Molecular basis for the interaction between rabies virus phosphoprotein P and the dynein light chain LC8: dissociation of dynein-binding properties and transcriptional functionality of P

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The lyssavirus phosphoprotein P is a co-factor of the viral RNA polymerase and plays a central role in virus transcription and replication. It has been shown previously that P interacts with the dynein light chain LC8, which is involved in minus end-directed movement of organelles along microtubules. Co-immunoprecipitation experiments and the two-hybrid system were used to map the LC8-binding site to the sequence 139RSSEDKSTQTTGR151. Site-directed mutagenesis of residues D143 and Q147 to an A residue abolished binding to LC8. The P–LC8 association is not required for virus transcription, since the double mutant was not affected in its transcription ability in a minigenome assay. Based on the crystal structure of LC8 bound to a peptide from neuronal nitric oxide synthase, a model for the complex between the peptide spanning residues 140–150 of P and LC8 is proposed. This model suggests that P binds LC8 in a manner similar to other LC8 cellular partners.

Members of the genus Lyssavirus have a single-stranded negative-sense RNA genome and belong to the family Rhabdovidae. On the basis of phylogenetic studies, seven genotypes of lyssaviruses have been identified, among which genotype 1 [rabies virus (RV), strains PV and CVS] and genotype 3 (Mokola virus) are the most divergent (Bourhy et al., 1993; Tordo et al., 1993). Lyssaviruses are highly neurotropic, migrating from inoculation point to the central nervous system (CNS) through peripheral nerves. The mechanisms involved in axonal transport of the virus remain unclear.

Lyssavirus ribonucleoproteins (RNP) contain the genomic RNA tightly encapsidated by the viral nucleoprotein (N) and the RNA polymerase complex, consisting of the large protein (L) and its co-factor, the phosphoprotein (P) (Emerson & Wagner, 1972). Both L and P proteins are involved in transcription and replication. During transcription, a positive-stranded leader RNA and five mRNAs are synthesized. The replication process yields nucleocapsids containing full-length antisense genomic RNA, which in turn serves as a template for the synthesis of positive-sense genomic RNA.

RV P protein is a non-catalytic co-factor and a regulatory protein: it associates with the L protein in the polymerase complex and interacts with both soluble and genome-associated N proteins. We have demonstrated previously the existence of two N protein-binding sites on the P protein: one located between amino acids 69 and 139 and the other located in the carboxy-terminal region comprising amino acids 268 to 297 (Chenik et al., 1994). We have shown also that the major L-binding site resides within the first 19 residues of P (Chenik et al., 1998). In addition, four other amino-terminally truncated products (PA2, PA3, PA4 and PA5) translated from P mRNA have been found in purified virus, infected cells and cells transfected with a plasmid encoding the complete P protein. Translation of these proteins is initiated from internal in-frame AUG initiation codons by a leaky scanning mechanism (Chenik et al., 1995). Their potential role in the virus cycle remains to be determined.

We have identified recently the cytoplasmic dynein light chain LC8 as a strong interacting partner of the P protein of two lyssaviruses, RV and Mokola virus, in a yeast two-hybrid screen (Iacob et al., 2000; Raux et al., 2000). The P–LC8 interaction was confirmed both in cells transfected with a plasmid encoding the P protein and in infected cells by co-
Fig. 1. Mapping of the LC8-binding site on P. (A) BSR cells were infected with vTF7-3 and transfected with plasmids encoding P (lane 1) or P\(\Delta N_{139-172}\) (lane 2), according to the methods described by Fuerst et al. (1986) and Rose et al. (1991). The plasmid pCDM8 encoding P\(\Delta N_{139-172}\) differed from pCDM8-P (Chenik et al., 1994) by a deletion of 102 bp from nucleotides 444 to 546 created by PCR gene fusion in the P gene. At 24 h after infection, cells were lysed on ice in 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.5% NP40 with an anti-protease cocktail (2 \(\mu\)g/ml leupeptin, 2 \(\mu\)g/ml antipain, 2 \(\mu\)g/ml pepstatin, 2 \(\mu\)g/ml chymostatin and 16 \(\mu\)g/ml aprotinin). Nuclei were eliminated from the lysate and the cytoplasmic cell extracts were immunoprecipitated with the murine polyclonal anti-P antibody. Immune complexes were collected with protein A-Sepharose and were analysed by Western blotting (16% SDS–PAGE). Blots were immunostained with a rabbit polyclonal anti-LC8 antibody and with an anti-rabbit peroxidase-labelled secondary antibody. An aliquot of uninfected BSR cell extract was used to indicate the position of LC8 within the gel (lane 3). Identical samples of BSR transfected cell extracts from the experiment described above were analysed by Western blotting (12% SDS–PAGE) with the murine polyclonal anti-P antibody (lanes 4 and 5). BSR cells infected with vTF7-3 were cotransfected with plasmids encoding the N and P (lane 6) or P\(\Delta N_{139-172}\) (lane 7) proteins. At 24 h after infection, proteins were labelled with \(1–85\) MBq of \(^{35}\)S\-methionine and \(^{35}\)S\-cysteine (PRO-mix, sp. act. \(>37\) TBq/mmol) (Amersham) for 2 h. Cells were lysed and the cytoplasmic fractions were immunoprecipitated with the anti-P antibody. The immunoprecipitates were analysed by 12% SDS–PAGE followed by autoradiography (lanes 6 and 7). (B) BSR cells were infected with vTF7-3 and transfected with plasmids encoding P, P\(\Delta N_{139-151}\), P(D\(^{143A–Q^{147A}}\)), P(D\(^{143A}\)) or P(Q\(^{147A}\)) (lanes 1–5). The plasmid pCDM8 encoding P\(\Delta N_{139-151}\) differed from pCDM8-P by a deletion of 36 bp from nucleotides 444 to 480 created by PCR gene fusion in the P gene. The plasmid encoding P\(D^{143A–Q^{147A}}\), P\(D^{143A}\) or P\(Q^{147A}\) carried either two substitutions or one substitution, respectively, which were introduced by the QuikChange Site-Directed Mutagenesis kit (Stratagene). At 24 h after infection, samples of cytoplasmic cell extracts, as prepared in (A), were analysed by Western blotting (12% SDS–PAGE) with the murine polyclonal anti-P antibody (upper gel). Cell extracts corresponding to the same transfected cells described above were immunoprecipitated with the anti-P antibody. Immune complexes were analysed by Western blotting (16% SDS–PAGE). The blot was then immunostained with a rabbit polyclonal anti-LC8 antibody and with an anti-rabbit peroxidase-labelled antibody (lower gel, lanes 1–5). (C) Quantification of the interaction of LC8 with wild-type P and P mutants. The interaction between LC8 and P was assessed by assaying the \(\beta\)-galactosidase activity of yeast grown in liquid medium. L40 yeast cells were co-transformed with the DNA encoding LC8 fused to the GAL4 activation domain (GAL4AD-LC8) (Raux et al., 2000) and the different P mutants fused to the DNA-binding domain (BD) of LexA. Quantitative results were obtained from three independent yeast co-transformants assayed using ONPG as the substrate (Guerente, 1993). \(\beta\)-galactosidase activity was expressed in units and calculated using the following formula: \((A_{420} \times 1000) / (A_{600} \times T \times V)\), where \(A_{420}\) is the absorbance of the reaction mixture, \(A_{600}\) is the cell density of the culture, T is the reaction time (in min) and V is the volume (in ml) used for the assay. BD-lamin encoding plasmid and activation domain (AD) without insert are used as negative control. (D) Comparison of transcriptional activity between wild-type P and P(D\(^{143A–Q^{147A}}\)) proteins. BSR cells grown in 24-well plates were infected with vTF7-3 and co-transfected using polyethylenimine (Sigma) with 0.5 \(\mu\)g of pDI-luc, 0.2 \(\mu\)g of plasmid L and 1 \(\mu\)g of plasmid N from PVCGJC.

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immunoprecipitation (Raux et al., 2000). Co-localization of the two proteins was demonstrated also by confocal microscopy (Jacob et al., 2000). Dynein is a microtubule-associated motor protein complex involved in minus end-directed movement of organelles along microtubules (Bowman et al., 1999; Pazour et al., 1998). The P–LC8 interaction could explain the propagation of the virus from the site of entry, such as a bite on the skin, to the CNS, i.e. via the long nerve axons. The LC8 protein, which forms dimers (Benashski et al., 1997; Liang et al., 1999), is suggested also to be an inhibitor of neuronal nitric oxide synthase (nNOS) (Jaffrey & Snyder, 1996) and NO changes in the CNS have been proposed to explain some of the neuropathogenic events occurring during RV infection (Akaike et al., 1995). LC8 homologues from evolutionarily distant species, such as human, rat, fly, nematode, fruit and green alga, display a remarkable degree of sequence identity and are constitutively produced in various cell types (King et al., 1996).

In order to define precisely the LC8-binding domain on P, we first constructed a mutant lacking residues 139–172 (PAN139–172), residues that were suspected to contain the LC8-binding site (Raux et al., 2000; Jacob et al., 2000). This mutant was tested for its ability to interact with LC8. Proteins from transfected cells were immunoprecipitated from cell extracts using a polyclonal anti-P antibody (Raux et al., 1997). The proteins present in the immune complexes were then detected on a Western blot using a rabbit polyclonal anti-LC8 antibody (R4058) (King & Patel-King, 1995). As expected, protein P interacted with the endogenous LC8, whereas PAN139–172 did not (Fig. 1A, lanes 1 and 2). However, PAN139–172 was expressed efficiently (Fig. 1A, lanes 4 and 5) and was able to bind to N protein in cells co-transfected with both plasmids, as shown after immunoprecipitation with the anti-P antibody (Fig. 1A, lanes 6 and 7), suggesting that PAN139–172 was correctly folded.

The lack of interaction between PAN139–172 and LC8 was confirmed in the yeast two-hybrid system. Quantitative results were obtained by assaying the β-galactosidase activity of yeast grown in liquid medium (Fig. 1C). The lacZ reporter gene was activated if both P and LC8 were co-expressed. In contrast, no lacZ activation was observed with PAN139–172, indicating that the deletion of 33 amino acids in the central part of P impaired binding to LC8. These data confirm that the amino acids 139–172 of P contain the LC8-binding site (Raux et al., 1997). We then carried out site-directed mutagenesis of residues D143 and Q147, altering either one or both residues. We expressed these P gene mutants in BSR cells and yeast and tested their interaction with LC8 by using the methods described above. As shown in Fig. 1(B), the substitution of the two residues D143 and Q147 with an A residue abolished binding to LC8 (Fig. 1B, lane 3). This was confirmed by the quantitative β-galactosidase assay in the yeast two-hybrid system (Fig. 1C). One substitution (Q147A) resulted in a substantial loss of interaction, as shown by immunoprecipitation (Fig. 1B, lane 5), and this interaction was too weak to be detected by the quantitative β-galactosidase assay (Fig. 1C). In contrast, protein P(D143A) bound to LC8, but less efficiently than the wild-type P protein (Fig. 1B, lane 4). This was confirmed by the β-galactosidase assay (Fig. 1C). These results suggest strongly that both residues, D143 and Q147, are critical for the binding of P to LC8, although the D to A substitution reduced only weakly the binding of P to LC8. This does not exclude the possibility that neighbouring amino acids could also be involved in stabilizing the interaction.

These results, taken together with the sequence alignment indicate that P and nNOS bind LC8 in a similar manner. Thus, the complex between the peptide residues S140–G150 of phosphoprotein P and LC8 was modelled using the structure of the complex between the 13 residue peptide of nNOS and LC8 from amino acids 139 to 151 is similar in sequence to other LC8-binding partners (Jacob et al., 2000). Thus, we constructed the deletion mutant PAN139–151 in pCDM8 and pLex in order to test its ability to bind to LC8 by co-immunoprecipitation and with the two-hybrid system, as described above. The results obtained with both methods demonstrated that PAN139–151 did not bind to LC8 (Fig. 1B, lane 2 and Fig. 1C), but interacted efficiently with the N protein (data not shown).

The structure of the LC8 protein bound to a 13 residue peptide from nNOS has been solved by X-ray diffraction (Liang et al., 1999). In the crystal structures, the nNOS peptide lies in a deep groove formed between the monomers of the LC8 dimer. Eleven residues of the peptide (225EMLDQTGVDQ237) participate as a β-strand structure in the formation of a central β-sheet comprising six anti-parallel β-strands. Four strands derive from one monomer, the fifth strand from the other monomer and the sixth from the peptide. Among these residues, D230 and Q234 make hydrogen bonds via their side chains with the LC8 dimer. These residues correspond to those present at positions 143 and 147 of the P sequence, which may then be important for the binding of P to the LC8 dimer.

We then carried out site-directed mutagenesis of residues D143 and Q147, altering either one or both residues. We expressed these P gene mutants in BSR cells and yeast and tested their interaction with LC8 by using the methods described above. As shown in Fig. 1(B), the substitution of the two residues D143 and Q147 with an A residue abolished binding to LC8 (Fig. 1B, lane 3). This was confirmed by the quantitative β-galactosidase assay in the yeast two-hybrid system (Fig. 1C). One substitution (Q147A) resulted in a substantial loss of interaction, as shown by immunoprecipitation (Fig. 1B, lane 5), and this interaction was too weak to be detected by the quantitative β-galactosidase assay (Fig. 1C). In contrast, protein P(D143A) bound to LC8, but less efficiently than the wild-type P protein (Fig. 1B, lane 4). This was confirmed by the β-galactosidase assay (Fig. 1C). These results suggest strongly that both residues, D143 and Q147, are critical for the binding of P to LC8, although the D to A substitution reduced only weakly the binding of P to LC8. This does not exclude the possibility that neighbouring amino acids could also be involved in stabilizing the interaction.

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Fig. 2. (A) Schematic diagram of the interactions between peptide S140–G150 from modelled RV phosphoprotein P and LC8 protein (both in colour-coded atoms) using LIGPLOT (Wallace et al., 1995). The peptide P(S140–G150) (atom bonds in green) mainly interact with one subunit of the LC8 dimer (atom bonds in black). Residues of the other subunit of the LC8 dimer hydrogen-bonded with the Q147 side chain from the peptide are indicated with a hash. (B) Molecular surface representation of the LC8 dimer colour-coded to show the surface area hidden (in yellow) by the peptide P(S140–G150). The modelled peptide (colour-coded atoms) fits well within the binding channel of the dimeric LC8 protein. The figure was prepared with GRASP (Nicholls et al., 1993). Co-ordinates of the LC8 dimer were taken from the protein database (code 1CMI).

(protein database code 1CMI) (Liang et al., 1999) as the starting point. The side chains of the amino acids from the sequence EMLDTGIQVDR of the peptide nNOS were substituted with the side chains of the sequence SSEDKSTQTTG of P(S140–G150). The conserved amino acids D143 and Q147 were used to anchor the peptide and the conformations of their side chains were kept unmodified from those of the equivalent nNOS residues. The peptide P(S140–G150) binds mainly to one monomer of the LC8 protein through the hydrogen-bonding scheme of the β-sheet and also through side chain interactions (Fig. 2A). Despite the hydrophobic groove formed by the LC8 dimer and the polar properties of amino acids of the peptide P(S140–G150) between D143 and Q147, no steric hindrance between the peptide and the LC8 dimer was observed (Fig. 2B).

The interaction of LC8 with RV P, which is the co-factor of the viral RNA-dependent RNA polymerase, raises the question of the potential role of LC8 in the transcription process. To address this possibility, the transcriptional functionalities of wild-type P and P(D143A–Q147A) proteins were compared in a reverse genetics assay (Le Mercier, personal communication). Briefly, T7 recombinant vaccinia virus (vTF7-3)-infected BSR cells were used to simultaneously express viral N, P and L proteins as well as a minigenomic RNA, where the luciferase gene is under the control of virus transcriptional sequences. Luciferase activity is related directly to the transcriptional
was determined (Fan et al., 2000). Recently, a nuclear magnetic resonance structure of similar to the one that we have defined on the RV P protein was developed using the PV strain of RV. Thus, we verified first that the CVS strain of RV P protein was compatible with this system. Heterogeneous RNP complexes with CVS strain P displayed a slightly lower transcriptional activity than that seen with the homologous PV strain system (data not shown). However, P(D143A–Q147A) shared a similar activity to the CVS strain P. As P(D143A–Q147A) is an LC8 interaction-defective protein, this result demonstrates that the mutants are transcriptionally active and that the P–LC8 interaction is not required for virus transcription.

In summary, we have defined precisely the LC8-binding domain on P between the acidic amino acids at positions 139–151 and demonstrated that two residues, D143 and Q147, are essential for this interaction. Other ligands of LC8 have been described recently and the LC8-binding sites identified (Puthalakath et al., 1999; Lo et al., 2001). These sequences are similar to the one that we have defined on the RV P protein (Table 1). Recently, a nuclear magnetic resonance structure of LC8 bound to the peptide from Bim, a Bcl-2 family member, (Table 1). Interestingly, some of them contain the sequence motif DKSTQ to LC8 is in accordance with our model proposed in Fig. 2. Thus, it appears that the P protein binds to LC8 in a manner similar to many natural cellular partners of the protein.

We have extended the analysis of putative targets of LC8 to viral proteins that may potentially bind to the dynein motor complex (Table 1). Interestingly, some of them contain the sequence involved in the LC8-binding domain, with amino acids D and Q spaced by three residues. For example, the protein VP4 of rotavirus, which has been shown to bind to microtubules (Nejmeddine et al., 2000), contains this sequence motif. Some viruses use the retrograde transport machinery to move the viral capsid to the site of replication, including two picornaviruses, coxsackievirus and poliovirus, whose VP2 capsid proteins contain the putative LC8 target sequence. We have also found this sequence in proteins of herpesviruses (herpes simplex virus type 1) and adenoviruses, which have been shown to recruit dynein for retrograde virus movements (Suomalainen et al., 1999; Ye et al., 2000), although these viral proteins have not been documented to interact directly with dynein or microtubules. These observations suggest that the interaction of viral capsid proteins with the LC8 dynein light chain may be a common virus event. Based on these data, further experiments will be aimed at understanding the molecular basis of the interaction between viral capsid proteins and dynein.

We thank Hélène Raux for helpful advice in the yeast two-hybrid experiments. This work was supported by the CNRS UPR 9053 (D. Blondel), the Patrick and Catherine Weldon Donaghue Medical Research Foundation and the NIH (GM 51293) grants to S. King.

### References


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<th>Table 1. Sequence alignment of the LC8-interacting domains</th>
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<td>Data analysis was performed using the PATTERNp software (<a href="http://www.infobiogen.fr/services/menuserv.html">http://www.infobiogen.fr/services/menuserv.html</a>). Target sequences found in the VP4 protein of human, bovine and porcine rotaviruses and in the VP2 protein of poliovirus (types 1–3) are shown. Putative interacting proteins are indicated in italics and conserved residues D and Q are shown in bold.</td>
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Received 7 June 2001; Accepted 30 July 2001