Characterization of the nuclear transport of a novel leucine-rich acidic nuclear protein-like protein

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Abstract We previously reported that the nuclear localization signal (NLS) peptides stimulate the in vitro phosphorylation of several proteins, including a 34 kDa protein. In this study, we show that this specific 34 kDa protein is a novel murine leucine-rich acidic nuclear protein (LANP)-like large protein (mLANP-L). mLANP-L was found to have a basic type NLS. The co-injection of Q69LRan-GTP or SV40 T-antigen NLS peptides prevented the nuclear import of mLANP-L. mLANP-L NLS bound preferentially to Rch1 and NPI-1, but not to the Qip1 subfamily of importin α. These findings suggest that mLANP-L is transported into the nucleus by Rch1 and/or NPI-1.

Key words: Nuclear protein import; Nuclear localization signal; Importin α; Leucine-rich acidic nuclear protein; Ataxin-1

1. Introduction

Protein import into the cell nucleus occurs via nuclear pore complexes (NPCs). The NPC consists of diffusion channels that permit the passive diffusion of small molecules, such as ions and proteins, which are smaller than 20–40 kDa. Karyophilic proteins can be actively transported through the NPC by a selective, mediated process, even though they are larger than the diffusion channel. This selective, active nuclear protein transport is mediated by a nuclear localization signal (NLS). The best characterized 'classical' NLS is the SV40 T-antigen, which contains a cluster of basic amino acids [1].

The NLS-mediated import process involves multiple sequential steps. Soluble factors required for nuclear protein import have been identified by using the NLS of SV40 T-antigen as a substrate, mainly via the use of an in vitro transport assay [2]. The NLS triggers the formation of a stable heterotrimeric complex containing importin α and β [3–5]. This complex binds to the NPC via importin β, then translocates through the NPC into the nucleus. After the translocation, the GTP-bound form of a small GTPase Ran interacts with importin β to trigger the dissociation of the complex (for reviews [6–9]).

Although little is known about how the import machinery is regulated, data suggesting the existence of a relationship between nuclear protein import and protein phosphorylation have accumulated [10–14]. Kurihara et al. found that synthetic NLS-containing peptides stimulate the phosphorylation of several cellular proteins both in vivo and in vitro and that a murine 34 kDa protein was found to be preferentially phosphorylated in an NLS-dependent manner, suggesting the existence of a protein kinase which is specifically activated by the NLS [15].

In this study, we purified this specific 34 kDa protein from mouse Ehrlich ascites tumor cells and isolated its cDNA clone. The protein was found to have approximately 58% amino acid identity with the leucine-rich acidic nuclear protein (LANP). The LANP was first isolated from rat cerebellar proteins and immunohistochemical studies revealed that the protein is localized mainly in the nuclei of Purkinje cells [16]. Although LANP has been extensively studied, its function is not well understood. We report the sequence of a novel murine LANP-like protein and the characterization of its nuclear transport pathway.

2. Materials and methods

2.1. Molecular cloning of mLANP-L

A 34 kDa protein was partially purified as described previously [15], and the partially purified samples were applied to gel filtration and then subjected to SDS–PAGE. The protein band corresponding to a 34 kDa protein (mLANP-L) was cut from the gel and digested with a lysyl endopeptidase, and the digested peptides of the protein were separated by reverse-phase HPLC. The sequences of two fragments were determined by a peptide sequencer. These sequences were used as queries for FASTA homology searches against the EST database. Clones AA212094 (mouse) and AA051736 (mouse) almost coincided with these partial sequences. A 6-week-old C57BL6 mouse (male) brain cDNA library in λZAPII was screened by PCR products, generated using oligonucleotide probes.

2.2. Expression and purification of the recombinant proteins

The recombinant mLANP-L proteins were generated as follows. mLANP-L was cloned into pGEX6p1 to produce a GST fusion protein. A FLAG tag was fused into the *Bam*HI site. A fragment encoding the entire amino acid sequence was generated by PCR using oligonucleotides (5′-TACTACGGATCCATGGAGATGAAGAAGAAGATTACC and 3′-CCCCTCCTTCTGCTGCTAATCCTTAAGCATCAT) and cloned into the *Bam*HI-*Eco*RI site of pGEX6p1, to produce an in-frame fusion with GST. The expression vectors described above were transformed into *Escherichia coli* strain BL21, and puri-

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Abbreviations: NLS, nuclear localization signal; WGA, wheat germ agglutinin; LANP, leucine-rich acidic nuclear protein; NPC, nuclear pore complex

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fied to homogeneity using glutathione-Sepharose (Pharmacia) following the manufacturer's recommendations.

2.3. Transfection experiments

Plasmids for transient expression, consisting of full-length mLANP-L or mLANP-L(1–245), were inserted into pEGFP-GFP-C1 (pEGFPx2). A fragment encoding the entire amino acid sequence of mLANP-L was generated by PCR using oligonucleotides (5′-GCCGGCGAATTCATGGAGATGAAGAAGAAG and 3′-CTCTTCCTGCTGCTAATCTCCAGGTAGCTA or 3′-CCAGAAGCCCCTCTCATCCCTAGGTAAATT) and cloned into the *Eco*RI-*Bam*HI site of pEGFPx2. mLANP-L was shortened by *Eco*RI-*Pst*I digestion, and mLANP-L(1–140) was cloned into the *Eco*RI-*Pst*I site of pEGFPx2. mLANP-L(140–260) was cloned into the *Pst*I-*Eco*RI site of pEGFPx2-C1a. mLANP-L(1–245) was shortened by *Pst*I-*Bam*HI digestion, and the mLANP-L(140–245) was cloned into the *Pst*I-*Bam*HI site of pEGFPx2a. To obtain LANP-L(245–250), a fragment encoding the entire amino acid sequence was generated using oligonucleotides (5′-GATCCGAGAAGAGAAAGCGAGATCCC and 3′-GCTCTTCTCTTTCGCACTAGGG) and the PCR product was cloned into the *Bgl*II-*Sma*I site of pEGFPx2. These cDNA clones were introduced transiently into HeLa cells by the microinjection method.

2.4. Synthetic peptides

Synthetic peptides containing SV40 large T-antigen wild-type NLS (CYGGPKKKRKVEDP), its reverse type mutant NLS (CYGGPDEVKRKKKP) and mLANP-L NLS (CGGGEKRKRD) were purchased from the Peptide Institute (Osaka, Japan).

3. Results and discussion

3.1. Identification of a murine 34 kDa protein specifically phosphorylated in an NLS peptide-dependent manner

In a previous study, we reported that a 34 kDa protein was preferentially phosphorylated in an NLS peptide-dependent manner in vitro. In order to identify this protein, we attempted to purify the 34 kDa protein from Ehrlich ascites tumor cells extract [15]. After purification, we determined the internal amino acid sequences of two fragments derived from the purified protein. Clones AA212094 (mouse) and AA051736 (mouse) matched these partial amino acid sequences. The cloned cDNA encodes a novel polypeptide consisting of 260 amino acids with a calculated molecular mass of 29.6 kDa. The clone was found to have 58, 57, 57 and 58% amino acid identity with mLANP [18], rLANP [16], PHAPI [20] and APRIL [21], respectively (Fig. 1). Therefore, we designated this protein murine LANP-like large protein (mLANP-L).

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mLANP     MEMDKRIYLELRNRTPSDVKELVLDNCKSIEGKIEGLTDEFEELEFLSTINVGLTSISNL
rLANP     MEMDKRIYLELRNRTPSDVKELVLDNCRSIEGKIEGLTDEFEELEFLSTINVGLTSISNL
PHAPI     MEMGRRIHLELRNRTPSDVKELVLDNSRSNEGKLEGLTDEFEELEFLSTINVGLTSIANL
APRIL     --MKRRIHLELRNRTPAAVRELVLDNCKSNDGKIEGLTAEFVNLEFLSLINVGLISVSNL
mLANP-L   MEMKKKINMELKNRAPEEVTELVLDNCLCVNGEIEGLNDTFKELEFLSMANVELSSLARL
            * ::* :**:**:*  * ******. . :*::***.  * :*****  ** * *::.*

mLANP     PKLNKLKKLELSENRISGDLEVLAEKCPNLKHLNLSGNKIKDLSTIEPLKKLENLKSLDL
rLANP     PKLNKLKKLELSENRISGDLEVLAEKCPNLKHLNLSGNKIKDLSTIEPLKKLENLKSLDL
PHAPI     PKLNKLKKLELSDNRVSGGLEVLAEKCPNLTHLNLSGNKIKDLSTIEPLKKLENLKSLDL
APRIL     PKLPKLKKLELSENRIFGGLDMLAEKLPNLTHLNLSGNKLKDISTLEPLKKLECLKSLDL
mLANP-L   PSLNKLRKLELSDNIISGGLEVLAEKCPNLTYLNLSGNKIKDLSTVEALQNLKNLKSLDL
          *.* **:*****:* : *.*::**** ***.:*******:**:**:*.*::*: ******

mLANP     FNCEVTNLNAYRENVFKLLPQVMYLDGYDRDNKEAPDSDVEGYVE------DDDEEDEDE
rLANP     FNCEVTNLNAYRENVFKLLPQVMYLDGYDRDNKEAPDSDVEGYVE------DDDEEDEDE
PHAPI     FNCEVTNLNDYRENVFKLLPQLTYLDGYDRDDKEAPDSDAEGYVEGL----DDEEEDEDE
APRIL     FNCEVTNLNDYRESVFKLLPQLTYLDGYDREDQEAPDSDAEVDGVDEEEEDEEGEDEEDE
mLANP-L   FNCEITNLEDYRESIFELLQQITYLDGFDQEDNEAPDSEEEDDDDE-----DGDEDEEDE
          ****:***: ***.:*:** *: ****:*::::*****: *          :  *::***

mLANP     EEYDEYAQLVEDEEEEDEEEEGEEEDVSGEEEE----------DEEGYNDGEVDDEEDE
rLANP     EEYDEYAQLVEDEEEEDEEEEGEEEDVSGEEEE----------DEEGYNDGEVDDEEDE
PHAPI     EEYDEDAQVVEDEEDEDEEEEGEEEDVSGEEEE----------DEEGYNDGEVDDEEDE
APRIL     DDEDGEEEEFDEEDDEDEDVEGDEDDDEVSEEE----------EEFGLDEEDEDEDEDE
mLANP-L   DEDEAGPPEGYEEEEDDDEDEAGSEVGEGEEEVGLSYLMKDEIQDEEDDDDYVDEGEEEE
          :: :       :*:::*:: *. .:  . .**           :* . ::   : :*:*

mLANP     EEAGEEEGSQKRKREPDDEGEEDD-
rLANP     EDAAEEEGSQKRKREPDDEGQEDD-
PHAPI     EELGEEERGQKRKREPEDEGEDDD-
APRIL     EEE-EGGKGEKRKRETDDEGEDD--
mLANP-L   EEEEEGLRGEKRKRDAEDDGEEDDD
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Fig. 1. Multiple sequence alignments among LANP. The amino acid sequence, which is indicated with the single-letter notation, are sites separated by gaps (–) to achieve maximum homology by the CLUSTAL W (1.8) software. Asterisks and dots below the alignment indicate positions which are occupied by identical amino acids and chemically conserved amino acids among all the sequences, respectively. Sequence data from mLANP = mouse LANP (AF022957), rLANP = rat LANP (D32209), PHAPI = human PHAPI (X75090) = mapmodulin = pp32, APRIL = human APRIL (Y07969), mLANP-L = murine LANP-like large protein.

3.2. In vitro phosphorylation of recombinant mLANP-L proteins

In order to confirm that the obtained clone encodes the 34 kDa protein which is preferentially phosphorylated in an NLS peptide-dependent manner, we examined whether the phosphorylation of the recombinant protein was specifically activated in vitro by the addition of NLS peptides. mLANP-L was actually phosphorylated in an NLS peptide-dependent manner (data not shown), suggesting that the 34 kDa protein, which was phosphorylated preferentially by the addition of NLS peptides, is mLANP-L.

3.3. mLANP-L migrates into the nucleus in a temperature-dependent, WGA-sensitive and Ran-GTP-sensitive manner in living cells

LANP was reported to be primarily localized in the nuclei of Purkinje cells [16]. Using yeast two-hybrid experiments, LANP has been shown to bind to ataxin-1, the spinocerebellar ataxia type 1 (SCA1) gene product [18]. It was found that mutant ataxin-1 with a polyglutamine tract causes a redistribution of the nuclear matrix-associated structures, which may be involved in SCA1 pathogenesis. The ataxin-1/LANP complex was found to be, in fact, localized in the nuclei of Purkinje cells [19]. From these findings, it was proposed that cerebellar LANP is involved in the pathogenesis of SCA1. However, the specific pathway for how the LANP family proteins are transported into the nucleus has not yet been elucidated.

Therefore, we attempted to examine the nuclear import of mLANP-L in more detail. Purified GST-FLAG-mLANP-L, when injected into the cytoplasm of living HeLa cells, migrates into the nucleus within 30 min (Fig. 2). Moreover, the nuclear migration of mLANP-L was temperature-dependent and was strongly inhibited by a lectin, wheat germ agglutinin (WGA) (Fig. 2), indicating that the nuclear migration

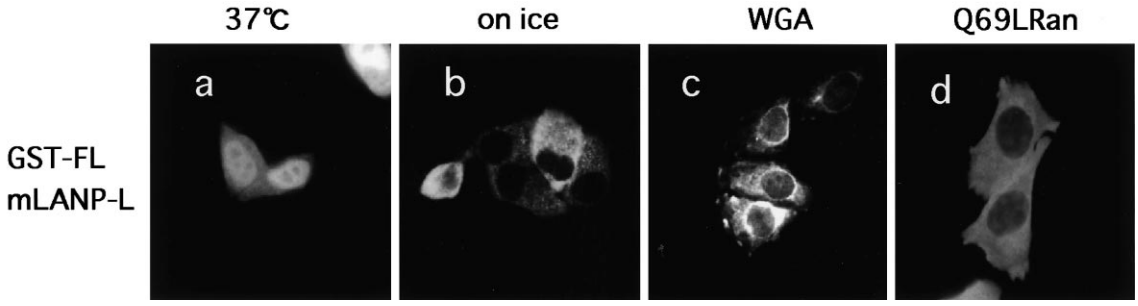

Fig. 2. mLANP-L migrates into the nucleus of living cells in a temperature-dependent and WGA-sensitive manner and the migration is inhibited by mutant Ran. Purified recombinant GST-FLAG-mLANP-L (0.1 mg/ml) was injected into the cytoplasm of HeLa cells with (c) or without (a, b) 1 mg/ml WGA. GST-FLAG-mLANP-L was co-injected with 8 mg/ml Q69L Ran-GTP (d). After incubation for 30 min at 37°C (a, c, d) or on ice (b), cells were fixed with 3.7% formaldehyde in PBS(−). Injected GST-FLAG-mLANP-L was visualized by indirect immunofluorescence with anti-FLAG mouse monoclonal antibody and Alexa488-labeled anti-mouse goat IgG. The localization of GST-FLAG-mLANP-L was examined by Axiophot microscopy (Zeiss).

of mLANP-L is an active process, although mLANP-L is sufficiently small to passively diffuse into the nucleus.

To further characterize the transport pathway, we co-injected Q69LRan-GTP, which is deficient for GTP hydrolysis [22]. It is known that Q69LRan-GTP, when co-injected into the cytoplasm, dominant-negatively inhibits the nuclear import mediated by importin β-like transport factors. As shown in Fig. 2, the nuclear import of mLANP-L was blocked by Q69LRan-GTP, indicating that the nuclear import of mLANP-L occurs in a Ran-dependent manner.

3.4. Identification of the NLS of mLANP-L

In order to identify the NLS of mLANP-L, we constructed several deletion mutants and expressed these transiently. When mLANP-L(1–140) was expressed in HeLa cells, it localized to the cytoplasm. In contrast, when mLANP-L(140–260) was expressed, it localized to the nucleus. These results indicate that the C-terminal portion is necessary for the nuclear localization (Fig. 3). Furthermore, the fact that mLANP-L(140–245) localized to the cytoplasm suggests that amino acids 246–260 contain the functional NLS of mLANP-L. This portion contains a basic amino acid cluster, ^{246}KRKR.

In order to better understand whether this sequence KRKR acts as a functional NLS of mLANP-L, we tested the nuclear import of recombinant mLANP-L(245–250) tagged with the GFP epitope at its C-terminus. As shown in Fig. 4, the purified recombinant GST-mLANP-L NLS-GFP, when injected into the cytoplasm of living cells, migrates into the nucleus within 30 min. The nuclear migration of GST-mLANP-L NLS-GFP was temperature-dependent and was potently inhibited by WGA and Q69LRan-GTP (Fig. 4), which faithfully reproduces the behavior of the full-length mLANP-L protein (cf. Fig. 2). These results indicate that amino acid sequence 245–250, DKRKRE, represents a functional NLS of mLANP-L. Moreover, the fact that the sequence KRKR is well conserved among other LANP family molecules suggests that KRKR is critical for the nuclear import of all the LANP family molecules.

3.5. Possible nuclear import pathway of mLANP-L

In order to verify the nuclear import pathway of mLANP-L in vivo, we examined the issue of whether the import is competitively inhibited in the presence of an excess amount of the NLS peptides. As shown in Fig. 5, the nuclear import of mLANP-L NLS-containing substrates was strongly inhibited by the SV40 T-antigen NLS peptides as well as mLANP-L NLS peptides, but not by reverse-type T-antigen mutant NLS

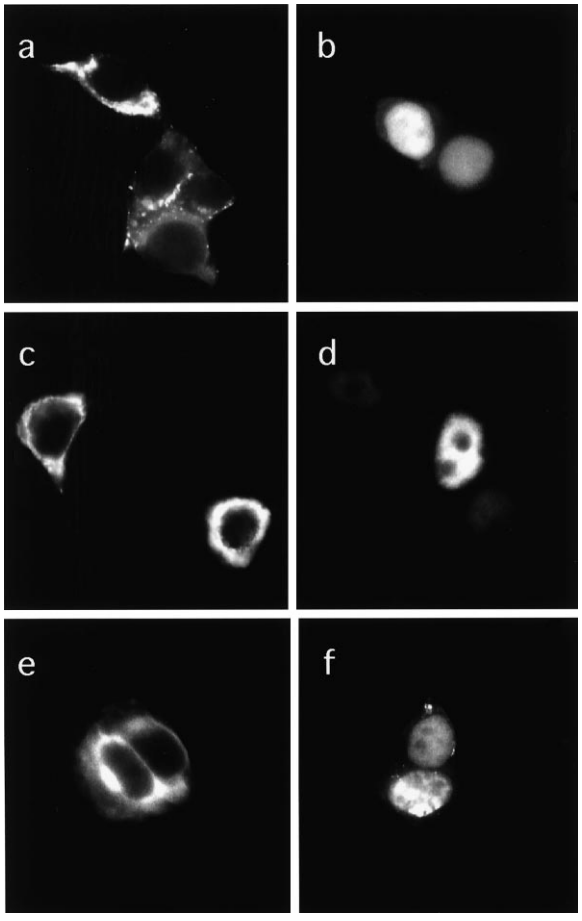

Fig. 3. The subcellular distribution of GFPx2-mLANP-L in transient transfection to HeLa cells. HeLa cells were transfected with GFPx2 alone (a), GFPx2-mLANP-L(1–260) (b), GFPx2-mLANP-L(1–140) (c), GFPx2-mLANP(140–260) (d), GFPx2-mLANP-L(140–245) (e) or GFPx2-mLANP-L(245–250) (f). After incubation for 6 h at 37°C, cells were fixed with 3.7% formaldehyde in PBS(−). The localization of GFPx2-mLANP was examined by Axiophot microscopy (Zeiss).

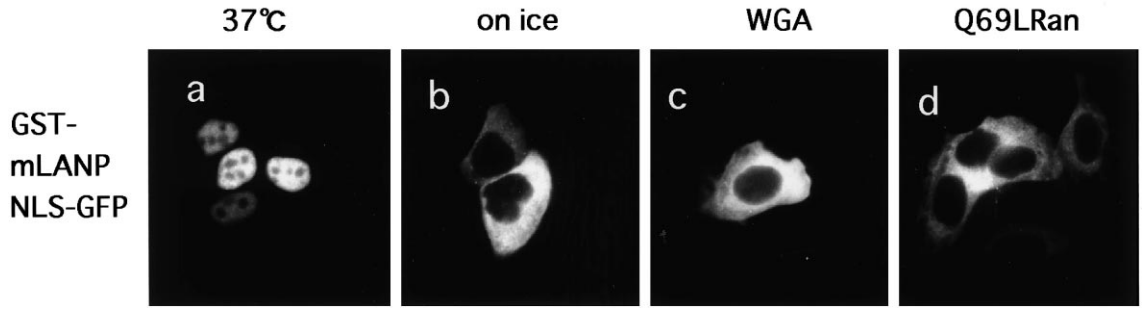

Fig. 4. mLANP-L NLS migrates into the nucleus of living cells in a temperature-dependent and WGA-sensitive manner and the migration is inhibited by mutant Ran. Purified recombinant GST-mLANP-L NLS-GFP (2 mg/ml) was injected into the cytoplasm of HeLa cells with (c) or without (a, b) 1 mg/ml WGA. GST-mLANP-L NLS-GFP was co-injected with 8 mg/ml Q69LRan-GTP (d). After incubation for 30 min at 37°C (a, c, d) or on ice (b), cells were fixed with 3.7% formaldehyde in PBS(−). The localization of GST-mLANP-L NLS-GFP was examined by Axiophot microscopy (Zeiss).

peptides. These data suggest that the nuclear import of mLANP-L most likely occurs via the classical importin α/β pathway.

3.6. Interaction between importin α family and mLANP-L NLS in vitro

In mammalian cells, the importin α family proteins are classified into three major classes, namely Rch1, NPI-1 and Qip1. Miyamoto et al. demonstrated that these NLS receptors have specificity in the recognition of various substrates [17]. We analyzed the binding affinity of each importin α family molecule with mLANP-L NLS using native gel electrophoresis, in which complex formation between two proteins gives rise to a new band. A mixture of the GST-mLANP-L NLS-GFP with Rch1 or NPI-1 efficiently gave a new band with a mobility shift from each protein alone (Fig. 6, lanes 2, 3 and 5, 6). In contrast, a mixture of the GST-mLANP-L NLS-GFP with Qip1 gave no new band (Fig. 6, lanes 7 and 8). These results indicate that the mLANP-L NLS preferentially binds to Rch1 and NPI-1, but not to the Qip1 of importin α family molecules, suggesting that the nuclear import of mLANP-L is mediated by Rch1 and/or NPI-1, but not Qip1. Moreover, in view of a previous report that NPI-1 is significantly expressed in the cerebellar cortex [23], it can be predicted that the nuclear import of mLANP-L is primarily mediated by the NPI-1 family of importin α in Purkinje cells, although the issue of how the LANP proteins contribute to the nuclear localization of ataxin-1 has not yet been elucidated.

3.7. Conclusion

We reported the identification of the 34 kDa protein, which is preferentially phosphorylated in the presence of the NLS

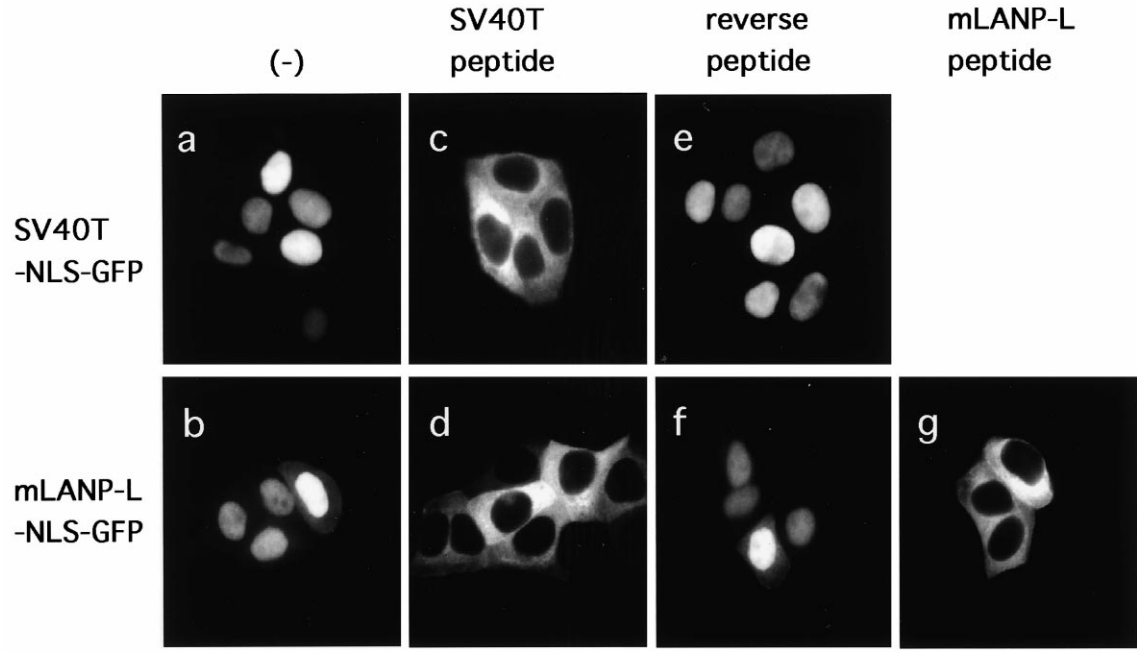

Fig. 5. Competitive in vivo nuclear import assay. A nuclear import assay was performed using HeLa cells. The GFP-tagged NLSs analyzed in this assay are indicated at the left, and competitor peptides, which were co-injected in excess (4 mg/ml) with GFP NLSs, are indicated at the top of the figure. After microinjection, cells were incubated for 30 min at 37°C. The localization of GFP-tagged NLSs was examined as in Fig. 4. The nuclear import of SV40T-NLS-GFP was competitively inhibited by excess SV40 T NLS peptide (c), but not with reverse type mutant T NLS peptides (e). mLANP-L-NLS-GFP was competitively inhibited by excess SV40 T NLS (d) or mLANP-L NLS (g) peptides, but not with reverse T peptides (f).

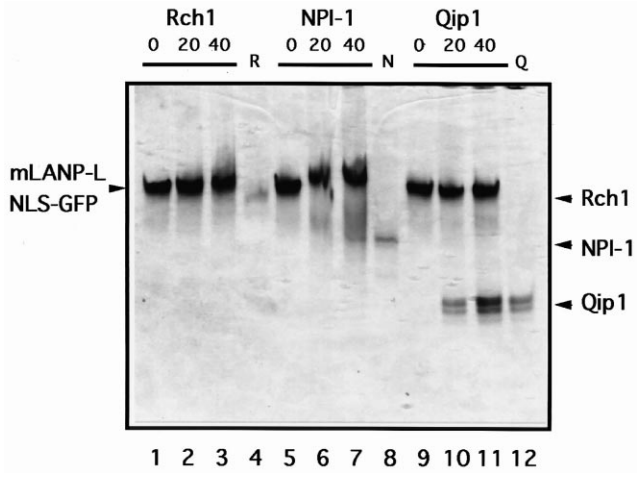

Fig. 6. The ability of mLANP-L NLS to bind the importin α family. Native gel electrophoresis showing Rch1, NPI-1, Qip1 and GST-mLANP NLS-GFP run separately or as a complex. A mixture of 20 pmol of the GST-mLANP-L NLS-GFP with 20 pmol or 40 pmol Rch1 and NPI-1 results in a new band with a mobility different from each protein alone (lanes 2, 3 and 6, 7). In contrast, a mixture of 20 pmol of the GST-mLANP-L NLS-GFP with 20 pmol or 40 pmol Qip1 gives no new bands (lanes 10 and 11). Migration of GST-mLANP-L NLS-GFP alone (lanes 1, 5, 9), Rch1 alone (lane 4), NPI-1 alone (lane 8), Qip1 alone (lane 12) are shown as a control.

peptides and designated it mLANP-L (murine LANP-like large protein) from the sequence homology with LANP proteins. Although the role of the phosphorylation of mLANP-L has not yet been elucidated, it was found that mLANP-L has a basic-type NLS, KRKR, in its C-terminal region and is actively transported into the nucleus by the Rch1 and/or NPI-1 family of importin α, probably in conjunction with importin β. Although the issue of whether the LANP proteins are actually involved in the nuclear import of ataxin-1 in Purkinje cells needs to be examined, further studies may elucidate the biological significance of the colocalization of ataxin-1 with LANP proteins.

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