleoporin localization, the FG-rich domain is conserved among eukaryotes and has probably been maintained for a critical function.

NLP-1 and hRIP/Rab are both ubiquitously expressed in humans and are probably conserved among species. Homologues of hRIP/Rab are likely to exist in mouse, quail, frog, fly, and even in yeast (15, 16). In the case of NLP-1, mouse cDNA sequences can be found in the expressed sequence tag database and even in yeast (15, 16). In the case of NLP-1, mouse cDNAalogues of hRIP/Rab are likely to exist in mouse, quail, frog, fly, humans and are probably conserved among species. Homo-

FIG. 6. NLP-1 interacts with CRM-1 in yeast and HeLa cells. A, yeast two-hybrid interaction assay. L40 cells were co-transformed with the indicated plasmids and spread on leucine, tryptophan, and histidine amino acids-depleted agar plates. Growth on selective plates is indicated by a plus sign. ND, not done. B, two-hybrid experiments in HeLa cells using reporter plasmid pSEAP LEX5X and the LexNLP-1 and VP16 or VP16CRM1 proteins expressing constructs. Reporter gene expression was evaluated by titrating SEAP activity in cell culture medium by chemiluminescence. The values are given as multiples of the number of relative light units obtained for cells transfected with pLexNLP-1 alone.

in other kinds of transport processes or nuclear functions involving CRM-1, similar to what has been shown for Rip1p (52).

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