Interactions amongst rabies virus nucleoprotein, phosphoprotein and genomic RNA in virus-infected and transfected cells

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Previous in vitro studies have indicated that rabies virus (RV) phosphoprotein (P), by interacting with the nucleoprotein (N), confers the specificity of genomic RNA encapsidation by N. In this study, interactions amongst N, P and the genomic RNA in virus-infected as well as in transfected cells were studied. The results showed that when N was expressed alone, it bound non-specific RNA, particularly the N mRNA. When N and P were co-expressed, they formed N–P complexes that did not bind to non-specific RNA. When N and P were co-expressed together with (mini-)genomic RNA, N–P complexes preferentially bound the (mini-)genomic RNA. This demonstrated that RV P, by binding to N, does indeed confer specificity of genomic RNA encapsidation by N in vivo. Furthermore, the role of N phosphorylation in the N, P and RNA interactions was investigated. It was found that only N that bound to RNA was phosphorylated, while N in the N–P complex prior to RNA encapsidation was not, suggesting that RV P, by binding to nascent N, prevents the immediate phosphorylation of de novo-synthesized N. However, mutation at the phosphorylation site of N did not alter the pattern of N–P and N–RNA interactions, indicating that N phosphorylation per se does not play a direct role in N–P interaction and RNA encapsidation.

INTRODUCTION

The genomic RNA of rabies virus (RV), like other non-segmented negative-strand RNA viruses, is tightly encapsidated by the nucleoprotein (N) and this N–RNA complex, together with the phosphoprotein (P) and the RNA-dependent RNA polymerase (RdRp or L), forms the ribonucleoprotein (RNP) complex (Rose & Whitt, 2000). Complex interactions amongst the RNP components lead to the initiation and regulation of viral RNA transcription and replication (Wunner, 1991). It has been shown in studies of vesicular stomatitis virus (VSV) that N plays an important role in the switch from RNA transcription to replication by encapsidating the de novo-synthesized genomic RNA (Banerjee & Chattopadhyay, 1990; Blumberg et al., 1983; Wertz et al., 1987). VSV P, in addition to being a co-factor for RdRp, plays a vital role in RNA replication by forming a complex with VSV N and keeping it in a suitable form for RNA encapsidation. Furthermore, previous in vitro studies have shown that VSV N alone is capable of binding to any RNA (Blumberg et al., 1983). It is P that, by binding to N, confers the specificity of N encapsidation of genomic RNA (Banerjee et al., 1989). Studies of RV N–P and N–RNA interactions in vitro have also revealed that N alone can bind to any RNA (Yang et al., 1998). When RV N and P were added simultaneously to the in vitro encapsidation reaction, it did not increase the affinity of N binding to genomic RNA. However, it dramatically reduced the ability of N to bind non-specific RNA, indicating that P, by binding to N, does indeed confer the specificity of N for genomic RNA encapsidation by eliminating non-specific binding to RNA (Yang et al., 1998). We postulated that these in vitro observations reflected the situation in RV-infected cells. When RV N is expressed alone in insect cells, it encapsidates cellular RNA to form nucleocapsid-like structures (Iseni et al., 1998). In contrast, when N is expressed in virus-infected cells, only genomic RNA is encapsidated, especially in purified virions (Wunner, 1991). However, the detailed interactions amongst N, P and genomic RNA in the process of RNA encapsidation in vivo have not been studied.

RV N is phosphorylated (Sokol & Clark, 1973) at serine 389 (Dietzschold et al., 1987) by casein kinase II (Wu et al., 2003). N phosphorylation has been reported to play a modulatory role in the process of viral RNA transcription and replication since mutation of the phosphorylated serine in N leads to a reduction in viral RNA transcription and replication (Wu et al., 2002). Because unphosphorylated N
binds more strongly to RNA than phosphorylated N (Yang et al., 1999), we proposed that N phosphorylation plays a role in the process of viral RNA transcription and replication via modulation of RNA encapsidation. It has also been reported that phosphorylated N is detected in nucleocapsids, but not in the N–P complex or as free N (Kawai et al., 1999). This indicates that newly synthesized N is not immediately phosphorylated but rather is associated first with P, which suggests that RV N phosphorylation occurs during the processes of or after RNA encapsidation.

In the present study, we investigated RV N, P and RNA interactions in vivo by expressing these components either individually or in combination in insect and mammalian cells. Our results showed that when RV N was expressed alone, it could bind to any RNA, particularly the N mRNA. When N and P were co-expressed in vivo, the N and P formed N–P complexes that did not bind to non-specific RNA. In the presence of RV (mini-)genomic RNA, the N–P complex bound to genomic RNA. Furthermore, the N in the N–P complex was not phosphorylated prior to encapsidation of the genomic RNA, suggesting that RV P, by binding to N, prevents phosphorylation of N.

**METHODS**

**Cells, viruses, plasmids and antibodies.** BSR cells were grown in Dulbecco’s MEM and insect cells (Hi-5) were cultured in Insect Express medium. RV strain L16 and recombinant baculoviruses expressing N (BRN) and P (BRP) were prepared as described previously (Fu et al., 1991, 1994; Wu et al., 2002). Recombinant vaccinia virus expressing T7 polymerase (vTF7-3) was prepared as described previously (Fuerst et al., 1986). Plasmids for expression of N (pRN), mutant N with serine 389 changed to alanine (pRN-SA) and P (pRP) have been constructed previously (Fu et al., 1994; Yang et al., 1998). RV minigenome (pSDI-CAT) was prepared as described previously (Conzelmann & Schnell, 1994). Monoclonal antibody to RV N (mAb 802-2) was obtained from the Monoclonal Antibody Research and Collation Centre for Disease Control and Prevention (Hamir et al., 1995) and anti-P polyclonal antibody was prepared in rabbits (Fu et al., 1994).

**Protein expression in insect cells.** Insect cells were infected with BRN, BRP or both as described previously (Fu et al., 1991, 1994). At 4 days post-infection, cells were harvested and disrupted in a Dounce homogenizer. After removing nuclei and cell debris by centrifugation, supernatants were used to purify N or N–RNA complexes.

**Protein expression in mammalian cells.** Transfection and labelling of BSR cells were performed as described by Wu et al. (2002). Briefly, cells were infected with recombinant vaccinia virus. One hour later, cells were transfected with pRN alone, pRN and pRP, pRN and pRP plus pSDI-CAT, or pRN and pSDI-CAT. Alternatively, pRN was replaced by pRN-SA in the transfection. Twelve hours after transfection, cells were washed with PBS and incubated with methionine-free medium for 1 h. Cells were then labelled with [35S]methionine (Amersham) for 8 h. Cells were harvested and processed as above.

**Purification of viral RNP, recombinant N and B-RNP.** Viral RNP was purified as described previously (Schneider et al., 1973). Cells infected with RV strain L16 were lysed with deionized H2O. After removing the cell debris and nuclei, supernatants were mixed with CsCl to a final concentration of 40 % (w/v) and centrifuged in an SW41 rotor at 37 000 r.p.m. overnight. The viral RNP band was collected and dialysed. Recombinant N was purified from insect cells by affinity chromatography as described (Fu et al., 1991). The RNP-like structure from baculovirus-expressed N (B-RNP) was purified the same way as for viral RNP by mixing the affinity-purified N with CsCl.

**Separation of N–P complexes from N–RNA or N–P–RNA complexes.** To separate further the N, P and N–P complexes from N–RNA complexes, discontinuous CsCl gradient centrifugation was performed as described previously (Spehner et al., 1997). Briefly, protein samples were collected from insect or mammalian cells were loaded on to a discontinuous CsCl gradient [20–40 % (w/v)] in NTE buffer (0-2 M NaCl, 10 mM Tris/HCl, pH 8-0, 0-1 mM EDTA). After centrifugation in an SW41 rotor at 37 000 r.p.m. overnight, the gradient was fractionated from top to bottom into 18 fractions. Each of the fractions was dialysed against NTE buffer before use.

**In vitro RNA encapsidation and gel-shift assay.** Viral leader RNA was transcribed and labelled as described previously (Yang et al., 1998). RNA encapsidation was performed as follows: recombinant RV N or B-RNP was allowed to react with in vitro-transcribed RV leader RNA (106 c.p.m. per reaction). The RNA–protein mixtures were subjected to digestion with micrococcal nuclease and RNase A at 37 °C for 30 min. The products were analysed by electrophoresis on a 4 % polyacrylamide gel containing 5 % glycerol.

**SDS-PAGE, Western blotting and immunoprecipitation.** Dialysed samples were subjected to electrophoresis on a 12 % polyacrylamide/10 % SDS gel and the separated proteins were electrophlated on to nitrocellulose membrane and incubated with anti-N or anti-P antibodies. After incubation with biotinylated secondary antibody, the proteins on the membrane were detected with DAB substrate (Vector Lab). For immunoprecipitation, samples were incubated with anti-N or anti-P antibodies overnight at 4 °C. Protein A–Sepharose was then added and the reaction incubated for 2 h at 4 °C. Immune complexes were precipitated, washed three times in NTE buffer and analysed by SDS-PAGE.

**RNA extraction, Northern blotting and dot-blot hybridization.** RNA extraction was performed either from purified viral RNP, B-RNP, baculovirus-expressed N (B-N), different fractions of the CsCl gradient or from immunoprecipitated preparations using Trizol. RNA samples were subjected to electrophoresis and blotted to membrane for Northern hybridization. Alternatively, RNA was directly dot blotted on to nylon membrane and fixed by UV cross-linking. Genomic RNA, a genomic analogue or N mRNA was detected by hybridization with digoxigenin-labelled N- or CAT-specific probes as described previously (Wu et al., 2002).

**Phosphorylation of RV N protein.** To study the status of N phosphorylation, BSR cells were labelled with [32P]P phosphoric acid (Amersham) as described previously (Wu et al., 2002). Cells were harvested and subjected to immunoprecipitation with anti-N antibodies followed by SDS-PAGE.

**RESULTS**

**RV N expressed in insect cells exists as RNA-free and RNA-bound forms.** To investigate whether RV N purified by affinity chromatography (B-N) (Fu et al., 1991) was associated with RNA, we subjected B-N to centrifugation in 40 % (w/v) CsCl. As a control, viral RNP was prepared from RV-infected cells. A band was formed at the same position (two-thirds of the...
way down the tube) as the RNP band from virus-infected cells. This band (termed B-RNP) was removed and the refractive index measured. The refractive index of the B-RNP was 1.3665, very similar to that of the viral RNP (1.3655), which corresponded to densities of 1.345 and 1.329, respectively. These data indicated that the affinity-purified N from insect cells contained RNA and formed nucleocapsid-like complexes.

To analyse whether recombinant N expressed in insect cells existed in forms other than the N–RNA complex, we collected nine fractions from the cushion. An aliquot from each of the fractions (fractions 5–8 contained B-RNP) was analysed by SDS-PAGE followed by Coomassie blue staining. As shown in Fig. 1(a), N was found in every fraction. To analyse the RNA content, the B-RNP portion and the rest of the cushion (fractions 1–4) were pooled and RNA was extracted and subjected to electrophoresis. As shown in Fig. 1(c), many RNA species were observed ranging from approximately 100–800 nt in the B-RNP portion (lane 2), but very little RNA was observed in the other fractions (lane 3). These data indicated that, although N was purified to near homogeneity by affinity chromatography (Fu et al., 1991), both RNA-free N (approx. 40%) and RNA-bound N (B-RNP, approx. 60%) were found.

**RV N expressed in insect cells encapsidates N mRNA**

To investigate whether RV N expressed in insect cells encapsidated N mRNA, RNA was prepared from each of the fractions shown in Fig. 1(a) and subjected to Northern blot hybridization. As shown in Fig. 1(b), N RNA was detected only in the B-RNP fractions (fractions 5–8), but not in fractions 1–4, further indicating that N expressed in insect cells exists as both RNA-free and RNA-bound forms. RNA preparations were also made from equal amounts of B-RNP, B-N (before CsCl centrifugation) or viral RNP and subjected to Northern blot hybridization using an N-specific probe (Wu et al., 2002). As shown in Fig. 2(a), N RNA was detected in all samples, with the most intense RNA band from viral RNP and the least intense band from B-N. The N RNA in the RNP sample presumably represented genomic RNA and the N RNA in the B-RNP and B-N represented N mRNA. These data indicated that at least part of the RNA encapsidated by recombinant N expressed in insect cells was N RNA. To test the ability of B-RNP to encapsidate RNA, the same amount of B-RNP and B-N (by weight) was used to encapsidate in vitro-synthesized leader RNA. As shown in Fig. 2(b), B-N encapsidated 15 times more RNA than B-RNP as measured by densitometry. These results indicated that B-RNP had already

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**Fig. 1.** Recombinant N expressed in insect cells exists as both RNA-free and RNA-bound N. Recombinant RV N expressed in insect cells and purified by affinity chromatography (Fu et al., 1991) was subjected to centrifugation on a 40% CsCl cushion. The cushion was then fractionated into nine fractions (fractions 5–8 contained the banded material, B-RNP). Aliquots from each of the fractions (numbered from top to bottom) were analysed by SDS-PAGE and Coomassie blue staining (a). MW, molecular mass marker. RNA was extracted from each of the fractions, subjected to electrophoresis and then hybridized with an N cDNA probe (b). RNA was also extracted from B-RNP (fractions 5–8) or from other fractions (1–4) and subjected to electrophoresis on a 2% agarose gel (c). Lane 1, molecular mass marker; lane 2, RNA extracted from B-RNP (fractions 5–8); lane 3, RNA extracted from fractions 1–4.
encapsidated RNA in the insect cells and, as expected, its ability to encapsidate in vitro-synthesized RNA further was dramatically reduced.

**RV N and P form complexes that prevent N from binding to RNA in insect cells**

To investigate whether the N–P complex formed in insect cells could prevent non-specific RNA encapsidation, RV N and P were expressed either individually or in combination using recombinant baculoviruses (Fu *et al*., 1991, 1994). RV-infected cells were used as a control. The results are summarized in Fig. 3(a). In RV-infected cells, both N and P were detected in fractions 11–15 and again in fractions 17–18, indicating that most of N formed complexes with P in infected cells. In insect cells expressing N alone (BRN), most of the N was detected in fractions 16–18, near the bottom of the gradient. A minor portion of N was detected in the upper fractions 10–14. In insect cells expressing P alone, all of P was detected in the upper fractions 5–12 and no P was detected in the bottom fractions. In insect cells expressing both N and P, most of N was shifted from the bottom fractions to the upper fractions 8–14. P was detected in the upper fractions 5–14, mostly in fractions where N was also detected, presumably forming an N–P complex. However, no P was detected in the bottom fractions (16–17) although some N was detected in these fractions.

**Fig. 2.** Recombinant N encapsidates N mRNA. Viral RNP, recombinant B-N and B-RNP (equal amounts by weight) were subjected to RNA extraction, electrophoresis on a 1% agarose gel and hybridization with an N sequence-specific cDNA probe (a). B-RNP and B-N (equal amounts by weight) were allowed to encapsidate in vitro-synthesized RV leader RNA L-70 (Yang *et al*., 1998). After RNase digestion, the N–RNA complex was analysed by a gel-shift assay on a 4% polyacrylamide gel (b).

**Fig. 3.** RV N and P expressed in insect cells. Insect cells were infected with either BRN or BRP, individually or in combination, and then harvested. Soluble proteins were subjected to CsCl gradient [20–40% (w/v)] centrifugation. After fractionation, the protein in each fraction was subjected to SDS-PAGE and Western blotting using either anti-N or anti-P antibodies (a). BSR cells infected with RV strain L16 were processed as controls. RNA was also prepared from each of the fractions and subjected to electrophoresis and dot-blot hybridization using an RV N sequence-specific cDNA probe (b).
To determine whether N in any of these fractions was associated with RNA, each of the fractions was subjected to RNA extraction and the RNA was spotted on to membranes for dot-blot hybridization with an N-specific probe. As shown in Fig. 3(b), RNA was only detected in the bottom fractions, particularly where N was also abundant, indicating that RNA-bound N sediments to the bottom fractions. In infected cells, N, together with P and genomic RNA, formed RNP complexes and sedimenting to the bottom fractions of the gradient. In insect cells expressing N alone, most of the N bound non-specific RNA, forming a nucleocapsid-like structure (B-RNP) and sedimenting to the bottom fractions. In insect cells expressing both N and P, most of the N bound to P to form N–P complexes, which did not encapsidate any RNA. As a consequence, very little RNA was detected in the bottom fractions. All these data suggested that P, by binding to N, prevents N from encapsidating non-specific RNA.

**RV P confers the specificity of genomic RNA encapsidation in vivo**

To investigate whether the N–P complex confers specificity of encapsidation of genomic RNA, we employed the RV minigenomic system. BSR cells were transfected with pRN alone or in combination with pRP. Alternatively, RV N alone or N and P were expressed together with the minigenomic RNA (pSDI-CAT). As shown in Fig. 4(a), the N and P profiles in BSR cells transfected with pRN or pRN plus pRP were similar to those in insect cells expressing N and N plus P, respectively. Most of the N in cells expressing N alone was detected in the bottom fractions 16–18. In contrast, most of the N in cells expressing both N and P shifted to the upper fractions 6–15. A minor portion of N was detected in the bottom fractions 16–17; however, very little or no P was detected in the bottom fractions. In cells expressing N, P and the minigenomic RNA, the majority of N was detected in the bottom fractions 16–18 and P was clearly detectable in these fractions as well. Thus, the protein profiles in cells expressing N, P and genomic RNA showed similar patterns to those in cells infected with RV (see Fig. 3a). To investigate whether the ratio of N : P affected the N–P interaction, we used an N : P ratio of either 10 : 1 or 1 : 10 with or without minigenomic RNA. However, similar protein profiles were observed (data not shown) regardless of the N : P ratio, indicating that the N : P ratio does not affect the N–P or N–P–RNA interactions.

To investigate whether N or N–P complexes expressed in mammalian cells encapsidated RNA, the gradient fractions were collected into three pools, pool 1 (fractions 1–4), pool 2 (fractions 5–14) and pool 3 (fractions 15–18). RNA was extracted from each of the pools, dot blotted and hybridized with either N or CAT (minigenomic RNA) probes. As shown in Fig. 4(b), RNA was only detected in fractions 15–18, similar to the results found in insect cells. No RNA was detected in the top fractions, further confirming that the N–P complex in the top fractions did not encapsidate any RNA. In cells expressing N, P and the minigenomic RNA, CAT RNA was preferentially encapsidated over N RNA. To make certain that the steady state of the N RNA was similar in cells transfected with each of the...
combinations, total RNA was subjected to Northern blot hybridization with an N-specific probe. A similar level of N RNA was detected (Fig. 4b, middle panel). All these data indicated the specificity of the N–P complex in encapsidation of genomic RNA.

**RV P, by binding to N, prevents N from being phosphorylated**

To investigate whether N phosphorylation was involved in the RNA encapsidation process, we studied the phosphorylation status of N. As shown in Fig. 5, phosphorylated N was only detected in the bottom fractions 16–18 from either infected or transfected cells. In cells expressing N and P, very little phosphorylated N was detected and very little N was found in the bottom fractions as shown in Fig. 4(a). N in the top fractions, where it is bound to P (Fig. 3a), was not phosphorylated (Fig. 5). These data indicated that RV N (mostly in N–P complexes) was not phosphorylated before RNA encapsidation. It was phosphorylated either during or after RNA encapsidation. These results suggested that P, by binding to N, prevented N from being phosphorylated.

**Mutant N interacts with P and RNA in the same way as native N**

Since RV N phosphorylation occurred during or after RNA encapsidation, we investigated whether mutant N with the phosphorylated serine changed to alanine (Yang et al., 1999) could alter the patterns of N–P and N–RNA interactions. As shown in Fig. 6(a), the mutant N and P distribution profiles were similar to those in cells expressing native N.

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**Fig. 5. Phosphorylation of RV N in infected and transfected cells.** BSR cells were infected with recombinant vaccinia virus vTF7-3 and then transfected with pRN, pRN and pRP, pRN and pRP plus pSDI-CAT, or pRN and pSDI-CAT. Cells were incubated with [32P]phosphate and harvested at 48 h after transfection. Alternatively, cells were infected with RV strain L16 and incubated with [32P]phosphate. 32P-labelled proteins were solubilized for immunoprecipitation with anti-N antibodies and the immunoprecipitated proteins were subjected to SDS-PAGE followed by autoradiography.

**Fig. 6. Effect of mutant N on RNA encapsidation.** BSR cells were infected with recombinant vaccinia virus vTF7-3 and then transfected with pRN-SA, pRN-SA and pRP, pRN-SA and pRP plus pSDI-CAT, or pRN-SA and pSDI-CAT. Cells were labelled with [35S]methionine and harvested at 48 h after transfection. Proteins were solubilized for immunoprecipitation with anti-N or anti-P antibodies and the immunoprecipitated proteins were subjected to SDS-PAGE followed by autoradiography (a). RNA was prepared from pooled fractions and subjected to electrophoresis and dot-blot hybridization using an N- or CAT-specific cDNA probe (b). Pools 1–3 were as described in Fig. 4.
and P (see Fig. 4). Furthermore, only mutant N in the bottom fractions 16–18 contained RNA (Fig. 6b). Thus, the unphosphorylated (mutant) N interacted with P and RNA in a similar way to native N.

DISCUSSION

The rhabdovirus N plays an important role in the switch from viral transcription to replication by encapsidating genomic RNA (Banerjee & Chattopadhyay, 1990; Blumberg et al., 1983; Wertz et al., 1987). Although only the genomic RNA is encapsidated by N and packaged into virions (Banerjee & Chattopadhyay, 1990; Wunner, 1991), previous in vitro studies have shown that N is capable of binding non-specific RNA (Blumberg et al., 1983; Yang et al., 1998). It is P that, by binding to N, confers the specificity of genomic RNA encapsidation by N (Banerjee et al., 1989; Yang et al., 1998). In the present study, we have presented in vivo evidence using the RV model that it is indeed P that, by binding to N, prevents N from encapsidating non-specific RNA, thus conferring the specificity of genomic RNA encapsidation. When N was expressed alone, it bound to non-specific RNA to form nucleocapsid-like structures. When N was co-expressed with P in transfected cells, it formed N–P complexes, which did not bind to non-specific RNA. When N, P and minigenomic RNA were expressed simultaneously in transfected cells, N bound to P to form N–P complexes that encapsidated the minigenomic RNA.

Different methods have been used to purify RV N expressed in insect cells (Fu et al., 1991; Iseni et al., 1998; Prehaud et al., 1990). Using antibody affinity chromatography, we purified N to near homogeneity (Fu et al., 1991) and this purified N was capable of interacting with P (Fu et al., 1994) and binding RNA in vitro (Yang et al., 1998). Using glycerol gradient sedimentation velocity centrifugation, Iseni et al. (1998) purified N and found that the recombinant N formed ring-like structures. Further analysis (Iseni et al., 1998; Schoehn et al., 2001) indicated that these ring-like structures contained 9–11 N monomers, which encapsidated RNA of 80–90 nt. In the present study, we also found that recombinant N encapsidated low-molecular-mass RNA. All these studies indicate that RV N encapsidates RNA and forms nucleocapsid-like structures (termed B-RNP in this study) in insect cells. These studies also raised the question of how recombinant N purified by antibody affinity chromatography (Fu et al., 1991) was still capable of encapsidating RNA in vitro (Iseni et al., 1998; Yang et al., 1998). To address this, we further analysed N by centrifugation in 40% CsCl and found that recombinant N expressed in insect cells existed as both RNA-free and RNA-bound forms. In vitro encapsidation assays revealed that the ability of RNA-bound N (B-RNP) to encapsidate in vitro-synthesized RNA decreased 15-fold when compared with RNA-free N (B-N). These results indicated that recombinant N in the B-RNP was no longer capable of encapsidating RNA. The RNA-binding activity of the affinity-purified N (Yang et al., 1998) was not due to the removal of RNA from the B-RNP by alkaline pH during the purification process as suggested by Iseni et al. (1998). Rather, a portion of N purified by affinity chromatography exists as RNA-free N and thus is capable of encapsidating in vitro-synthesized RNA.

By sequencing some of the RNA associated with the recombinant N, Iseni et al. (1998) identified cellular tRNA, suggesting that RV N expressed in insect cells encapsidated cellular RNA. However, our in vitro encapsidation assay showed that RV N bound more strongly to N mRNA than to non-specific and non-viral RNA (Yang et al., 1998). It could thus be assumed that, in insect cells, RV N would encapsidate N mRNA as well as other RNA species. To this end, we prepared RNA samples from B-RNP or nucleocapsid-like structures in mammalian cells for Northern blot hybridization using an N-specific probe. It was found that all the RNA samples hybridized with the N-specific probe, indicating that RV N encapsidated N mRNA in addition to cellular tRNA. Green et al. (2000) also detected N mRNA encapsidated by VSV N when it was expressed in prokaryotes, further indicating that rhabdovirus N is capable of encapsidating N mRNA when expressed alone.

RV P, as in other negative-strand RNA viruses, plays multiple roles in the virus replication cycle. In addition to being the co-factor for RdRp, P interacts with N (Banerjee & Chattopadhyay, 1990; Wertz et al., 1987). The N–P interaction has many facets in the process of viral transcription and replication. P, by binding to N, not only keeps N soluble for RNA encapsidation (Banerjee & Chattopadhyay, 1990; Wertz et al., 1987) but also confers the specific encapsidation of genomic RNA (Banerjee et al., 1989). Previous in vitro studies have amply demonstrated the latter function of P (Banerjee et al., 1989; Wertz et al., 1987; Yang et al., 1998). However, few in vivo studies have been carried out to confirm these in vitro studies. In one report, Spehner et al. (1997) expressed measles virus N and P either individually or in combination using recombinant vaccinia virus. They reported similar findings to those observed for RV N and P in the present paper. When N was expressed alone, most bound to non-specific RNA and formed nucleocapsid-like structures. When N was co-expressed with P, it interacted with P to form N–P complexes that prevented N from encapsidating non-specific RNA. However, Spehner et al. (1997) did not express genomic RNA in the system. When we expressed N, P and minigenomic RNA together, we observed that the N–P complexes preferentially encapsidated the minigenomic RNA. All these data demonstrate that, although N by itself is capable of encapsidating any RNA species to form nucleocapsid-like structures, P, by binding to N, eliminates the encapsidation of non-specific RNA. A small amount of N and RNA was always detected in the bottom fractions in cells expressing N and P. This could be due to some of the N that has not yet formed a complex with P and is thus still capable of binding to non-specific RNA. Furthermore, non-specific binding of N RNA was also detected in cells expressing N, P and...
minigenomic RNA. This was also due to the fact that not all of N bound to P immediately after synthesis and thus a portion of the free N bound to non-specific RNA. This condition not only exists in cells expressing N, P and minigenomic RNA, but also in virus-infected cells. Binding of mRNA by N has been reported in RV-infected cells as mRNP (Wunner, 1991). Together these results confirm previous in vitro findings that P, by binding to N, confers specific encapsidation of genomic RNA by eliminating non-specific RNA encapsidation (Yang et al., 1998).

In addition to the functions reported previously, we found another function for P, at least in the RV model. RV P, by binding to N, prevents N from becoming phosphorylated. RV N is phosphorylated in virus-infected cells (Sokol & Clark, 1973), as well as when N is expressed alone in eukaryotic cells (Prehaud et al., 1990; Yang et al., 1999). N phosphorylation has been reported to play a modulatory role in the process of RV transcription and replication (Wu et al., 2002; Yang et al., 1999). Since unphosphorylated or dephosphorylated N binds to RNA more strongly than phosphorylated N, we assumed that RV N phosphorylation may be involved in the RNA encapsidation process. In the present study, we examined the phosphorylation status of RV N in virus-infected cells, as well as in cells expressing N alone, N and P, or N and P plus the minigenome. We found that N was not phosphorylated before RNA encapsidation, and only RNA-bound N was phosphorylated. These findings are in agreement with the report by Kawai et al. (1999) that phosphorylated N is detected only in the nucleocapsid, but not in the N–P complex or as RNA-free N. Together, these studies indicate that N is phosphorylated either during or immediately after RNA encapsidation. The fact that only RNA-bound N was phosphorylated suggests that N phosphorylation per se is not important in the process of RNA encapsidation. However, these data suggest that P, by binding to N, prevents N from being phosphorylated.

Previously, we showed that unphosphorylated N had a higher affinity for RNA than phosphorylated N (Yang et al., 1999). Thus, it is advantageous for N not to be phosphorylated before encapsidating RNA. Here, we have shown definitively that RV N is not phosphorylated before RNA encapsidation, regardless of whether N is expressed alone or in virus-infected cells. During or after RNA encapsidation, N may go through conformational changes that enable it to be phosphorylated. We have hypothesized that, following phosphorylation, the charge repulsion between the negatively charged phosphoserine of N and the negatively charged genomic RNA may weaken the interaction between N and RNA, thus facilitating the initiation of the next round of viral RNA transcription and replication (Wu et al., 2002). Recently, Toriumi & Kawai (2004) reported that N phosphorylation enhances N–P interactions in the nucleocapsid. Thus, although N phosphorylation may not be important in the RNA encapsidation process, it plays a role in subsequent replication cycles.

Another interpretation for the fact that N is not phosphorylated in N–P complexes may be that RV N in N–P complexes prior to RNA encapsidation is in a unique conformation. In such a conformation, N cannot be phosphorylated. Furthermore, we hypothesize that in such a unique conformation N can only encapsidate genomic RNA. However, the mechanisms by which P, by binding to N, keeps N in a unique conformation for specific encapsidation of genomic RNA are not clear. Recently, it has been shown that the N : P ratio in the N–P complex before RNA encapsidation is 1 : 2 (Mavrakis et al., 2003). However, it has been reported that the N : P ratio is 2 : 1 in the purified

![Fig. 7. Proposed model of N, P and RNA interactions and N phosphorylation. When N is expressed alone, it binds to any RNA and thus becomes phosphorylated (a). When N is co-expressed with P, P binds to N to form N–P complexes, which eliminates the non-specific RNA encapsidation. Because no RNA has been encapsidated, N stays unphosphorylated (b). When N, P and genomic RNA are expressed together, N and P form N–P complexes that encapsidate the genomic RNA and N becomes phosphorylated (c).](https://example.com/fig7.png)
virions (Wunner, 1991). These observations suggest that the mode of N–P interaction before RNA encapsidation is different from that after RNA encapsidation.

Based on all the available data, we propose the following model (Fig. 7) to explain the detailed N, P and RNA interactions and N phosphorylation. When N is expressed alone, it binds to any RNA (Fig. 7a). During the process of N–RNA interaction, the RNA-bound N undergoes a conformational change and becomes phosphorylated. When N is co-expressed with P, P binds N to form the N–P complex, which eliminates non-specific RNA encapsidation. Because no RNA has been encapsidated, N has not undergone any conformational change and thus stays unphosphorylated (Fig. 7b). When N, P and genomic RNA are expressed together, N can encapsidate genomic RNA and thus becomes phosphorylated (Fig. 7c). Although the mechanisms by which RV N and P interact with each other to eliminate the binding of non-specific RNA are not understood, we offer the following explanations. It is possible that P binds to N and keeps N in a unique conformation. Due to steric hindrance, in such a conformation N cannot encapsidate non-specific RNA, but can still encapsidate genomic RNA. Because N is in such a conformation, it cannot be phosphorylated. Alternatively, RV P and RNA may share the same binding site. Due to some unknown characteristics, the genomic RNA, but not non-specific RNA, can displace P, thus encapsidating the genomic RNA. Because the phosphorylation site (aa 389) is close to the putative RNA-binding domain (aa 289–352) (Kouznetzoff et al., 1998), at this particular site, P might prevent N from phosphorylation. After displacing P and encapsidating RNA, N undergoes a conformational change, and thus becomes phosphorylated. Clearly, further studies are needed to investigate the dynamic interactions between N and P during the RNA encapsidation process, which could shed light on the mechanisms by which P, by binding to N, keeps N in the unique conformation that is able to encapsidate the genomic RNA.

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REFERENCES


