Mapping of domains on the human parainfluenza virus type 2 nucleocapsid protein (NP) required for NP–phosphoprotein or NP–NP interaction

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The epitopes recognized by 41 monoclonal antibodies directed against the nucleocapsid protein (NP) of human parainfluenza virus type 2 (hPIV-2) were mapped on the primary structure of the hPIV-2 NP protein by testing their reactivities with deletion mutants. By Western immunoblotting using these monoclonal antibodies, the analysis of deletion mutants of the hPIV-2 NP protein was performed to identify the region essential for NP–NP interaction and phosphoprotein (P)-binding sites on the NP protein. The results indicate that the N-terminal 294 aa of the NP protein are all required for NP–NP self-assembly, and that two C-terminal parts of the NP protein are essential for NP–P binding: one region, aa 295–402, is required for binding to the C-terminal part of the P protein and another region, aa 403–494, to the N-terminal part of the P protein.

Introduction

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory pathogens and a member of the genus Rubulavirus in the family Paramyxoviridae. Its RNA genome is approximately 15 kb in length and, like other paramyxoviruses, encodes seven structural proteins; the nucleocapsid (NP), phospho- (P), V, matrix, haemagglutinin–neuraminidase, fusion and large (L) proteins. The NP protein of paramyxoviruses is the template for both mRNA synthesis and genome replication by the viral RNA-dependent RNA polymerase, which consists of the P and L proteins (Horikami et al., 1992; Curran et al., 1993). Although the three RNA-associated proteins thus play important roles in the replication cycle of the virus, the functional domains of each protein and the domains that participate in protein–protein interactions among these three polypeptides have yet to be defined completely.

The hPIV-2 P gene encodes the V protein, and the P protein mRNA is produced by the addition of two nontemplated G residues, which results in a frameshift and the expression of the P protein as a fusion protein with the N-terminal 164 aa of the V protein (Ohgimoto et al., 1990). We have reported previously that two noncontiguous regions of the hPIV-2 P protein are required for binding to the NP protein; one is located in the N-terminal region, aa 1–46, which is in the P–V common domain, and the other is in the C-terminal part, aa 357–395, which is required for granule formation of the NP–P complex (Nishio et al., 1996). We have also demonstrated that the trimerization site of the P protein is located between aa 211 and 248, which is predicted to form a coiled coil (Nishio et al., 1997). We have shown that the N-terminal domain, aa 1–46, of the hPIV-2 V protein is involved in binding to the NP protein (Watanabe et al., 1996b), and that two noncontiguous regions in the V protein, aa 1–46 and aa 175–196, are required for nuclear localization and retention (Watanabe et al., 1996a).

The NP proteins of paramyxoviruses play a central role in the replication of the viral genomic RNA (Horikami et al., 1992; Buchholz et al., 1993, 1994; Bankamp et al., 1996; Myers et al., 1997; Spehner et al., 1997). Based on these known functions, the NP protein of paramyxovirus must have at least three domains: one or more binding site(s) for the P protein to form the NP–P encapsidation complex, an NP–NP interaction site(s) for the assembly of nucleocapsid, and an RNA-binding domain(s) for initiation and elongation during packaging. However, none of these functional sites has been mapped in hPIV-2. To begin the characterization of the functional domains of the hPIV-2 NP protein, we mapped the regions recognized by 41 monoclonal antibodies (MAbs) directed against the NP protein. By Western blot assay using these MAbs, the analysis
of deletion mutants of the hPIV-2 NP protein was performed to identify the region essential for NP–NP interaction and P-binding sites on NP protein in vitro.

Methods

Cells and MAbs. Transfections were performed in COS cells as described previously (Nishio et al., 1997). Forty-one MAbs directed against the hPIV-2 NP and two MAbs directed against the hPIV-2 P (85A, 335) were produced as described previously (Tsududome et al., 1989). The epitope recognized by the 85A anti-P antibody was located between 111 and 164 aa, and the epitope recognized by the 335A antibody was located between 357 and 359 aa as described previously (Nishio et al., 1997).

Constructions of subclones. A cDNA fragment encoding the hPIV-2 NP, P or deleted P mutants was inserted into the expression vector pcDL-SRα296 to obtain plasmids pDS-NP, pDS-P, pPAc118 and pPAc, respectively, as described previously (Takebe et al., 1988; Nishio et al., 1996, 1997). Most of the NP deletion mutants detailed in Fig. 1 were generated by PCR amplification using the wild-type NP gene as template as described previously (Nishio et al., 1996, 1997). The mutant pNPABglIII was derived from pDS-NP by excision of BglII restriction enzyme recognition sites. All plasmids were characterized by restriction analysis and sequenced across the fusion junctions.

Transfection. COS cells were grown in six-well plates at 37 °C in Dulbecco’s modification of Eagle’s tissue culture medium (DMEM) supplemented with 10% foetal calf serum (FCS). The cells were washed twice with warm DMEM, and then transfected with various plasmids (3 μg each) in lipofectin (Gibco). After incubation for 7 h at 37 °C, DMEM containing 10% FCS was added.

Immunoprecipitation and Western blot assay. After 2 days of transfection, cell extracts were prepared with lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.1% NP40) containing 1 mM DTT. The extracts were incubated overnight with 150 μl MAbs at 22 °C, 50 μl of 50% protein A–Sepharose in PBS was added and incubation was continued for 4 h at 22 °C. Immune complexes were washed three times with lysis buffer containing 1 mM DTT and once with PBS, then analysed by LDS (lithium dodecyl sulfate)–PAGE. Electrophoretic transfer of virus polypeptides from gels onto nitrocellulose membranes was carried out as described previously (Nishio et al., 1996).

Results

Mapping of epitopes recognized by anti-NP MAbs

To define the epitope recognized by each anti-NP MAb, we constructed a set of deletion mutant genes encoding truncated NP proteins (Fig. 1) and examined the reactivities of 41 MAbs with a series of deleted NP proteins. COS cells were transfected with various plasmids encoding the NP protein and truncated NP proteins, lysed by sonication, and then the cell lysates were analysed by LDS–PAGE and Western blotting. Fig. 2(a) shows the reactivities of MAb 20A with the NP protein (lane 1), NPΔ100 (lane 2), NPΔ200 (lane 3), NPΔ300 (lane 4) and NPΔBglIII (lane 5). MAb 20A reacted with the NP, NPΔ100 and NPΔ300 proteins. On the other hand, MAb 20A did not react with NPΔ200 and NPΔBglIII. These results indicated that the epitope recognized by MAb 20A was located between aa 101 and 200. Fig. 2(b) shows the results using MAb 26A and the NP protein (lane 1), NPΔ100 (lane 2), NPΔ200 (lane 3), NPΔ50 (lane 4) and NPΔC100 (lane 5). MAb 26A reacted with NP, NPΔ100, NPΔ200 and NPΔ50, but not with NPΔC100, indicating that the epitope recognized by MAb 26A was located between aa 444 and 494. As shown in Fig. 2(c), MAb 159-1 reacted with NP (lane 1), NPΔ100 (lane 2) and NPΔ200 (lane 3), but not with NPΔC50 (lane 4). Thus it was concluded that the epitope recognized by MAb 159-1 was located between aa 495 and 545. The epitopes recognized by the other 38 MAbs were determined using the same assay, and anti-NP MAb-binding sites on the NP protein are summarized in Table 1.

Localization of the site essential for NP–NP oligomerization

When the NP protein and C-terminal truncated NP protein NPΔC50, NPΔC143, NPΔC250 or NPΔC300 were coexpressed in COS cells, each of the proteins was efficiently expressed in transfected cells (Fig. 3a, lanes 1, 2, 5 and 6). When the lysates of the cells cotransfected with NP and C-terminal truncated NP cDNAs were immunoprecipitated with MAb 159-1, which
Table 1. Mapping of epitopes recognized by MAbs directed against the hPIV-2 NP protein

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Non-reactive deletion mutants</th>
<th>Region (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1, 13A, 16-1, 126A, 138A</td>
<td>NPΔan47, NPΔan100, NPΔ444–494</td>
<td>1–47</td>
</tr>
<tr>
<td>20A, 30B-1, 318-1, 330-1, 366-1, 369</td>
<td>NPΔan200, NPΔBglII</td>
<td>101–200</td>
</tr>
<tr>
<td>28A, 38S1, 47-1A, 53-1A, 64-2A, 95-1A, 146A, 154A, 159-1, 325</td>
<td>NPΔc50, NPΔc100, NPΔc143, NPΔc250, NPΔc300</td>
<td>495–545</td>
</tr>
</tbody>
</table>

Fig. 2. Reactivities of MAbs. COS cells were transfected with various plasmids encoding the NP protein and deleted NP proteins, lysed by sonication and then the cell lysates were analysed by LDS–PAGE and Western blotting. (a) The reactivities of MAb 20A with NP protein (lane 1), NPΔan100 (lane 2), NPΔan200 (lane 3), NPΔc300 (lane 4) and NPΔBglII (lane 5). (b) The reactivities of MAb 26A with NP protein (lane 1), NPΔan100 (lane 2), NPΔan200 (lane 3), NPΔc50 (lane 4) and NPΔc100 (lane 5). (c) The reactivities of MAb 159-1 with NP protein (lane 1), NPΔan100 (lane 2), NPΔan200 (lane 3) and NPΔc50 (lane 4).

The complex of the hPIV-2 full-length NP and P proteins was detected in the immunoprecipitates obtained with either anti-NP MAb 20A or anti-P MAb 85A (Fig. 4 a, lanes 1 and 2). In a previous study, we identified two domains on the P protein of hPIV-2 responsible for binding to the NP protein: one is located in the N-terminal part of the protein, aa 1–47, and the other is in the C-terminal part, aa 357–359 (Nishio et al., 1996). Therefore, we first examined the region on the NP protein of hPIV-2 required for interaction with the N-terminal part of the P protein. PΔc118, which has the only N-terminal binding region of the P protein, and C-terminally truncated NP protein NPΔc50 or NPΔc143 were coexpressed in COS cells. The cell lysates were immunoprecipitated with anti-NP MAb 20A or anti-P MAb 85A, and then the immunoprecipitates were analysed by Western blot assay. As shown in Fig. 4 (b), complex NPΔc50–PΔc118 was detected in the immunoprecipitates obtained with either anti-NP or anti-P antibody (lanes 1 and 2), but complex NPΔc143–PΔc118 could not be detected (lanes 3 and 4). These results suggested that region aa 403–494 on the NP protein was critical for interaction with the N-terminal binding site of the P protein. However, since NPΔc100 coigrated with the immunoglobulin heavy chain in LDS–PAGE, a further analysis could not be carried out.

Subsequently, we determined the region on the NP protein required for interaction with the C-terminal part of the P protein, PΔn, which has only the C-terminal binding region, and C-terminally truncated NP protein NPΔc50, NPΔc143 or NPΔc250 were coexpressed in COS cells and the cell lysates were immunoprecipitated with either NP MAb 20A or anti-P MAb 335A. As shown in Fig. 4 (c), complexes NPΔc50–PΔn and NPΔc143–PΔn were detected with either anti-NP or anti-P antibody (lanes 1–4). However, neither anti-NP antibody 20A nor anti-P antibody 335A immunoprecipitated complex missing the aa 49–242 region, did not bind to the NP protein either. Therefore, these results indicated that the N-terminal 294 aa of NP are all required for NP–NP self-assembly.

**Characterization of domains on the NP protein involved in NP–P interaction**

The complex of the hPIV-2 full-length NP and P proteins was detected in the immunoprecipitates obtained with either anti-NP MAb 20A or anti-P MAb 85A (Fig. 4 a, lanes 1 and 2). In a previous study, we identified two domains on the P protein of hPIV-2 responsible for binding to the NP protein: one is located in the N-terminal part of the protein, aa 1–47, and the other is in the C-terminal part, aa 357–359 (Nishio et al., 1996). Therefore, we first examined the region on the NP protein of hPIV-2 required for interaction with the N-terminal part of the P protein. PΔc118, which has only the N-terminal binding region of the P protein, and C-terminally truncated NP protein NPΔc50 or NPΔc143 were coexpressed in COS cells. The cell lysates were immunoprecipitated with anti-NP MAb 20A or anti-P MAb 85A, and then the immunoprecipitates were analysed by Western blot assay. As shown in Fig. 4 (b), complex NPΔc50–PΔc118 was detected in the immunoprecipitates obtained with either anti-NP or anti-P antibody (lanes 1 and 2), but complex NPΔc143–PΔc118 could not be detected (lanes 3 and 4). These results suggested that region aa 403–494 on the NP protein was critical for interaction with the N-terminal binding site of the P protein. However, since NPΔc100 coigrated with the immunoglobulin heavy chain in LDS–PAGE, a further analysis could not be carried out.

Subsequently, we determined the region on the NP protein required for interaction with the C-terminal part of the P protein, PΔn, which has only the C-terminal binding region, and C-terminally truncated NP protein NPΔc50, NPΔc143 or NPΔc250 were coexpressed in COS cells and the cell lysates were immunoprecipitated with either NP MAb 20A or anti-P MAb 335A. As shown in Fig. 4 (c), complexes NPΔc50–PΔn and NPΔc143–PΔn were detected with either anti-NP or anti-P antibody (lanes 1–4). However, neither anti-NP antibody 20A nor anti-P antibody 335A immunoprecipitated complex...
NPΔc250–PΔN, though NPΔc250 and PΔN were precipitated (lanes 5 and 6). These results indicated that the aa 295–402 region of the NP protein is essential for interaction with the C-terminal binding site of the P protein. Thus, both the binding sites of the NP protein are located in the C-terminal part of the NP protein.

**Discussion**

Paramyxovirus RNA replication and transcription are complex processes requiring specific interactions between the L, P and NP proteins, and sequences in genomic RNA. This requires that there are multiple functional domains on these proteins. In a previous study, we identified two independent domains in the hPIV-2 P protein responsible for binding to the NP protein (Nishio et al., 1996) and the region essential for the trimerization of the P protein (Nishio et al., 1997). In addition, we have shown that the hPIV-2 V protein binds to the NP protein (Watanabe et al., 1996b), and that two noncontiguous regions in the V protein are required for nuclear localization and retention (Watanabe et al., 1996a).

In the present study, we mapped the epitopes recognized by 41 MAbs directed against the hPIV-2 NP protein by testing their reactivity with lysates of COS cells expressing various deleted NP proteins. Using these MAbs, we have determined the sites on the hPIV-2 NP protein responsible for the NP–NP and NP–P interactions by Western blotting.

Many studies on nonsegmented negative-stranded viruses have indicated that the NP protein folds into a globular body with an exposed C terminus and that this exposed region
Fig. 5. A schematic model of domains in the NP, P and V proteins of hPIV-2. The various domains identified from this and previous works are indicated by boxes. Amino acid residues 1–46 on the P/V common region are required for binding to the aa 403–494 region on the NP protein. Residues 357–395 on the C-terminal domain of P protein are required for binding to the aa 295–402 region on the NP protein. Residues 211–248 on the P protein are required for trimerization. The N-terminal region aa 1–294 on the NP protein is required for self-assembly. Two hatched boxes on the V protein are identified as nuclear localization signals. The arrows above mark the site at which the P protein is fused in place by mRNA editing.

interacts with the P protein (Homan et al., 1991; Curran et al., 1993, Ryan et al., 1993; Bankamp et al., 1996). In the two-hybrid system in yeast, the NP–P interaction was directly demonstrated in Sendai virus (SeV) (Horikami et al., 1996) and in bovine respiratory syncytial virus (Krishnamurthy et al., 1998). More recently both the central conserved region (CCR, aa 258–357) and the N-terminal 255 aa have been found to contain sequences important for NP–NP self-assembly by using the fusion proteins between the maltose-binding protein and different regions of the SeV NP protein. In addition, the specific residues in the CCR were proven to be important for binding to RNA (Myers et al., 1997). We identified the domain on the hPIV-2 NP protein responsible for multimerization to the whole N-terminal region aa 1–294. It is conceivable that almost the whole globular domain of the NP protein is required for the NP–NP interaction, suggesting that the deletions within the globular body compromise the overall structure of the polypeptide and consequently abolish the NP–NP interaction. The sequences of the NP protein required for interaction with the P protein are ill defined in most nonsegmented negative-stranded RNA viruses. Large regions in the NP proteins of vesicular stomatitis virus (Takacs et al., 1993), human respiratory syncytial virus (Garcia-Barreno et al., 1996) and SeV (Homann et al., 1991) have been shown to be essential for interacting with the P protein.

In this report, we have also demonstrated the existence of two independent NP–P-binding sites which can interact with different parts of the P protein. Region aa 403–494 on the NP protein is essential for the interaction with the N-terminal binding site of P protein, and the C-terminal binding site of P protein interacts with region aa 295–402 on the NP protein, which includes part of a domain that is highly conserved among the parainfluenza NP proteins (Miyahara et al., 1992). These results suggest that both P-binding sites are located in the C-terminal part of the hPIV-2 NP protein.

The domains on the hPIV-2 NP, P and V proteins identified in this work and our previous works are summarized in Fig. 5. In SeV and measles viruses, the N-terminal two-thirds are important in NP–NP interactions and RNA encapsidation, and the C-terminal one-third is important in binding to polymerase complex (P–L) (Curran et al., 1993; Buchholz et al., 1993, 1994; Bankamp et al., 1996). A similar situation exists in the hPIV-2 NP protein. In a previous study, we demonstrated by using immunofluorescence staining that the interaction of the hPIV-2 NP protein with the N-terminal region of the P protein was different from that with the C-terminal region of the P protein. We have also shown that the C-terminal region of the P protein was required for the NP–P complex to form granules, which were detected in hPIV-2-infected cells. The C-terminal region of the P protein binds to the NP protein near the NP–NP interaction site and part of the CCR which might be a domain important for a common function of the paramyxovirus NP proteins. Thus it might be that the binding between the NP and C terminus of P proteins affects the structure of the NP protein, resulting in granule formation. When NPAn200 and P proteins of hPIV-2 were coexpressed in COS cells, granule formation was not found, although the mutant NP protein did bind to the P protein (data not shown), suggesting that NP protein self-assembly is required for the NP–P complex to form granules.

In conclusion, we propose that the N-terminal part of the NP protein is important for NP–NP self-assembly, and two C-terminal regions of the NP protein contribute to the NP–P interaction. Future experiments will be required to elucidate these relationships and the importance of other residues of these proteins.

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References


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