Structural relationship between nucleocapsid-binding activity of the rabies virus phosphoprotein (P) and exposure of epitope 402-13 located at the C terminus

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The structural changes of the nominal phosphoprotein (P) of rabies virus using a monoclonal antibody, mAb #402-13, was investigated. This mAb recognized a linear epitope that was mapped roughly to a C-terminal region of the P protein, ranging from aa 256 to 297. The P gene products were detected by the mAb in immunoblot assays, the products of which were produced either in BHK-21 cells or in Escherichia coli cells. The mAb, however, detected very low levels of P gene products in immunoprecipitation assays. The mAb recognized the nucleocapsid (NC)-associated P proteins but recognized free P protein and free N–P complex produced in the infected cells much less efficiently. When the P proteins were released from the NC, however, they were no longer recognized by the mAb. Similar results were obtained from BHK-21 cells co-transfected with P and N cDNAs. Furthermore, studies with C-terminally truncated P protein mutants revealed that the NC-binding ability of the P protein was dependent on the presence of the C-terminal epitope region. From these results, it is thought that the 402-13 epitope region is concealed when the P protein is present in a free form or free N–P complex but is exposed when it is associated with the NC. The C-terminal epitope region seemed to be essential for the P protein to be associated with the NC but not for the formation of free N–P complexes with newly synthesized N protein.

Introduction

The nominal phosphoprotein (P) of rabies virus is thought to collaborate in viral RNA synthesis with a large catalytic protein (L) of the virus, based on the structural and functional similarities with the phosphoprotein (P/NS) of vesicular stomatitis virus (VSV), another member of the Rhabdoviridae family (Kawai & Morimoto, 1994). The nature and properties of the rabies virus P protein are summarized briefly below. The rabies virus P protein is composed of several species of subcomponents produced by different grades of phosphorylation as well as various amino acid truncations from the N terminus caused by the initiation of protein synthesis from different in-frame AUG codons (Chenik et al., 1995; Takamatsu et al., 1998). Phosphorylation of the P protein seems to be performed by certain protein kinases of host-cell origin, as shown by their different susceptibility to protein kinase inhibitors (Takamatsu et al., 1998; Gupta et al., 2000).

The P protein is thought to bind to the nucleoprotein (N) to play its roles in viral RNA synthesis; however, structural requirements seem to be different for its appropriate interactions with the viral N protein, probably depending on the different situations of the N protein (i.e. before, during and after the encapsidation of viral genomic RNA). For instance, Chenik et al. (1994) suggested the existence of at least two independent N protein-binding sites on the P protein, one located in the C-terminal part and the other located between aa 69 and 177. Fu et al. (1994) reported that the N–P interaction of the N-terminal domain of the P protein occurs when the N protein is newly synthesized or during the period of N and P protein synthesis, while the C-terminal-binding site is capable of interacting with the N protein even when both proteins are synthesized separately. Very recently, Jacob et al. (2001) reported that binding through the C-terminal half of the P protein was much stronger than the interaction through its N-terminal half. In addition to N protein binding, the rabies virus
P protein was shown recently to associate with the light chain (LC8) of cellular dynein, which was suggested to be involved in transportation of the viral nucleocapsid (NC) through the neuronal axons (Jacob et al., 2000; Raux et al., 2000).

Detailed studies for understanding the function-related conformations and conformational changes of the P protein and its precise roles in the virus replication cycle at the molecular level are, however, lacking. For such kinds of studies to be performed, it would be helpful to use monoclonal antibodies (mAbs) that recognize specific structures or conformations of the protein.

Concerning this problem, we found in our stocks of mAbs, which were selected for their ability to recognize rabies virus-induced cytoplasmic inclusion bodies (Kawai et al., 1999), a mAb (#402-13) that recognized the P protein. In this study, this mAb was shown to recognize a linear epitope of the protein that was exposed only when the protein took a specific conformation. Using this mAb, we could further differentiate in the cell at least two forms of the P protein, the 402-13 epitope-positive and -negative forms or conformations. We will discuss a possible role of the 402-13 epitope-positive P protein.

Methods

■ Viruses and cell culture. We used the BHK-adapted HEP-Flury strain of rabies virus (Kawai et al., 1975). Working stocks of the virus were prepared by a single passage of plaque-purified virus isolates through BHK-21 cell monolayer cultures. Infectivity titres were assayed by plaque formation on agarose-suspended BHK-21 cells, as noted previously (Kawai & Takeuchi, 1992). The recombinant vaccinia virus RVV-T7 (a kind gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science, Tokyo) is a modified virus that contains the RNA polymerase gene of T7 bacteriophage. RVV-T7 was used to provide T7 RNA polymerase for promoting the expression in animal cells of a foreign gene inserted downstream of the T7 promoter of prokaryotic and eukaryotic expression vectors pET3a and pCDM8. Working stocks of RVV-T7 were prepared and plaque-assayed using BHK-21 cell monolayers.

BHK-21 cells were propagated in Eagle’s minimal essential medium (MEM) supplemented with 5% calf serum and 10% tryptophan phosphate broth (Difco). Virus-infected cultures were maintained in MEM containing 3% foetal bovine serum.

■ Expression of P cDNA in Escherichia coli. For production of the P protein analogues in E. coli, full-length P cDNA was inserted into the Ndel site of an inductive expression vector, pET3a (Novagen), as described previously (Takamatsu et al., 1998). Expression of cDNA was induced in E. coli BL21 (DE3) pLysS cells (Novagen) by adding IPTG at a final concentration of 1 mM, according to Studier et al. (1990). P gene products were lysed in SDS–PAGE sample buffer.

■ Expression of P and N cDNAs in animal cells. Reconstructed expression vectors for P and N proteins (pCDM8-P and pCDM8-N) were described in our previous report (Takamatsu et al., 1998; Kawai et al., 1997). Transfections of P and N cDNAs to BHK-21 cells were done using the calcium phosphate method, as described previously, for which RVV-T7 was used to provide the T7 RNA polymerase (Kawai et al., 1999).

■ Preparation of C-terminally deleted mutants of the P protein. The P cDNA of rabies virus (HEP-Flury strain) was cut with one of the following restriction enzymes: HincII, HindIII, BamHI or Mbol. It was then ligated with an universal Ndel linker [d(CTAGCTAGCTAG)] to introduce stop codons in all translational frames and subsequently transferred into an expression vector, pET3a. By this process, the P cDNA was made to produce C-terminally truncated P protein analogues, termed PΔC166, PΔC89, PΔC42 and PΔC22, lacking 166, 89, 42 and 22 aa from the C terminus, respectively. In this study, the prokaryotic expression vector pET3a was used for P gene expression in BHK-21 cells with the help of T7 phage RNA polymerase encoded by recombinant vaccinia virus (RVV-T7).

■ Antibodies. Hybridoma cells were the same as those described previously (Kawai et al., 1997, 1999). mAbs were screened originally according to their recognition of the viral antigens located in the virus-induced cytoplasmic inclusion bodies by fluorescent antibody (FA) staining (Kawai et al., 1997). Among those, a mAb (#402-13, isotype IgM) was found to recognize the SDS-denatured P protein from rabies virus-infected cells (Fig. 1A). We also used two anti-N mAbs, 5-2-26 and 5-1-11, which recognize a phosphorylation-dependent linear epitope and a conformational epitope of rabies virus N protein, respectively (Kawai et al., 1997, 1999; Anzal et al., 1999).

Rabbit polyclonal antibodies (pAbs) against the rabies virus P and N proteins were the same as those described previously (Kawai et al., 1997; Takamatsu et al., 1998). Antibody titres were determined by FA staining of rabies virus-infected cells.

■ SDS–PAGE. Assay samples were dissolved in SDS–PAGE sample lysis buffer (125 mM Tris–HCl, 4% SDS, 10% 2-mercaptoethanol, 0.005% bromophenol blue and 20% glycerol; pH 6.8) and applied to 10 or 12% polyacrylamide gels prepared using Laemmli’s discontinuous buffer system (Laemmli, 1970). After electrophoresis, protein bands were subjected to immunoblotting (see below) or simply stained with Coomassie brilliant blue R-250 (CBB) (Merck).
**Immunoblotting.** Cell lysates were prepared by lysing mock-infected and virus-infected cells with SDS–PAGE sample buffer and applied to SDS–PAGE on a 10% polyacrylamide gel. After separation, the proteins were electrically blotted onto a nitrocellulose membrane using a semi-dry-type blotting apparatus. After blocking, the membrane was stained with murine or rabbit anti-P antibody and then incubated with peroxidase-conjugated second antibody. Colour was developed by adding H₂O₂ and chloronaphthol. To estimate the apparent molecular mass of the proteins, a set of molecular markers (Dr Western, Oriental Yeast) was co-electrophoresed.

**Radioactive labelling.** After preincubation of the cultures with methionine-free medium for 30 min, the cultures were metabolically labelled with l-[³⁵S]methionine (final concentration of 10 µCi/ml; 0.37 MBq/ml) for various periods, as described in the text. Then, the radionlabelled cells were washed three times with PBS and lysed in 100 µl RIPA (I) buffer (140 mM NaCl, 50 mM Tris–HCl, 1% Nonidet P-40, 20 nM okadaic acid, 1 mM pefabloc, 25 µg/ml leupeptin; pH 7.4) for immunoprecipitation studies.

**Fractionation of rabies virus-infected BHK-21 cells.** Rabies virus-infected BHK-21 cells were lysed in deoxycholate (DOC)-free RIPA (I) buffer. After centrifuging briefly (10 min at 12000 r.p.m.) in a refrigerated microfuge, the lysates were applied to 8–10% SDS–PAGE and autoradiography. The NC fraction was exposed to 1% DOC in the presence of 0.5 M NaCl in a buffer composed of 5 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 1 mM pefabloc and 25 µg/ml leupeptin for 10 min at 4°C and then placed onto a 20% sucrose cushion, followed by ultracentrifugation for 120 min at 200000 g. The dissociated P proteins were recovered from the top of the tube and subjected to immunoprecipitation with anti-P mAb and pAb and then to SDS–PAGE and autoradiography.

**Dissociation and recovery of the P protein from the NC.** The NC fraction was exposed to 1% DOC in the presence of 0.5 M NaCl in a buffer composed of 5 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 1 mM pefabloc and 25 µg/ml leupeptin for 10 min at 4°C and then placed onto a 20% sucrose cushion, followed by ultracentrifugation for 120 min at 200000 g. The dissociated P proteins were recovered from the top of the tube and subjected to immunoprecipitation with anti-P mAb and pAb and then to SDS–PAGE and autoradiography.

**Immunoprecipitation and autoradiography.** Radionlabelled cell lysates were subjected to immunoprecipitation with mAbs or rabbit antibodies against rabies virus N or P protein. In brief, 1–2 µl of antibody solution was added to 10–20 µl of the lysates and placed on ice for 1–2 h, followed by precipitation with Pansorbin cells (commercial product of insoluble protein A-containing formalin-fixed Staphylococcus aureus cells) in PBS for 2 h at 4°C. When precipitated with murine mAbs, the precipitates were recovered using the second antibody (rabbit) against the murine immunoglobulin for efficient recovery with Pansorbin cells. The immune complexes recovered with Pansorbin cells were then dissolved in 50 µl of SDS–PAGE sample buffer and applied to 8–10% polyacrylamide gels for SDS–PAGE. Wide range molecular mass standards (Bio-Rad) were co-electrophoresed in SDS–PAGE. After being stained with CBB, the gels were dried onto 3 mm filter paper (Whatman) and exposed to an imaging plate for autoradiography in a Bio-Imaging Analyser BAS2000 (Fuji Photo Film).

### Results

**Characterization of the epitope for mAb #402-13**

Among more than 10 of our mAbs, which we screened originally according to their ability to recognize the rabies virus-induced inclusion bodies in the cytoplasm (Kawai et al., 1999), we found a mAb (#402-13) that recognized the SDS-denatured P protein of rabies virus (HEP-Flury strain). As shown in Fig. 1, immunoblotting with this mAb equally recognized both the SDS-denatured P proteins obtained from virus-infected cells and the P gene products expressed in E. coli. This result suggests that the mAb recognizes a linear epitope of the P protein without any special post-translational modifications that would occur in the infected cells.

The epitope region was mapped roughly as described. Some C-terminal deletion mutants of the P protein were prepared by cutting the P cDNA at one of the unique restriction sites and ligating it to an universal NheI linker, as described in Methods. Fig. 2 shows that mAb #402-13 did not recognize the mutants that lacked the C-terminal 42 aa (from positions 256 to 297) or more, and recognized very weakly the mutant that lacked 22 aa (from positions 276 to 297), implying that the presumed epitope region is located at around Gln²⁷⁷.

**Further studies on antigen recognition by mAb #402-13**

We performed further immunoprecipitation studies with this mAb. Although enough amounts of the P gene products were present in the lysates of the P cDNA-transfected BHK-21 cells, as shown by immunoprecipitation with anti-P pAb (Fig. 3, lane 2), the mAb precipitated very small amounts of the P gene products (Fig. 3, lane 4). Consistent with this observation, immunofluorescence studies resulted in the detection of very dark fluorescence of the P gene products in the P cDNA-transfected BHK-21 cells (data not shown). From these results, we assumed that the linear 402-13 epitope was not mostly
were precipitated by mAb
a small portion of the P proteins from virus-infected cell lysates
were radiolabelled with L-[35S]methionine (10 µCi/ml; 0.37 MBq/ml) for 6 h from 36 h after infection or for 4 h from 16 h after the transfection. Then, cells were lysed in DOC-containing RIPA (I) buffer and subjected to immunoprecipitation with rabbit anti-P pAb (lanes 1 and 2) and mAb 
402-13 (lanes 3 and 4). The precipitates obtained were analysed by SDS–PAGE on a 10% polyacrylamide gel and autoradiography using the Bio-Imaging Analyser BAS2000. Marker proteins were broad range SDS–PAGE molecular mass standards (Bio-Rad), which were CBB-stained after electrophoresis. Some additional bands of faster mobilities than that of the P protein (37 kDa) are the N-terminally truncated P proteins produced by translation initiation from different in-frame AUG codons (Takamatsu et al., 1998).

Exposed on the P gene products in the P cDNA-transfected cells and that the epitope would be exposed only under certain limited conditions.

Similar observations were obtained when immunoprecipitation assays were done for the P proteins produced in rabies virus-infected BHK-21 cells. As shown in Fig. 3 (lane 3), only a small portion of the P proteins from virus-infected cell lysates were precipitated by mAb 
402-13, which suggested its specific recognition of a limited form or structure of the P protein. In relation to this assumption, we noticed that certain amounts of viral N and L proteins were co-precipitated with the P proteins. This observation implied an intimate association of the 402-13 epitope-positive P protein with the NC, with which the L protein might also be associated (this point will be discussed again later). To examine this possibility further, we next performed studies with fractionated cell lysates.

Identification of the epitope-positive P protein in the cell

(1) Cell fractionation and detection of the NC-associated P protein. Radiolabelled, infected cell lysates were fractionated into the top soluble and the middle NC-containing fractions by ultracentrifugation in a sucrose density gradient, as noted in Methods. As described in our previous study (Kawai et al., 1999), the top fraction contained free P proteins and free N–P complexes as well as other soluble viral proteins and the NC fraction contained the NC and NC-associated proteins, including the P and L proteins, which could be co-precipitated by either anti-N or anti-P pAbs.

As shown in Fig. 4 (lanes 2), the top and NC fractions contained the N and P proteins, which were co-precipitated with anti-P pAb. When these fractions were subjected to immunoprecipitation with mAb 
402-13, the P protein was recovered mostly from the NC fraction with co-precipitated N and L proteins (Fig. 4B, lane 3), while little P or N protein was recovered from the top fraction by the mAb (Fig. 4A, lane 3; about 6% of the total P protein was detected in the band, as estimated by comparing the radioactivity of the bands). From these results, we assume that mAb 
402-13 recognized the epitope that is mostly exposed on the P proteins that are associated with NC.

(2) Studies on the P proteins found in the top fraction. As for the reason to explain why mAb 
402-13 precipitated only small amounts of the P protein from the top fraction, we can suppose three possibilities: (i) the epitope is masked due to N protein binding (most P proteins in this fraction exist as free N–P complexes) (Kawai et al., 1999); (ii) the epitope region is structurally hidden by conformational change(s) of the P protein molecule or by homo-multimer formation and is exposed by structural change(s) occurring after or during their involvement in the encapsidation of viral RNA (NC formation); and (iii) the epitope site is concealed after synthesis but exposed by certain post-translational modifications (e.g. phosphorylation) of the P and/or N proteins occurring after or during NC formation. The first possibility was eliminated as described below.

As noted in our previous report (Kawai et al., 1999), P proteins comprising the free N–P complex in the top fraction could be dissociated by treatment with sodium DOC.
Structural changes of rabies virus P protein

Fig. 5. Studies on the reactivity with mAb #402-13 of the P proteins dissociated from the free N–P complex and NC. (A) The soluble top fraction described in Fig. 4 was divided into four aliquots, two of which were treated with 1% DOC (lanes 2 and 4) and two of which were mock-treated (lanes 1 and 3). Then, samples were subjected to immunoprecipitation with rabbit anti-P pAb (lanes 1 and 2) and mAb #402-13 (lanes 3 and 4), followed by SDS–PAGE on a 10% polyacrylamide gel and autoradiography. (B) The NC fraction shown in Fig. 4 was treated with 1% DOC in a high-salt buffer (1% Nonidet P-40, 0.5 M NaCl, 5 mM Tris–HCl, 1 mM pefabloc, 25 µg/ml leupeptin; pH 7–4). Then, the sample was divided into two parts and applied to immunoprecipitation with rabbit anti-P pAb (lane 1) and mAb #402-13 (lane 2), followed by SDS–PAGE on a 8% polyacrylamide gel and autoradiography.

Accordingly, we examined the dissociated P proteins for their reactivity with mAb #402-13. As shown in Fig. 5(A), no DOC-dissociated P proteins were precipitated by the mAb, implying that the 402-13 epitope region was not masked by binding to the newly synthesized free N protein.

(3) Studies of NC-associated P proteins. To check the second possibility described above, we examined the antigenicity of the P proteins that were released from the NC. A large portion of the P proteins could be dissociated from the NC by a brief treatment with 1% DOC under high-salt conditions (see Methods) and the dissociated P proteins were no longer recognized by the mAb (Fig. 5B). This result suggests that the epitope-exposed, NC-associated P proteins would revert to the original epitope-negative form when they are released from the NC.

As to the third possibility mentioned above, we think that, from the immunoblotting studies shown in Fig. 1 as well as from the results shown in Fig. 5(B) (if the DOC-mediated dissociation did not cause any change in the P protein modifications), additional modifications (e.g. proteolytic cleavage, phosphorylation, etc.) are not necessary for the P protein to be recognized by mAb #402-13, even if protein modification occurs during or after the encapsidation process.

Conditions required for exposing the 402-13 epitope

We next investigated the conditions required for the P protein to expose the 402-13 epitope region using the P cDNA transfection system in animal cell cultures. The P and N gene products produced in the N and P protein cDNAs co-transfected cells were metabolically radiolabelled with [35S]methionine and lysed in RIPA (I) buffer for immunoprecipitation with rabbit anti-P pAb (lanes 1–4) and mAb #402-13 (lanes 5–8). The precipitates obtained were analysed by SDS–PAGE on a 10% polyacrylamide gel and autoradiography using the Bio-Imaging Analyser BAS2000.

Fig. 6. Immunoprecipitation of the P proteins produced by the cells co-transfected with P and N cDNAs. BHK-21 cells were transfected with N + P cDNAs (lanes 1 and 5), N cDNA (lanes 2 and 6), P cDNA (lanes 3 and 7) or no cDNA (mock-transfected, lanes 4 and 8). After incubation for 20 h, cells were radiolabelled with [35S]methionine for 4 h and lysed in RIPA (I) buffer for immunoprecipitation with rabbit anti-P pAb (lanes 1–4) and mAb #402-13 (lanes 5–8). The precipitates obtained were analysed by SDS–PAGE on a 10% polyacrylamide gel and autoradiography using the Bio-Imaging Analyser BAS2000.

Interaction of the C-terminally deleted P protein mutants with the NC

Finally, we checked the structural relationship between the NC-binding ability of the P protein and exposure of its 402-13
Fig. 7. Immunoprecipitation studies on the NC-binding ability of the C-terminally truncated mutants of the P protein. BHK-21 cells were co-transfected with cDNAs encoding the N and P proteins or one of the C-terminally deleted mutants described in Fig. 2 (PΔC22, PΔC42, PΔC89 and PΔC166). Cells were radiolabelled in a manner similar to that described in Fig. 6 and lysed in RIPA (I) buffer for immunoprecipitation studies with anti-N pAb (lanes 1–3), mAb g1-7-11 (lanes 4–6) and anti-P pAb (lanes 7–9). (A) Lanes 1, 4 and 7, P (wt); lanes 2, 5 and 8, PΔC22; lanes 3, 6 and 9, PΔC42. (B) Lanes 1, 4 and 7, P (wt); lanes 2, 5 and 8, PΔC89; lanes 3, 6 and 9, PΔC166. The asterisk in (A, lane 5) indicates a faint band of PΔC22.

Fig. 8. Schematic illustration of mapping of the 402-13 epitope and structural requirement for the NC-binding of the P protein. (A) The bar on the top indicates the restriction sites on the P cDNA that were used for inserting an universal NheI linker to prepare the C-terminally deleted mutants (see Methods). Open bars illustrate wt and four C-terminally deleted mutants of the P protein. Results of antigenicity assays of NC-binding ability are described to the right of each bar (see text). (B) The long bar depicted at the bottom indicates the whole P protein (297 aa), while short bars indicate: a, possible epitope region recognized by mAb #402-13; b, strong N-binding domain located at the C-terminal side (aa 209–215), proposed by Jacob et al. (2001); c, a region (aa 251–273) essential for NC-binding, as suggested by Fu et al. (1994); d, C-terminal region (aa 268–297) required for N-binding, as proposed by Chenick et al. (1994).
epitope. The wild-type (wt) P protein and four C-terminally deleted mutants (PAc22, PAc42, PAc89 and PAC166) were co-expressed with the N protein in BHK-21 cells and radiolabelled for immunoprecipitation studies with anti-N and anti-P pAbs as well as the anti-N mAb #1-7-11 (this recognizes a conformational epitope that is exposed on the NC-comprising mature form of the N protein but not on the N–P complex) (Kawai et al., 1999). As shown in Fig. 7, all mutants were detected by anti-P pAbs and were co-precipitated with the anti-N pAb but not with anti-N mAb #1-7-11, except for one mutant PAC22, whose very faint band was detected when co-precipitated with mAb #1-7-11.

From these results, we think that the C-terminally deleted mutants were not capable of binding to the NC-like structures when they lacked more than 22 aa from the C terminus but could bind to newly synthesized N proteins to form free N–P complexes. We also assume that the NC-binding ability of the P protein is dependent on the presence of the 402-13 epitope region located at a certain distance from the presumed NC-binding domain, indicated recently by Jacob et al. (2001). These considerations are summarized in Fig. 8 (see Discussion).

Discussion

Rabies virus P protein-specific mAbs have also been reported from other laboratories (Raux et al., 1997; Nadin-Davis et al., 2000) and describe mostly the antigenic sites on the P protein, although further studies have not yet been described with these mAbs. We described here some studies on the structural changes of the rabies virus P protein, which could be recognized by our anti-P mAb, mAb #402-13.

We demonstrated that mAb #402-13 recognizes a linear epitope that is located at the C-terminal region of the rabies virus P protein, while the epitope region is exposed only when the P protein is associated with the viral NC. The epitope-containing region of newly synthesized P protein would mostly be hidden immediately after synthesis, so, the epitope was undetectable on the free N–P complex and on the free P protein; the former case was not due to masking of the epitope region by the N protein binding. We conclude that mAb #402-13 recognizes a linear epitope exposed on the P protein that is intimately associated with the viral NC and that the epitope is concealed again when released from the NC.

Free N–P complexes are involved in viral RNA synthesis to encapsidate newly synthesized viral RNA to form the NC. If the P proteins still remain associated with the NC after RNA encapsidation, the amount of NC-associated P proteins would theoretically correspond to that of the P proteins that are co-precipitated by our conformational epitope-specific anti-N mAbs (e.g. #1-7-11) (Kawai et al., 1999). In virus-infected cells as well as in N + P DNA-transfected cells, however, such NC-associated (402-13 epitope positive) P proteins were detected in very small amounts in comparison with those precipitated by anti-P pAbs. These results suggest strongly that, after RNA encapsidation, N–P binding was disrupted and most P proteins were released from the newly formed NC. Dissociated P proteins may undergo structural changes to restore the original form and conceal the epitope region and might be pooled again in the cell for recycled uses through the free N–P complex formation with newly synthesized N proteins.

Exposure of the epitope region seems to be dependent on structural or conformational change(s) of the P protein, which would occur mostly during its association with the NC under certain conditions, but not due to the post-translational modifications. Concerning this, we think as follows: during or after the encapsidation process, the N proteins undergo conformational changes that could be recognized by anti-N mAbs (e.g. #1-7-11). And, the conformational change(s) of the N protein might also affect the N–P association on the newly formed NC; most of the P proteins would be dissociated from the NC. Some such P proteins, however, might undergo (by chance?) certain structural changes to keep their NC-associated state and expose concomitantly the 402-13 epitope.

The P protein may display various types of association with the N protein in relation to its multiple functions, which might be reflected in its different structures or conformations. The P protein may be present in either of three different forms, which are as follows: (i) free P protein; (ii) free N–P complexes used for encapsidation of viral RNA; and (iii) the NC-associated P protein thought to be involved in initiation of viral RNA synthesis or elongation. Concerning the latter two situations, two research groups reported previously that both the N- and C-termini of the P protein are involved differently in the formation of free N–P complexes and NC–P binding, respectively (Chenik et al., 1994, 1999).

Chenik et al. (1994) suggested that there may be at least two independent N-binding sites on the P protein, one located in the C-terminal part, the other in a region spanning from aa 69 to 177. Fu et al. (1994) reported that the N-terminal domain of the P protein interacts with the N protein only when the N and P proteins were synthesized simultaneously, while the C-terminal site of the P protein is capable of interacting with the N protein even though both proteins are synthesized separately. From these results, they suggested that the N-terminal-binding site is involved in free N–P complex binding and that the C-terminal domain is required for NC–P binding. Our present observations provide novel evidence that would support the proposal by Fu et al. (1994). At present, we can say that the 402-13 epitope site is not the NC-binding site itself because this site is recognized by the mAb. Very recently, Jacob et al. (2001) described that one of two N-binding domains (probably the NC-binding domain) is mapped to a small region ranging from aa 209 to 215 (Fig. 8).

When the N and P proteins are co-expressed in animal cells, N and P proteins assemble to form free N–P complexes, as seen in infected cells. The N–P complexes are used for constructing the NC-like structures that could be recognized by anti-N mAbs (e.g. #5-2-26 and #1-7-11) (Kawai et al., 1999). All of the
C-terminally deleted mutants showed similar ability of N–P complex formation, which is consistent with a suggestion that the N-terminal half of the P protein contains a region required for the formation of free N–P complexes with newly synthesized N protein.

On the other hand, since the C-terminally deleted mutant PAC22 showed very weak antigenicity to the mAb and weak NC-binding ability, it was suggested that the epitope-containing the C-terminal region is required for the P protein to display NC-binding activity. Structural changes to expose the epitope region would also be important for the P protein to strongly associate with the NC, whereby the P protein would become competent for working in viral RNA synthesis.

In other negative-stranded RNA viruses, similar studies have been done to define two different N protein-binding sites on the N- and C-terminal domains of the P/NS protein (Takacs et al., 1993; Harty & Palese, 1995): the C-terminal of VSV P protein is involved in NC–P binding (Gill et al., 1986). Ryan & Kingsbury (1988) reported that the C-terminal region of the Sendai virus P protein is required for NC binding, while Curran et al. (1995) described that both the N- and the C-termini of the P protein are required for P–NP complex formation (which may correspond to rabies virus N–P complex formation). It has also been reported that the C-terminal region of the respiratory syncytial virus P protein is involved in the N–P interaction (Garcia-Barreno et al., 1996). All of these descriptions concerning the C-terminal domain implicate its possible involvement in a certain important function common to P proteins, although one which has not yet being defined precisely.

As to the possible role(s) of the 402-13 epitope-positive P protein, we have to remember the fact that the C-terminal epitope-containing region is one of the highly conserved regions of the P protein when compared with some rabies virus strains (Tordo et al., 1986; Larson & Wunner, 1990; Conzelmann et al., 1990; Takamatsu et al., 1998). A similar situation has also been described for the VSV P protein, which has a highly conserved region composed of 21 aa at the C terminus (Gill & Banerjee, 1985). Consistent with these considerations, we observed that the C-terminal sequence mimicked in synthetic oligopeptides specifically inhibited VSV transcriptase activity in vitro by reducing the frequency of RNA synthesis initiation (Yamashita & Kawai, 1990). Based on these considerations, we think that exposing the C-terminal epitope-containing region on the NC-associated P protein allows the P protein to be ready for accepting the L protein, as implicated from the results shown in Fig. 4(B). Possible roles of the C-terminal region of the P protein are now under investigation from the viewpoint of NC binding of the P and L proteins as well as the possible involvement of other viral and cellular components.

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References


Structural changes of rabies virus P protein


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