Identification of a Role for Nucleolin in Rabies Virus Infection

S. Oksayan, a,b J. Nikolic, c T. C. David, a D. Blondel, c D. A. Jans, b G. W. Moseley a

Viral Pathogenesis Laboratory, Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, Australia a; Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia b; Institute for Integrative Biology of the Cell (I2BC), Université Paris-Saclay, CEA, CNRS, Université Paris-Sud, Gif-sur-Yvette, France c

Lyssaviruses, including rabies virus (RABV) and Australian bat lyssavirus, cause over 55,000 human deaths worldwide annually (1). The RABV phosphoprotein (P-protein) has critical roles in genome transcription/replication and antagonism of interferon (IFN)-dependent antiviral responses (2–9). In infected cells, the P gene produces full-length P-protein (P1) and several N-terminally truncated isoforms, predominantly P2 and P3, via a ribosomal leaky scanning mechanism (7, 10). Although RABV replication occurs in the cytoplasm, the nucleocytoplasmic distribution of P-protein in infected cells is dependent on cellular nuclear export mechanisms, indicating that P-protein undergoes active nuclear trafficking during infection (7).

P-protein isoforms have distinct nuclear trafficking properties whereby P1 and P2 localize mainly in the cytoplasm, while P3 can accumulate in the nucleus (11–13) (Fig. 1). This depends on specific nuclear localization (NLS) and nuclear export (NES) sequences, including an N-terminal NLS (N-NLS) (Fig. 1) that is active only in P3, indicative of specific intranuclear functions (7, 11–13). Notably, a number of viruses with cytoplasmic replication cycles express proteins able to enter the nucleus and to interact with nucleoli and core nucleolar proteins, such as nucleolin (NCL) and fibrillarin (FBL), suggestive of key roles in infection potentially related to functions in ribosome biogenesis, RNA processing, and regulation of cell cycle progression and apoptosis (14–18). Possible roles for nucleoli/nucleolar proteins in lyssavirus infection, however, have not been investigated.

To identify proteins that interact with P-protein, we immunoprecipitated total P-protein from SK-N-BE cells infected with challenge virus standard (CVS) RABV for mass-spectroscopic analysis using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF). NCL was identified among the proteins co-precipitated (data not shown). To verify this interaction, we performed immunoprecipitation (IP) analysis using HEK293T cells transfected to express P1, P3, or P3(KR-N), in which the N-NLS residues K54 and R55 are replaced by asparagine to inhibit nuclear localization (11), fused to green fluorescent protein (GFP) (Fig. 1). As previously described (19), we observed that nucleolin was present as several forms in cell lysates (Fig. 2A and 3E, Input) with apparent molecular masses between ca. 55 and 110 kDa; this includes NCL modified posttranslationally, including through extensive phosphorylation, methylation, and acetylation (20–22), as well as NCL autocleavage products (19, 23, 24). NCL coprecipitated with P1 and P3(KR-N), despite the fact that NCL is strongly nuclear/nucleolar while P1 is cytoplasmic and P3(KR-N) is inhibited for nuclear import compared with wild-type P3 (Fig. 2A) (11). Notably, we also found that in all cases, P-protein co-precipitated specific forms of NCL (ca. 110 kDa and ca. 70 kDa or less) without precipitating forms between ca. 70 and 100 kDa (Fig. 2A; see also Fig. 3E); thus it appears that P-protein forms selective interactions with specific modified and/or cleaved forms of NCL.

We additionally analyzed IPs of GFP-P1 and P3 by mass spectrometry, identifying NCL as an interactor (data not shown); however, the core nucleolar protein FBL was not detected in this analysis, indicating that interaction with NCL is specific.

To examine the nucleolar interaction of P1, P3, and P3(KR-N) in intact cells, we transfected HeLa cells to express the GFP-fused proteins, before fixation and immunostaining with antibodies against the nucleolar marker FBL and analysis by confocal laser scanning microscopy (CLSM). This revealed that P3 localized throughout the nucleus, including within FBL-labeled nucleoli; this localization was substantially reduced for P3(KR-N) (Fig. 2B). Due to its exclusion from the nucleus, P1 did not colocalize with FBL/nucleoli (Fig. 2B). NCL can shuttle between the nucleus and cytoplasm but localizes principally in nucleoli (24). Immunostaining for NCL confirmed this distribution and indicated that P3 but not P1 can localize within NCL-labeled nucleoli (Fig. 2C). Expression of P1 or P3 did not appear to substantially affect the localization of NCL compared with mock-transfected cells. This suggests that P1 does not cause substantial relocalization of NCL to the cytoplasm such that their coimmunoprecipitation is likely to include associations formed postlys; however, since cellular NCL includes a cytoplasmic pool (25, 26), it is also likely that P1 interacts with cytoplasmic NCL in intact cells. Clearly, however, it remains possible that NCL is important for lyssavirus infection.
the data indicate that NCL interaction requires a sequence within residues 56 to 297 of P-protein.

To determine the region of P3 required for nucleolar localization and NCL interaction, we analyzed the localization of GFP-fused P34–174 (N-terminal region) and P174–297 (C-terminal domain [CTD]) (Fig. 1) in living cells (Fig. 3A and B) or cells immunostained for FBL or NCL (Fig. 3D), using CLSM. GFP-P34–174 was strongly nuclear due to N-NLS activity (11) but was excluded from nucleoli, consistent with the requirement for specific signals for nucleolar targeting (16) and suggesting that the nucleolar localization signal of P3 is within the CTD (Fig. 3A). Consistent with this GFP-P174–297, although less nuclear due to deletion of the N-NLS, clearly could localize in nucleoli (Fig. 3A).

To quantify the level of nucleolar localization of each of these proteins, we analyzed the nucleus of living cells expressing the different GFP-fused P-proteins or GFP alone, using differential interference contrast (DIC) microscopy to identify nucleoli and using CLSM to assess GFP localization within the nucleus/nucleoli (Fig. 3B). DIC typically identified 3 to 6 characteristic nucleolar structures within each nucleus. As expected, GFP was excluded from the nucleolus, and fusion to P3 or P174–297, but not P54–174, conferred the capacity to localize within the nucleolus (Fig. 3B). Calculation of the ratio of nucleolar to nuclear fluorescence corrected for background fluorescence (Fnu/Fn) in living cells was performed as previously described (27, 28); we also calculated the nuclear to cytoplasmic fluorescence ratio (Fn/Fc) (11) to determine the level of protein accumulation in the nucleus (Fig. 3C). This confirmed that GFP and GFP-P34–174 were excluded from nucleoli even though P34–174 conferred a significant (P < 0.0001) increase in nuclear accumulation. GFP-P3 and GFP-P174–297 showed significantly (P < 0.0001) increased nucleolar accumulation compared with GFP and GFP-P34–174, consistent with the presence of a nucleolar localization sequence (Fig. 3C). Nucleolar localization of GFP-P3 and GFP-P174–297 was also observed in fixed cells immunostained for NCL or FBL (Fig. 3D), and IP analysis identified coprecipitation of NCL with GFP-P174–297 but not GFP-P34–174 (Fig. 3E), indicating that the CTD is necessary and sufficient to target nucleoli and to interact with NCL. This suggests that in P3 the N-NLS mediates efficient nuclear import, and a CTD-localized nucleolar localization/NCL interaction signal mediates nucleolar association.

To examine the role of NCL in infection, we transfected U-373-MG cells with scrambled small interfering RNA (Scr-siRNA) or NCL-targeting siRNA using Dharmafect-1 reagent, according to the manufacturer’s instructions (Thermo Scientific). To exclude any off-target effects, we tested two siRNAs targeting distinct sequences of NCL (NCL1 [5′-UCCAAGGUAACUUUAAUUCUU-3′] and NCL2 [5′-UUCCUUUGACAGGCUCUCUUCCUU-3′]) (29) (Fig. 4 and data not shown). U373-MG cells (2 × 10^5) were transfected with siRNA (25 nM) on days 1 and 2, before infection with CVS RABV on day 3. Cells were lysed 16, 24, or 28 h later, and the expression of NCL, tubulin, and viral P- and N (nucleo) -proteins was analyzed by Western blotting (Fig. 4A and
NCL1 and NCL2, but not Scr-siRNA, depleted NCL in infected and mock-infected cells, but tubulin expression was unaffected, indicating that NCL depletion had no major effect on cell viability; we also confirmed directly that cell viability was unaffected by NCL siRNA transfection using a trypan blue exclusion assay (data not shown). P-protein was clearly detectable in infected Scr-siRNA-transfected cells, but in cells transfected with NCL1 or NCL2 siRNA, P-protein expression was strongly reduced (Fig. 4A and data not shown). Comparable effects on N-protein were observed (Fig. 4B). Inhibition of P-protein expression was observed at 16, 24, and 28 h postinfection and at multiplicities of infection (MOI) of 0.5, 2, and 5 (Fig. 4A and B and data not shown). To determine whether these effects correlated with altered infectious virus production, we collected supernatants from cells transfected with NCL1 or NCL2 and infected at MOI of 5 for 24 h, and determined viral titers by immunofluorescence using the Poisson distribution (30). NCL depletion by NCL1 or NCL2 resulted in a ca. 6-10-fold decrease in titer (Fig. 4C and data not shown).

In conclusion, our data show for the first time that RABV can interact with nucleoli and NCL via the P-protein and that NCL expression is important to infection. This identifies a novel host interface in lyssavirus infection, providing a potential new target for the development of attenuated vaccine strains and antivirals against these highly pathogenic viruses. Previous data have indicated roles for the nucleolus and nucleolin in the replication of viruses with nuclear replication cycles (e.g., retroviruses and DNA viruses), including direct roles for nucleoli as sites of genome replication (31–33). However, a number of studies have also indicated that certain cytoplasmic RNA viruses can interact with the nucleolus/nucleolar proteins with importance to infection (16, 18); our study now extends these observations to lyssaviruses.
These interactions are likely to relate to viral modulation of the multifunctional nucleolus/nucleolin, potentially including effects on ribosome biogenesis, nucleocytoplasmic transport, apoptosis, cell cycle and immunity (14, 34). P-protein has well established multifunctional nucleolus/nucleolin, potentially including effects on ribosome biogenesis, nucleocytoplasmic transport, apoptosis, cell cycle and immunity (14, 34). P-protein has well established roles in viral modulation of host cell biology, including innate immune responses (3, 4, 35–38), suggesting that it might target the nucleolus to facilitate these functions; this possibility will form the basis of future studies in our laboratory.

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REFERENCES


FIG 4 NCL is required for RABV infection. (A) U-373-MG cells transfected with Scr (control) or NCL-targeting siRNA (NCL1 [29]) were infected with CVS11 RABV at an MOI of 0.5 or 5, or mock infected, for 24 h. Cell lysates were then analyzed by IB using antibodies against NCL (rabbit antibody ab22758; Abcam). P-protein (mouse antibody 25C2 [39]), and tubulin (mouse antibody N3656; Amershams) and fluorescein-conjugated anti-rabbit and anti-mouse IgG secondary antibodies. The blot shown is representative of 2 assays. (B) Cell lysates from the assay represented in panel A were analyzed by IB using anti- IgG secondary antibodies. The blot shown is representative of 2 assays. (B) Cell lysates from the assay represented in panel A were analyzed by IB using anti- IgG secondary antibodies. The blot shown is representative of 2 assays. (B) Cell lysates from the assay represented in panel A were analyzed by IB using anti- IgG secondary antibodies. The blot shown is representative of 2 assays. (B) Cell lysates from the assay represented in panel A were analyzed by IB using anti- IgG secondary antibodies. The blot shown is representative of 2 assays.


