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Full Length Research Paper

An intermediate region in C-terminal of phosphoprotein is required for binding to nucleocapsid of Newcastle disease virus

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The phosphoprotein (P) of paramyxoviruses plays a central role in the viral genome replication and transcription. The P either binds to the unassembled nucleocapsid (NP_0) to assist in viral genomic replication or binds to assembled NP (NP-RNA or NP_{NC}) to transcribe genome to produce the sub-genomic mRNAs. In this study, the region of P that binds to NP_{NC} was mapped. To determine the binding region, 18 N- and C-terminally truncated P mutants were synthesized by *in vitro* translation in rabbit reticulocytes and mixed with purified NP (NP_{NC}). The mutants which did not bind to NP were considered as mutants and they contain deleted regions that may be involved in binding to NP. To identify the mutants that did not bind to NP, radioimmunoprecipitation and protein binding assays were used. Based on radioimmunoprecipitation analysis, it was shown that the region of P that binds to NP is located within the internal region of C-terminal half of P, from amino acids 243 to 279. In agreement with the radioimmunoprecipitation analysis, protein binding assay showed that the interactive domain was mapped to the internal region of the C-terminal half of P. However, a slightly bigger region of interactive domain (amino acids 224 to 279) was determined by the latter assay. As protein binding assay is considered as a more sensitive assay as compared to radioimmunoprecipitation assay, thus, the interactive region of P to NP_{NC} was located within C-terminal half of P between amino acids 224 to 279.

Key words: Phosphoprotein, nucleocapsid, binding domain, radioimmunoprecipitation, protein binding assay.

INTRODUCTION

Newcastle disease virus (NDV) is a member of the family Paramyxoviridae, which is known to cause fatal avian disease, Newcastle disease (ND). The virus infects a wide host range and remains to be a threat to the poultry industry. NDV infection has occurred worldwide through migration of wild birds and movements of pet birds and contaminated workers. Birds suffering from NDV infection are associated with increased respiratory rate and distress, neurological deterioration, bloody diarrhea, fever and sudden death, while humans may exhibit milder symptoms like headache, ‘flu-like’ symptoms and mild conjunctivitis.

NDV is spherical with rough surface and occasionally appears in a filamentous form. It contains single stranded (negative strand) RNA genome which encode for 6 proteins, namely nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), hemaglutinin-neuraminidase (HN) and RNA polymerase (L). Among these proteins, the NP, P and L proteins are involved in viral genomic transcription and replication. All these proteins interact with each other to show both transcription and replication of viral genome. In the present study, the interaction between P and NP was studied. P is a muiltifunctional

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protein that is highly phosphorylated in NDV (Lamb and Kolakofsky, 1996). It acts as a cofactor of the L protein that contains RNA polymerase activity. NP encapsidates the viral genome and has been regarded as the main regulator in viral genome replication (Das et al., 1999; Myers et al., 1999). Previous studies have shown that the interaction between P and NP is required in viral propagation (Lamb and Kolakofsky, 1996). In Sendai virus, interaction between P and assembled NP (NP-RNA or NP_{NC}) is required for transcription of viral RNA (Ryan et al., 1991). During the viral nascent RNA chain assembly, the P interacts with NP monomer (NP_O) and forms a P-NP_O complex. This complex acts as an active form of NP_O that is used to assemble nascent RNA chain during genome replication (Horikami et al., 1992). The formation of the P-NP_O complex also prevents the nonspecific aggregation of NP_O to cellular RNA (Master and Bannerjee, 1988; Curran et al., 1995b; Spehner et al., 1997).

In view of this, a comprehensive understanding of the multifunctional domains of the P that are involved in the P-NP interaction is fundamental towards a better understanding of the transcription and replication in NDV. By gaining the information of which P domain interacts with NP, the real mechanism of transcription and replication cellular events could be revealed. Previous studies have shown that the P interactive domain varies for different negative stranded RNA viruses, although the same terminal of P (C-terminal) is involved in the interaction. In vesicular stomatitis virus (Gill et al., 1986), Sendai virus (Ryan and Portner, 1990), measles virus (Liston et al., 1995) and La Piedad Michoacan Mexico virus (Svenda *et al*., 2002), the exact location of amino acids in C-terminal of P involved in the binding varies. Therefore, the present study was carried out to determine the domains of P that interact with NP, particularly NP_{NC} in NDV.

MATERIALS AND METHODS

Construction of truncated P genes

A total of 18 truncated P genes were amplified using a DNA template, pCITE-2a containing P gene which was obtained from the previous study conducted by Kho et al. (2002). Eleven 5'-end deleted P genes were amplified to produce truncated P mutants with 20, 51, 84, 145, 186, 205, 223, 233, 242, 252 and 279 amino acids deleted from the N-terminal end (Figure 1). Seven 3'-end deleted P genes were amplified to produce truncated P mutants with 27, 64, 116, 130, 143, 153 and 162 amino acids deleted from the C-terminal end (Figure 1). The amplification was performed in a thermocycler (PTC-200; MJ Research, USA) and the amplification programme was as follows: 94°C/5 min (94°C/1 min, 55°C/45 s, 72°C/2 min) for 30 cycles and a final extension cycle of 72°C/7 min.

To clone the truncated genes into pCITE-2a, both the PCR products and the plasmid were digested with *Bam H*I and *Sac* I (Promega, USA), purified from TAE agarose gel, ligated and transformed into *Escherichia coli* TOP10. After the positive clones were identified, they were grown in LB medium and their recombinant plasmids were extracted for *in vitro* transcription.

In vitro transcription and translation

In vitro transcription and translation reactions were carried out according to the method of Tan and Dyson (1999) to produce N- and C-terminal P mutants. Plasmid pCITE-2a containing the full length and truncated P genes were linearized with *Xho*I (Promega, USA) and later purified from TAE agarose gel using the QIAquick Gel Extraction kit (Qiagen). These purified linearized plasmids were utilized as templates for *in vitro* transcription. *In vitro* transcription mixtures containing linearized plasmid (~ 1 ug), 1X T7 transcription buffer (80 mM HEPES-KOH pH 7.5, 24 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT), 7.5 mM rNTPs and RNA polymerase mix (RNA polymerase, recombinant RNasin, yeast inorganic pyrophosphatase) (Promega, USA) were prepared and incubated at 37°C for 2 h. The resulting synthesized RNAs were aliquoted (2 µl each) and stored at -80°C. For the *in vitro* translation reaction, diluted RNA samples (1 µl; 5X dilution) were added to the translation mixtures (9 µl) containing 55% (v/v) rabbit reticulocyte lysate (Promega, USA), 20 µM amino acid mixture minus methionine, [^{35}S]-methionine (1 Ci/mol; Amersham, USA), 0.6 mM $Mg(OAC)_2$, 120 mM KCl, 2 mM DTT and 0.8 U/ul RNasin (Promega, USA). The mixtures were incubated at 30°C for 2 h before they were analysed by SDS-PAGE. SDS-PAGE was carried out according to Laemmli (1970).

The separated proteins on the gel were transferred by electroblotting to nitrocellulose membrane. The membrane was dried and autoradiographed against X-ray film (Kodak, Japan). After overnight exposure, the film was developed by soaking in a developer (Kodak, Japan) for 5 min, washed and fixed for 5 min in a fixer (Kodak, Japan).

Purification of NP

NP was purified from *E. coli* culture according to Kho et al. (2001) with slight modifications. Approximately 250 ml bacterial cultures were grown at 37°C in LB broth until the culture reached A_{600} of about 0.6 to 0.8. After induction with IPTG (1mM) for 5 h, the cells were harvested by centrifugation at 10,000 xg (J2-MI; Beckman, USA) for 10 min at 4°C. The pellets were lysed in lysis buffer [50 mM Tris (pH 7.9), 0.1% Triton X-100, 0.2 mg/ml lysozyme, 4 mM $MgCl_2$], treated with RNase (5 µg/ml) and DNase (5 µg/ml) (Amresco, USA) for 30 min at RT and followed by sonication (30 s) at high frequency for five times. The cell extracts were recovered by centrifugation at 20,000 xg for 20 min at 4°C and precipitated by ammonium sulphate (0 to 60% saturation). The precipitates were pelleted by centrifugation under the same conditions and dialysed extensively in dialysis buffer (50 mM Tris, 100 mM NaCl, pH 7.8). The dialysed solutions were carefully layered to 10 to 50% sucrose gradient and centrifuged at 110,000 xg (Optima L-90K; Beckman, USA) for 5 h at 4°C. After the centrifugation, fractions of 0.5 ml were collected and analysed by SDS-PAGE to determine which fractions contained NP. Fractions containing the NP were pooled, dialysed and then concentrated with a 100 kDa cut-off Centricon centrifugal filter (Milipore, USA) and kept at 4°C for radioimmunoprecipitation assay and protein binding assay.

Radioimmunoprecipitation

To identify the region of P that binded to NP_{NC}, radioimmunoprecipitation was carried out. An aliquot of 4 µl of *in vitro* translated P mutants diluted in NEP-gel buffer [50 mM potassium phosphate, pH 7.5, 150 mM, 0.1% (v/v) NP-40, 1 mM EDTA, 0.25% (w/v) gelatine, 0.02% (w/v) sodium azide] supplemented with 2 mM 1,4-dithiothreitol (DTT) were mixed with ~1 µg of purified NP (NP_{NC}) and 30 µl of mAbs, a2s (Ahmad-Raus et al., 2009) and rolled gently overnight at 4°C. The mixture was then added to the tube containing agarose protein G and rolling was continued for 2 h at room

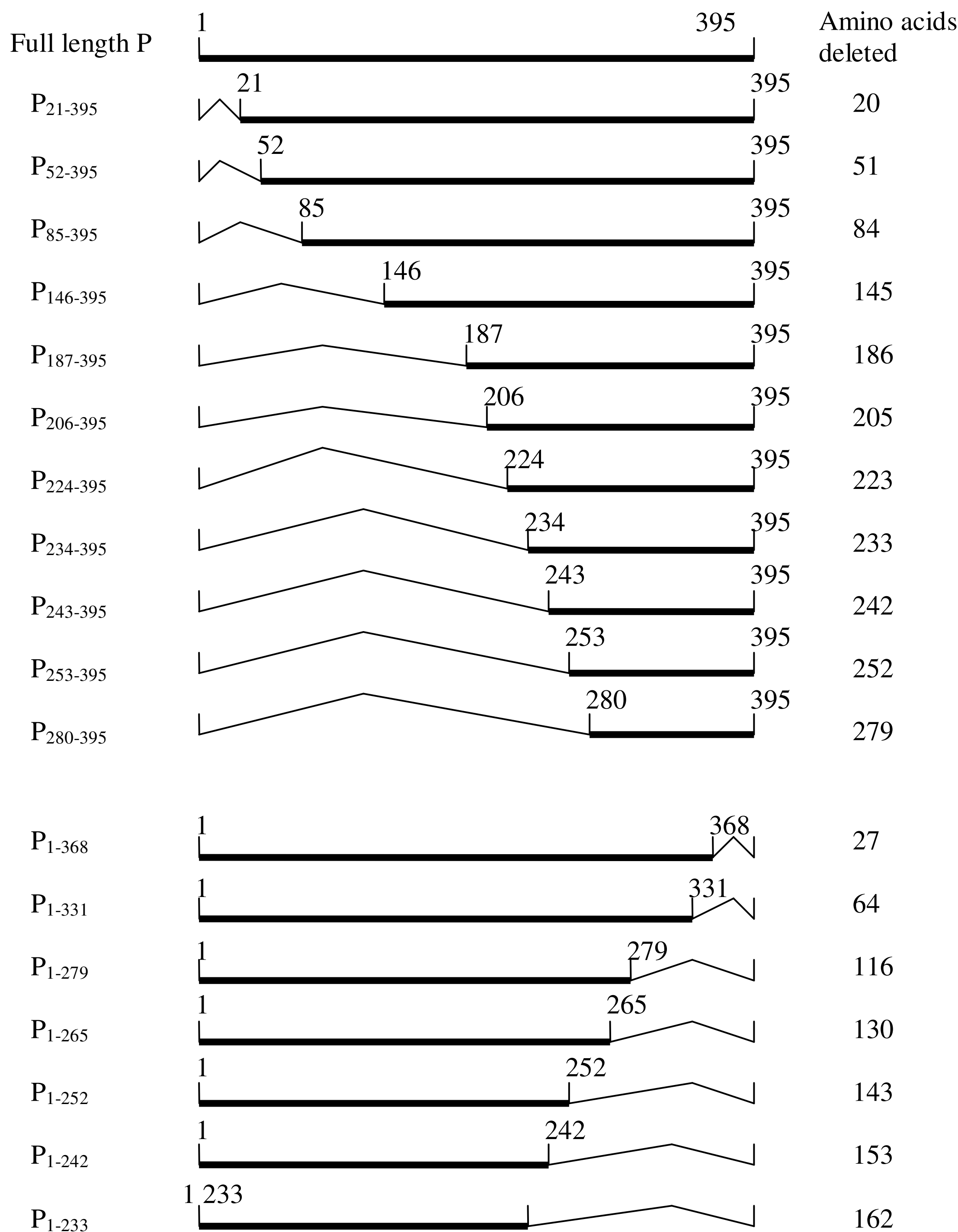

Figure 1. Schematic representation of the deletion mutants of P. Thick lines represent the protein product of each truncated P gene and the amino acid positions are indicated on top of theses lines. Angled lines indicate deleted regions.

temperature. To recover the immunoprecipitated complexes, the pellets were washed four times with NEP-gel buffer and one time wash with 10 mM Tris-Cl (pH 7.6) containing 0.1% (v/v) NP-40. They were then boiled together with 2X sample buffer and the immunoprecipitated complexes were removed from the agarose protein G by centrifugation. The supernatants were loaded onto SDS-polyacrylamide gel, transferred to nitrocellulose and autoradiographed to determine which of the P mutant products failed to be immunoprecipitated. P mutants which were not able to retain their binding capacity with the NP_{NC} were not visualized and considered as mutants that contain the deleted regions that may be involved in the binding to NP_{NC}.

Protein binding assay

To further confirm the region of P that interacted with NP_{NC} and also quantify the interaction, protein binding assay were carried out using the method of Kho et al. (2004). A 96 well pico plate (Packard, USA) was coated overnight with 10 µg/ml of purified NP protein in PBS buffer at 4°C with gentle agitation. The plates were washed 3 times with PBS and blocked with 3% (w/v) BSA (in PBS) for 2 h at 4°C with gentle agitation. The plates were then washed 6 times with 2% (w/v) BSA (in PBS) followed by the addition of *in vitro* translated wild type P and P mutants (or [^{35}S] methionine labeled P proteins) which were already diluted 100 fold in NEP-gel buffer containing 2 mM DTT, to the plates. After 5 h incubation with gentle shaking at room temperature, the plates were washed 5 times with the NEP-gel buffer containing 2 mM DTT. Finally, 200 µl of scintillation liquid were added to the plates for quantification of radioactivity with microplate scintillation counter (TopCount NXT; Packard, USA). A negative control was carried out with the same conditions but in the absence of *in vitro* translated products. The radioactivity (cpm) recorded between the radioactively labelled full length P and NP_{NC} was taken as 100% and the degree of interaction between the P mutants and NP_{NC} were expressed relative to that of the full length P protein.

RESULTS

In vitro translated P mutants

Figures 2 and 3 show the *in vitro* translated products of the 18 recombinant clones containing various truncated P genes. All the N- and C-terminally truncated P proteins were expressed ranging from approximately 13 to 51 kDa in which they displayed a stepwise decrease in size that corresponded to the sizes of the deletions. Nevertheless, protein bands other than the respected P mutants were observed in some of the lanes. The bands with smaller molecular weight than the P mutants were probably translated proteins from the internal AUG codon (start codon) of the inserts and the bands with higher molecular weight than the P mutants were probably the dimer of the internally translated proteins.

Immunoprecipitation analysis

Figures 2 and 3 show that the *in vitro* translated products of mutants $P_{21\text{-}395}$, $P_{52\text{-}395}$, $P_{85\text{-}395}$, $P_{146\text{-}395}$, $P_{187\text{-}395}$, $P_{206\text{-}395}$, $P_{224\text{-}395}$, $P_{234\text{-}395}$ and $P_{243\text{-}395}$ (lanes 2#, 5#, 8#, 11#, 14#, 17#, 20#, 23# and 26#) representing up to 61% deletion (242 amino acids) of the N-terminal end of P were immunoprecipitated in the presence of NP_{NC}. This suggests that the N-terminal half of P may not play direct role in the binding to NP_{NC}. However, any *in vitro* translated product obtained from deletions beyond the first 242 amino acids of P (from amino acids at position 243 to 279 in mutants, $P_{253\text{-}395}$, $P_{280\text{-}395}$) abolished the immunoprecipitation reaction (Figure 2; lanes 29# and 32#). These results indicate that the regions of P that were involved in the binding to NP_{NC} were probably located within the internal region of the C-terminal half of P (between amino acids 243 to 279).

To confirm this suggestion, a series of progressive C-terminal deletions were examined for their interactions with NP_{NC}. The results show that deletion of the last 116 amino acids from the C-terminal ($P_{1\text{-}368}$, $P_{1\text{-}331}$ and $P_{1\text{-}279}$) did not abolish the P-NP_{NC} interaction, indicating that the C-terminal amino acids 280 to 395 were not involved in the interaction (Figure 3; lanes 38#, 41# and 44#). On the other hand, deletions before the 280 amino acid location ($P_{1\text{-}265}$, $P_{1\text{-}252}$, $P_{1\text{-}242}$ and $P_{1\text{-}233}$) resulted in the abolishment of any binding between P and NP_{NC} (Figure 3; lanes 47#, 50#, 53# and 56#). Interestingly, three of these P mutants that failed to interact with NP_{NC} contained progressive deletions from the C-terminus within the 243 to 279 amino acids region that were initially suggested to be involved in binding interaction. This implied that the interaction region may be located within amino acids 243 to 279. In addition, this whole region may be required for the interaction as deletions of few amino acids within this region resulted in the failure of P to hold its binding capacity to NP_{NC}.

Protein binding assay analysis

To further confirm the data obtained by radioimmuno-precipitation, a protein binding assay was carried out using the same truncated P mutants. Figure 4 shows that deletion of the first 20 amino acids immediately from the N-terminal ($P_{21\text{-}395}$) end moderately reduced the interaction by approximately 20% of the intact protein. Surprisingly, further deletions to 51 and 84 amino acids from the N-terminal ($P_{52\text{-}395}$ and $P_{85\text{-}395}$) end, increased the interaction to more than two and four-folds higher as compared to that of the wild type P, respectively. However, when the deletion was extended to 186 amino acids ($P_{187\text{-}395}$) from the N-terminus of P, the interaction became approximately similar to that of the full length P. Thus, it confirmed the earlier observation made in immunoprecipitation analysis that the N-terminal 186 amino acids of P were not involved in the interaction with NP.

Intriguingly, a further deletion to 205 amino acids or 52% of the N-terminal deletion ($P_{206\text{-}395}$) did affect the interaction with NP. It reduced the interaction to 33% but this was quickly restored to the same efficiency as that of the wild type P when the deletion was extended to 223 amino acids or 56% of the N-terminal ($P_{224\text{-}395}$). At this stage, it was difficult to explain why 52% deletion from the N-terminus reduced the P-NP_{NC} interaction to less than half of the interaction of full length P which was then reverted back to approximately 100% interaction when 56% of N-terminus P was deleted. It was most likely that the deletion of N-terminal 205 amino acids of P formed an unstable secondary structure of P which then affected the interaction but somehow further deletion to 223 amino acids probably reverted the conformation of the P to almost its normal conformational structure, resulting in an interaction almost similar to that of the wild type P-NP_{NC}

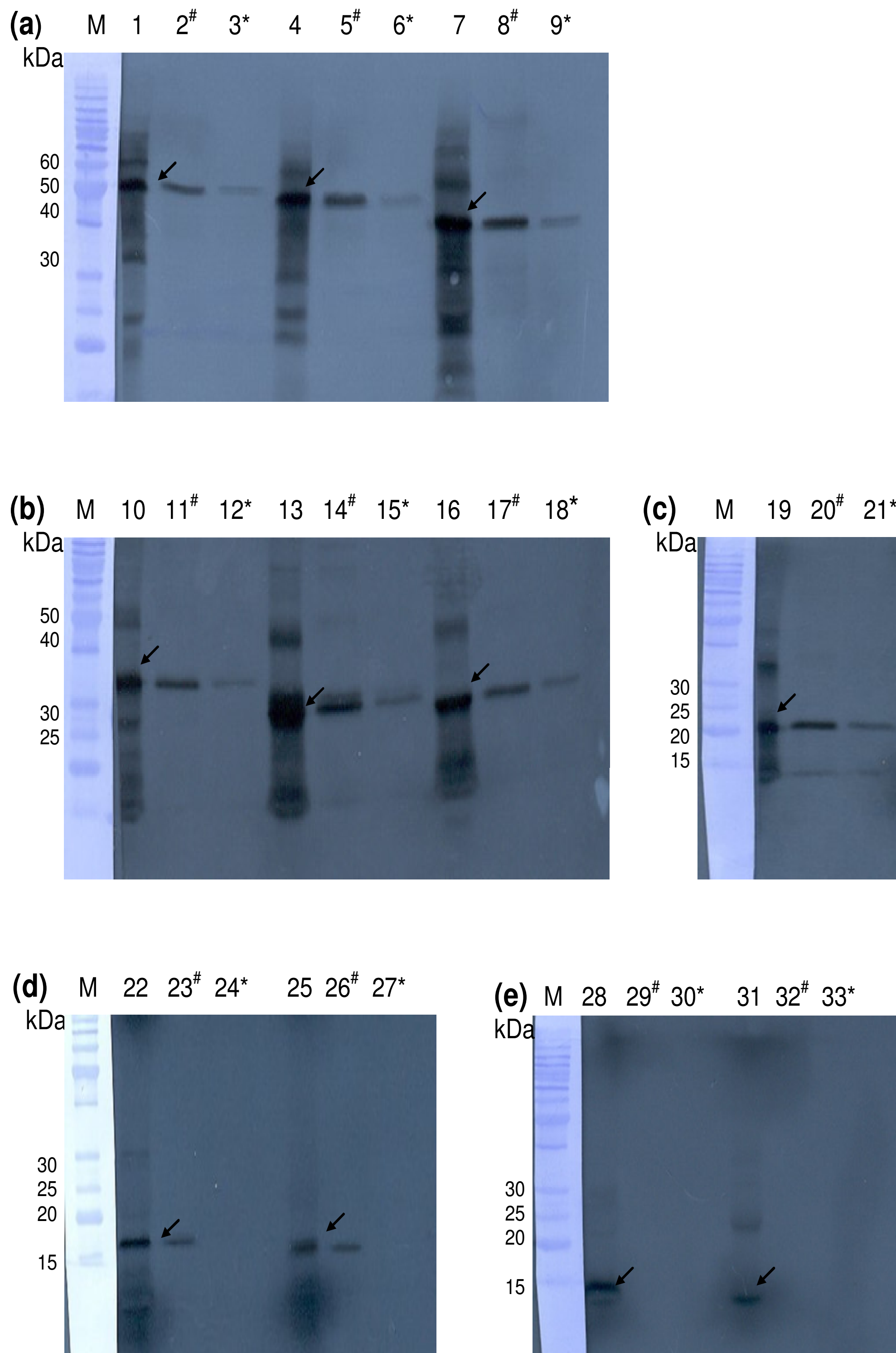

Figure 2. Radioimmunoprecipitation of *in vitro* translated products of N-terminal deletion P mutants. The respective P deletion mutants were immunoprecipitated by anti-NP mAb in the presence (lane number with hatch) and absence (lane number with asterisk) of purified NP. The immunoprecipitated complexes were separated on 12% SDS-PAGE, blotted onto nitrocellulose membrane and autoradiographed. The N-terminal deletion P mutants are shown by the arrowheads. (a) Lanes 1 to 3, $P_{21\text{-}395}$; lanes 4 to 6, $P_{52\text{-}395}$; lanes 7 to 9, $P_{85\text{-}395}$. (b) Lanes 10 to 12, $P_{146\text{-}395}$; lanes 13 to 15, $P_{187\text{-}395}$; lanes 16 to 18, $P_{206\text{-}395}$; (c) Lanes 19 to 21, $P_{224\text{-}395}$; (d) Lanes 22 to 24, $P_{234\text{-}395}$; lanes 25 to 27, $P_{243\text{-}395}$; (e) Lanes 28 to 30, $P_{253\text{-}395}$; lanes 31 to 33, $P_{280\text{-}395}$; lane M, Protein molecular markers in kDa were from Invitrogen, USA.

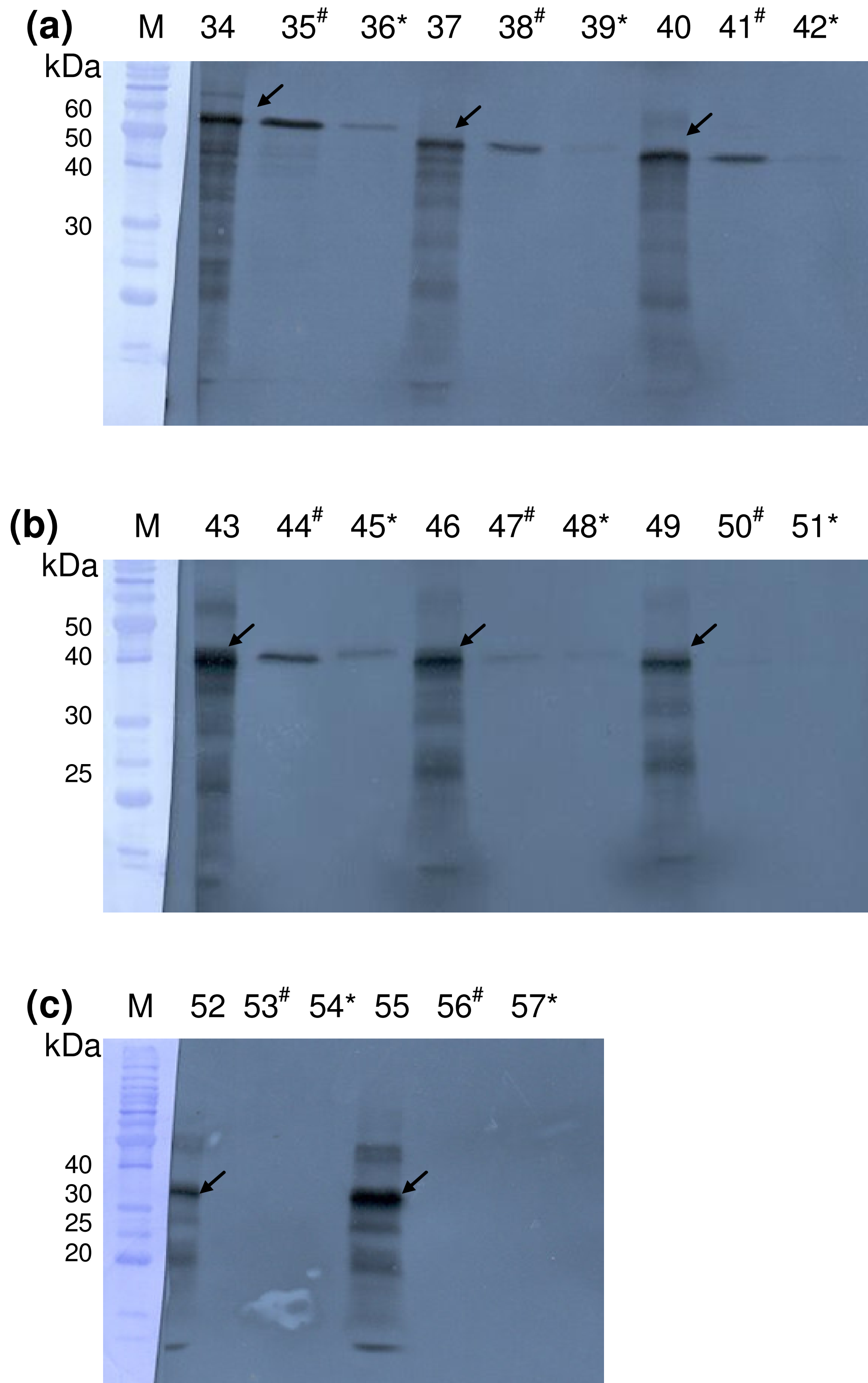

Figure 3. Radioimmunoprecipitaion of *in vitro* translated products of C-terminal deletion P mutants. The respected deleted P mutants were immunoprecipitated by anti-NP mAb in the presence (lane number with hatch) and absence (lane number with asterisk) of purified NP. The immunoprecipitated complexes were separated on 12% SDS-PAGE, blotted onto nitrocellulose membrane and autoradiographed. The C-terminal deletion P mutants are shown by the arrowheads. (a) Lanes 34 to 36, P full length; lanes 37 to 39, P_{1-368}; lanes 40 to 42, P_{1-331}; (b) Lanes 43 to 45, P_{1-279}; lanes 46 to 48, P_{1-265}; lanes 49 to 51, P_{1-252}; (c) Lanes 52 to 54, P_{1-242}; lanes 55 to 57, P_{1-233}; lane M, protein molecular markers in kDa from Invitrogen, USA.

	Amino acid positions	Amino acids deleted	% of deletion	% of radioactivity
Full length P	1–395	-	-	100
P_{21-395}	21–395	20	5	79 ± 7.8
P_{52-395}	52–395	51	13	293 ± 28
P_{85-395}	85–395	84	21	454 ± 41
$P_{146-395}$	146–395	145	37	88 ± 1.5
$P_{187-395}$	187–395	186	47	131 ± 13
$P_{206-395}$	206–395	205	52	33 ± 1.5
$P_{224-395}$	224–395	223	56	118 ± 4.4
$P_{234-395}$	234–395	233	59	29 ± 1.5
$P_{243-395}$	243–395	242	61	29 ± 1.5
$P_{253-395}$	253–395	252	64	0.4 ± 0.1
$P_{280-395}$	280–395	279	71	0.2 ± 0.1
P_{1-368}	1–368	27	7	63 ± 3.8
P_{1-331}	1–331	64	16	100 ± 8.6
P_{1-279}	1–279	116	29	48 ± 4.2
P_{1-265}	1–265	130	33	12 ± 0.6
P_{1-252}	1–252	143	36	4 ± 0.6
P_{1-242}	1–242	153	39	2 ± 0.6
P_{1-233}	1–233	162	41	4 ± 1

Figure 4. Protein binding assay of truncated P mutants (radioactively labelled P mutants). Radioactivity (cpm) of full length P is considered as 100% radioactivity. Average radioactivity from three experiments of each P mutant was determined, and expressed as a percentage of that of wild-type P. Thick lines represent the protein product of each truncated P gene and the amino acid positions are indicated on top of these lines.

interaction.

However, when deletions were extended up to 242 amino acids (61% deletion), the interaction between the two respective P mutants ($P_{234-395}$ and $P_{243-395}$) with NP_{NC} were reduced dramatically by 90% as compared to the previous mutant, $P_{224-395}$ (118%). This indicated that the

amino acids located between positions 224 and 242 played an important role in the interaction, although this region still recorded approximately 30% interaction. This region however has not been suggested by immunoprecipitation analysis to interact with the NP_{NC} but the bands observed for these two mutants were quite faint as compared to those of other P mutants which gave positive results (Figure 2; lane 23# and 26#). Further deletions beyond amino acids 242 totally abolished the binding as no interaction was recorded for both mutants, $P_{253-395}$ and $P_{280-395}$, indicating that another 37 amino acids (amino acids 243 to 279) may be involved in the P-NP_{NC} interaction. Interestingly, this region of amino acids has also been suggested in immunoprecipitation analysis to be involved in P-NP_{NC} interaction. Nevertheless, the N-terminal deletion analysis showed that N-terminal half of P did not interact with NP_{NC} and the interactive regions of P to NP_{NC} were probably located within the internal region of the C-terminal half of P. It possibly involved the region between amino acids 224 and 279 as $P_{234-395}$, $P_{243-395}$, $P_{253-395}$ and $P_{280-395}$ mutants showed low or no interaction with NP_{NC}.

To confirm this suggestion and investigate whether C-terminus of P might also have possible role in the interaction, seven C-terminal P deletion mutants were examined for their interactions with NP_{NC}. Deletions which removed the first 27 amino acids from the C-terminal end (P_{1-368}), gave a moderate reduction in binding strength (63% of the intact P). Further deletion to 64 amino acids of C-terminus (P_{1-331}) however, reverted the efficiency of binding to that of the intact P. When the deletion was extended to 116 amino acids from the C-terminal end (P_{1-279}), the interaction was reduced to approximately half of the interaction of full length P, indicating that the last 116 amino acids of the C-terminal (amino acids: 280 to 395) were not directly involved in P-NP_{NC} interaction but they were somehow required to maintain the integrity of the structure of C-terminal end of P to secure stable binding.

Deletions more than the earlier mentioned, however, abrogated the interaction as very low and no interactions were recorded for the following mutants: P_{1-265}, P_{1-252}, P_{1-242} and P_{1-233}. Interestingly, the same C-terminally deleted P mutants which did not interact with NP_{NC} were also observed in the immunoprecipitation analysis. These P mutants contain progressive deletions from the C-terminus within the P-NP_{NC} interactive domain, amino acids 224 to 279 that was suggested to be involved in the binding.

Based on both N- and C-terminal deletion analysis, it can be suggested that region between amino acids 224 and 279 may play a major role in the P-NP_{NC} interaction as deletion of a few amino acids within this region abolished the interaction between P with NP_{NC}. The region at amino acid position 280 to 395 may not be directly involved in the P-NP_{NC} interaction but it was required to form a conformational structure that probably helped in the binding of P to NP_{NC}.

DISCUSSION

It has been previously shown that P of NDV interacts with assembled NP, NP_{NC} (Kho et al., 2004), similar to that observed in other paramyxoviruses including Sendai virus (Portner and Murti, 1986), La Piedad Michoacan Mexico virus (LPMV) (Svenda et al., 2002), measles virus (Spehner et al., 1997) and vesicular stomatitis virus, VSV (Emerson and Schubert, 1987). Kho et al. (2004) found that the region of NP_{NC} that is responsible for the binding of P to NDV was located within the first 25 amino acids of the N-terminal of NP. However, the domain of P that binds to NP_{NC} was not determined in their study. In the present study, the interactive P domain that binds to NP_{NC} was identified by using radioimmunoprecipitation assay and protein binding assay.

Radioimmunoprecipitation was conducted by using the NP mAb to precipitate the N- and C- terminally deleted P mutants that were associated with NP_{NC} to determine the interaction region. Results from radioimmunoprecipitation assay showed that the whole N-terminal half and the immediate C-terminal end of P was not involved in the binding of P to NP_{NC}. Instead, the internal part of C-terminal half of P located within amino acids 243 to 279 were the residues that were involved in the interaction. In agreement with the results of radioimmunoprecipitation assay, the protein binding assay showed a similar region and another 19 amino acids (amino acid positions 224 to 242) adjacent to the N-terminal end of the interactive domain observed in the former assay was involved in the interaction between P and NPNC. A slightly larger region of interaction was determined by the latter assay (amino acid position 224 to 279) as compared to the former assay (amino acid position 243 to 279) was due to the different nature and sensitivity of both assays. In the latter assay, the interaction was recorded as values and in contrast, interaction was determined on the ability to visualize the immunoprecipitated products of deleted P mutants bands on autoradiographed film in the former assay. Thus, when amino acids 224 to 242 that were deleted ($P_{N234-395}$, $P_{N243-395}$), 90% reduction was recorded in interaction as compared to the previous deleted P mutants (118%, $P_{N224-395}$) region, that was considered to be critically involved in the interaction, although both mutants still had 30% interaction and faint bands could be observed in the radioimmunoprecipitation assay. In order to clarify this ambiguity, an internal deletion covering these two different interactive domains should be used. However, its construction is not within the scope of this study. Within the limitations in this study, it can be concluded that the region of P that was indispensable for the binding to NP_{NC} was located within amino acids 224 to 279.

Based on the protein binding assay, it was observed that when amino acids 21 to 84 were deleted in mutants $P_{N52-395}$ and $P_{N85-395}$, there was approximately 3 and 4.5 fold increase in the interaction as compared to that of the

wild type P. It is possible that this region negatively regulates the P-NP_{NC} interaction as removal of this region increased the interaction. Although, this assumption is theoretically possible, there have been no records on such an observation on other negative RNA stranded viruses. However, there are regions on P which down-regulated P-NP_O interaction (Zhao and Banerjee, 1995; Slack and Easton, 1998). Zhao and Banerjee (1995) found that a domain located between amino acids 63 and 403 negatively regulated the P-NP_O interaction, while Slack and Easton (1998) revealed that a region within residues 198 and 217 reduced the interaction between P and NP_O.

By analogy to P of Sendai virus and VSV, majority of phosphorylation sites of P protein were concentrated in the N-terminal half of P (Hsu and Kingsbury, 1982; Banerjee and Barik, 1992). As the P binding domain of NDV was not mapped on the N-terminal half of P, most probably, phosphorylation was not required for the interaction of P to NP_{NC}. Therefore, other mechanisms are probably responsible for the binding of P to NP_{NC}. It is probable that P trimerization is required to initiate the binding of P NDV to NP_{NC} as P binding to NP_{NC} in Sendai virus depends on P trimerization to permit contacts between multiple C-terminal ends of P and NP_{NC} (Curran, 1998). The same observation was also demonstrated in VSV where only multimeric form of P can interacts with both L and NP_{NC} but the interaction also requires the phosphorylation of P (Gao and Lenard, 1995).

In contrast to the other proteins of NDV, P is so unique and several non-structural proteins can be expressed from the P-gene via RNA editing (Steward et al., 1993; Locke et al., 2000). V and W proteins are translated through this process where non-templated single G and two Gs, respectively are inserted at the editing site which is located within the N-terminal half of P (Steward et al., 1993). As a result, both proteins share the same N-termini with P but differ at their C termini. Subsequently, it is tempting to suggest that both proteins might not interact with NP_{NC} as their C termini do not bear any sequence homology with the C-terminal half of P, the region responsible in the interaction with NP_{NC}. This possibility is supported by the observation demonstrated in Sendai virus where neither its V nor W proteins bound to NP_{NC} (Horikami et al., 1996). The two proteins of Sendai virus were also quite similar to that of NDV where they share a common N-terminal with their respective P but utilize different reading frames at their C termini (Galinski and Wechsler, 1991).

It is important to note that the negative binding results recorded in the present study might not directly reflect the actual P-NP_{NC} interaction site(s) located within the deleted domains. The progressive terminal deletions used in this study might disrupt the P structure rather than remove the sequences that are directly involved in P-NP_{NC} complex formation. A detailed study using internally deleted P mutants that might minimize the terminal deletion effects should be carried out in the future to provide a better understanding in determining the P-NP_{NC} interactive domain.

Finally, although the P-NP_{NC} interactive domain obtained by the two different techniques that was based on an *in vitro* system is slightly different, this is only logical as the sensitivity of each technique varies and both techniques are affected by different physiological factors. This has also been observed in localizing the P-NP_{NC} interactive domain in vesicular stomatitis virus using two different techniques (Gill et al., 1986; Emerson and Schubert, 1987). Immunoprecipitation of various deleted Ps-NP:RNA complex (Emerson and Schubert, 1987) to determine the P-NP_{NC} interactive domain demonstrate a larger P binding domain as compared to another technique that involved centrifugal sedimentation of the valued complex (Gill et al., 1986). Each technique, either using an *in- vitro* or *vivo* system has its own advantages and disadvantages that might generate different results for a particular purpose. Therefore, different techniques are required to be carried out in the future for confirmation purposes.

Conclusion

Both radioimmunoprecipitation and protein binding assay methods gave rise to data that are similar to each other, which revealed that the P interactive domain was located within the internal region of C-terminal half of P and; N-terminal half and immediate C-terminal end of P was not involved in the P-NP_{NC} interaction. A slightly larger region of interaction was determined by the protein binding assay (amino acid position 224 to 279) as compared to the radioimmunoprecipitation assay (amino acid position 243 to 279) which was due to the sensitivity of the former assay. The protein binding assay is a quantitative assay, while radioimmunoprecipitation assay is qualitative. Thus, the interactive region was located within C-terminal half of P between amino acids 224 and 279.

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