Interaction between nucleocapsid protein (NP) and phosphoprotein (P) of human parainfluenza virus type 2: one of the two NP binding sites on P is essential for granule formation

Machiko Nishio, Masato Tsurudome, Mitsuo Kawano, Noriko Watanabe, Shinji Ohgimoto, Moriiro Ito, Hiroshi Komada and Yasuhiko Ito

Department of Microbiology, Mie University School of Medicine, 2-174, Edobashi, Tsu-Shi, Mie-Ken, 514, Japan

The paramyxovirus phospho- (P) and nucleocapsid (NP) proteins are involved in transcription and replication of the viral genome. To study the interaction between NP and P proteins, we established HeLa cell lines that constitutively expressed the NP and/or P proteins of human parainfluenza virus type 2 (hPIV-2). Co-immunoprecipitation assays revealed that the NP and P proteins can form complexes in HeLa cells expressing both proteins (HeLa-NP+P cells) and in mixed cell lysates of HeLa-NP and HeLa-P cells. Deletion mutant analysis of the P protein was performed to identify the regions of P protein that interact with NP protein.

The results indicate that two independent NP-binding sites exist on P protein: one is located in the N-terminal part of the protein, aa 1–47, and the other in the C-terminal part, aa 357–395. In addition, cells co-expressing NP and P proteins with N-terminal deletions showed immunofluorescence staining patterns (granular pattern) similar to those found in hPIV-2-infected cells. However, cells co-expressing NP and P proteins with C-terminal deletions showed a different immunofluorescence staining pattern (diffuse pattern), indicating that the C-terminal region is required for granule formation.

Introduction

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory pathogens. It is a member of the genus Paramyxovirus in the family Paramyxoviridae, which are nonsegmented negative-stranded RNA viruses. Its RNA genome is approximately 15 kb in length and, like that of other paramyxoviruses encodes seven structural proteins: the nucleocapsid (NP), phospho- (P), V, matrix (M), haemagglutinin-neuraminidase (HN), fusion (F) and large (L) proteins. The NP protein is the most abundant component and encapsidates the genomic RNA to form the NP–RNA template, so maintaining its structural integrity. NP–RNA functions as the active template for transcription and replication. The L and P proteins form the RNA polymerase complex, which itself associates with the NP–RNA template.

Although the three RNA-associated proteins play important roles in the life-cycle of the virus, the functional domains of each protein and the domains that allow protein–protein interactions to occur between the three polypeptides have yet to be defined completely.

In the case of Sendai virus, Ryan & Portner (1990) showed that two non-contiguous regions, amino acids (aa) 345–411 and aa 479–568 in the C-terminal half of the P protein, are required for binding to NP protein. Curran et al. (1995) have shown that an N-terminal domain of the P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. Therefore, three regions of the P protein are known to be important for binding to NP protein: aa 345–411 and aa 479–568 are necessary and sufficient for binding to nucleocapsids, and aa 479–568 and aa 33–41 are required for binding to unassembled NP protein.

In the case of rabies virus, Chenik et al. (1994) showed that at least two independent nucleoprotein-binding sites exist on the phosphoprotein: one is located in the C-terminal part of the protein and another between aa 69–177.

We used a different expression system to express NP and P proteins in mammalian cells, and showed that these proteins associate with each other in vivo. Then, we constructed various deletion mutants and demonstrated the existence of two NP protein-binding sites on the P protein, one of which is located...
in the C-terminal domain and the other in the N-terminal domain. Furthermore, our immunofluorescence experiments showed that only the C-terminal domain is required for granule formation similar to that found in hPIV-2-infected cells.

**Methods**

- **Viruses and cells.** The viruses used in this study were hPIV-2 Toshiba strain and simian virus 41 (SV41) Toshiba strain. HeLa cells were grown in Eagle’s MEM supplemented with 5% calf serum.

- **Monoclonal antibodies (MAbs).** MAbs directed against the P (13-1A, 85A, 57-1A) and NP proteins (28A, 38S1, 159-1A) were produced as described previously (Tsurudome et al., 1989).

- **Plasmid construction**

(i) **Construction of wild-type NP and P genes.** A cDNA clone of the hPIV-2 NP or P gene was inserted into plasmid expression vector pcDL-SR~296 (Takebe et al., 1988) downstream of the SV40 early promoter and R-U5 sequence of the human T-lymphotropic virus (HTLV-1) LTR to obtain plasmids pDS-NP and pDS-P, respectively.

(ii) **Construction of deleted P genes.** Constructs pPan47 and pPan110 differed from pDS-P by deletions of 141 bp and 330 bp at the N terminus of the P gene, respectively. These deletions were introduced by PCR amplification of the wild-type P gene using synthetic oligonucleotides PA1, PA2 and PB. PA1 (ACTCGAGAACCTGCTTTCTACTGTGCA) contains the sequence corresponding to nucleotides 215–233 of the P mRNA, thus including an in-frame ATG (in bold letters). PA2 (ACTCGAGAAGATGGAGACATTGACATT) contains the sequence corresponding to nucleotides 404–421 of the P mRNA including an in-frame ATG (in bold letters). PB (ACTGCAGTTATGAGCGACTATTTATT) contains the sequence corresponding to nucleotides 1240–1261 of the P mRNA, at the 3’ end of the P gene. Constructs pPac39, pPac78 and pPac118 differed from pDS-P by deletions of 117 bp, 234 bp and 354 bp at the C terminus of the P gene, respectively. These deletions were introduced by PCR amplification of the wild-type P gene using synthetic oligonucleotides PB1, PB2, PB3 and PA. PB1 (ATTAAAGCTGCTCAGAAGTGTT) contains the sequence corresponding to nucleotides 1121–1141 of the P mRNA, PB2 (ATTACACCTGTTCGGCTGTGAA) contains the sequence corresponding to nucleotides 1007–1024 of the P mRNA, all including an in-frame stop codon. PA (ACTGCAGTTATGAGCGACTATTTATT) contains the sequence corresponding to nucleotides 74–88 of the P mRNA, including an in-frame initiation codon, ATG (in bold letters). Construct pPan47c39 was obtained by deletion of 141 bp from the N terminus and 117 bp from the C terminus of pDS-P using the oligonucleotides PA1 and PB1. Construct pPan47c78 was obtained by deletion of 117 bp from the N terminus and 234 bp from the C terminus of pDS-P using the oligonucleotides PA2 and PB1.

- **Establishment of HeLa-P, HeLa-NP and HeLa-NP+P cell lines which constitutively express hPIV-2 P, NP and NP+P proteins, respectively.** HeLa cell lines which constitutively express virus protein(s) were established as described previously (Nishio et al., 1994).

- **Transfection.** HeLa–NP cells were grown in 12-well plates at 37 °C in MEM containing 10% fetal calf serum. The cells were washed twice with warm MEM and then transfected with various plasmids (5 μg each) using lipofectin (Gibco). After incubation for 7 h at 37 °C, MEM with 10% calf serum was added.

- **Immunoprecipitation and Western blot assay.** After transfection for 2 days, cell extracts were prepared by lysis in NP40 buffer (50 mM-Tris·HCl, pH 7.5, and 0.5% NP40). The extracts were incubated overnight with 100 μl of MAbs at 22 °C. Then 50 μl of 50% protein A–Sepharose in PBS was added, and incubation continued for 4 h at 22 °C. Immune complexes were washed three times with NP40 buffer and once with PBS, then analysed by LDS–PAGE. Electrophoretic transfer of virus polypeptides from gels onto nitrocellulose membranes was carried out at 4 °C according to Towbin et al. (1979). The membranes were washed with PBS/T, blocked with 5% skim milk in PBS, treated with each MAb at room temperature for 1 h, washed three times with PBS/T, then treated with biotinylated horse immunoglobulin to mouse IgG (heavy and light chains) (Vector Laboratories) at room temperature for 1 h. After washing with PBS/T, the membranes were treated with avidin–biotin–peroxidase complex (Vector Laboratories) at room temperature for 1 h. After washing with PBS, the membranes were immersed in methanol–PBS (2:1) containing 4-chloro-1-naphthol (0.3%) and hydrogen peroxide (0.009%).

**Results**

**Expression of NP and P proteins in transfected cells**

The expression of virus-specific antigens in cells transfected with plasmid(s) and the proportions of cells expressing antigens were investigated by analysis of immunofluorescence-stained cells with a fluorescence microscope and a flow cytometer. Almost all HeLa–NP, HeLa–P and HeLa–NP+P cells were positive for immunofluorescence (Fig. 1). Fig. 2 shows the results of flow-cytometric analysis. The hPIV-2 NP and P proteins were expressed to almost the same extent in HeLa–NP, HeLa–P and HeLa–NP+P cells as in virus-infected HeLa cells.

**Interaction between NP and P proteins**

Incubation of cell extracts of hPIV-2-infected cells with anti-NP antibody precipitated both NP and P proteins (Fig. 3, lane 1). Similarly, incubation of HeLa–NP+P cell extracts with anti-NP antibody precipitated both NP and P proteins (Fig. 3, lane 2). To test whether such complexes could be detected in mixtures of extracts of HeLa–NP and HeLa–P cells, such mixtures were immunoprecipitated with specific mouse monoclonal anti-NP (mixture of 28A, 38S1 and 159-1A) or anti-P (85A) antibodies. When the immunoprecipitates were analysed
Fig. 1. Immunofluorescent staining of cell lines that constitutively express wild-type NP and/or P proteins. HeLa-NP (a, b), HeLa-P (c, d), HeLa-NP+P (e, f) and HeLa cells infected with hPIV-2 (g, h) were fixed with 10% paraformaldehyde for 15 min and permeabilized for 20 min with 0.05% Triton X-100 in PBS. Viral proteins were stained using anti-NP protein MAbs (MAbs 28A, 38S1, 159-1A) (a, c, e, g) or anti-P protein MAb (MAb 85A) (b, d, f, h) for 1 h and, then reacted with FITC-conjugated anti-mouse IgG goat serum.
Fig. 2. Expression of viral proteins in normal HeLa cells, HeLa cells infected with hPIV-2, and in HeLa-NP, HeLa-P and HeLa-NP+P cells. The level of expression of virus-specific antigen and proportions of expressing cells were investigated by flow cytometry. ---, cells stained with anti-NP or P MAb; ..., normal HeLa cells stained with anti-NP or P MAb.

Fig. 3. Detection of NP-P complexes by Western blot. HeLa cells infected with hPIV-2 (lane 1) or HeLa-NP+P (lane 2) were immunoprecipitated by anti-NP MAb. Mixtures of HeLa-NP lysate and/or HeLa-P lysate immunoprecipitated by anti-P MAb (lanes 3, 4 and 5) or anti-NP MAb (lanes 6, 7 and 8) were further immunostained with a mixture of anti-NP and anti-P MAb.

38S1 and 159-1A) precipitated both proteins as complexes (lane 8). These co-immunoprecipitations were due to specific precipitation of either the NP or P proteins, because under the same precipitation conditions, the anti-P antibody did not precipitate NP protein in the absence of P protein (lane 3) and the anti-NP antibodies did not precipitate P protein in the absence of NP protein (lane 7).

Characterization of domains on the P protein involved in NP-P interaction

To identify domains on the P protein required for binding to NP protein, we constructed a set of eight deletion mutant genes encoding truncated P proteins (Fig. 4) and transfected the various cDNAs into HeLa-NP cells. Each of the proteins was efficiently expressed in transfected cells and all were recognized by anti-P antibody (Fig. 5, lanes 1–5). The three C-terminally deleted P proteins (PΔc39, PΔc78 and PΔc118) were tested for their ability to form complexes by binding to NP protein in HeLa-NP cells (Fig. 5, lanes 1–3, 6–8). The two N-terminally deleted P proteins (PΔN47 and PΔN110) were also tested for their ability to bind to NP protein in HeLa-NP cells. These complexes were also detected in the immunoprecipitates obtained from each of the plasmid-transfected HeLa-NP cell extracts by the use of anti-NP or anti-P antibody (Fig. 5, lanes 4, 5, 9, 10).

The fact that the C-terminally deleted P proteins and the N-terminally deleted P proteins can bind to NP protein suggests two possibilities: one is that the middle portion of P protein contains the binding site for NP protein; the other is that P protein contains at least two binding sites for NP protein. Therefore, we constructed an additional set of three deleted genes truncated at both terminal domains (PΔN47c39, 2460
Fig. 5. Analysis by Western blot assay of the interaction between NP protein and C- or N-terminally truncated P proteins. HeLa-NP cells were transfected with plasmids encoding C-terminally truncated P proteins PΔC39 (lanes 1 and 6), PΔC78 (lanes 2 and 7) and PΔC118 (lanes 3 and 8), or N-terminally truncated P proteins PΔN47 (lanes 4 and 9) and PΔN110 (lanes 5 and 10). These lysates were immunoprecipitated with anti-P antibody (85A) (lanes 1–5) or anti-NP antibodies (28A, 38S1 and 159-1A) (lanes 6–10). Subsequently, these lysates were immunostained with a mixture of anti-NP and anti-P MAb.

Fig. 6. Analysis by Western blot assay of the interaction between NP protein and terminally truncated P proteins. HeLa-NP cells were transfected with plasmids encoding the terminally truncated P proteins, PAN47C78 (lanes 1, 4), and PAN47C39 (lanes 2, 5) or PAN110C39 (lanes 3, 6). These lysates were immunoprecipitated with anti-P antibody (85A) (lanes 1–3) or anti-NP antibodies (28A, 38S1, 159-1A) (lanes 4–6). Subsequently, these cells were immunostained with a mixture of anti-NP and anti-P MAb.

Anti-P antibodies prevent binding of NP proteins

We prepared 43 MAbs against the P protein of hPIV-2 (Tsurudome et al., 1989) and the epitopes recognized by these anti-P MAbs were determined by immunoprecipitation using COS cells transfected with the deletion mutants (unpublished data). We found that the epitopes recognized by MAbs 13-1A, 85A and 57-1A were located between aa 1–47 (N-terminal region), aa 111–164 and aa 357–395 (C-terminal region), respectively (data not shown). The regions recognized by 13-1A and 57-1A were considered to be necessary for interaction with NP protein. Therefore, we tested the ability of anti-P antibodies to block the interaction between NP and P proteins. HeLa–NP cell lysates were mixed and incubated at 22 °C for 4 h with HeLa–P cell lysates preincubated with anti-P MAb(s). Fig. 7 shows that MAb 57-1A, but not MAb 13-1A blocked the interaction between the P and NP proteins, indicating that the C-terminal epitope recognized by MAb 57-1A is related to NP–P complex formation.

Effect of P truncations on the pattern of immunofluorescence of the NP–P complex in cotransfected cells

We investigated the effect of P protein deletions on the intracellular distribution of the NP–P complex by using immunofluorescence staining. P protein in HeLa–P cells and NP protein in HeLa–NP cells showed diffuse staining throughout the cytoplasm (see Fig. 1 b, c). However, when the NP and P proteins were co-expressed in HeLa–NP + P cells, their distribution changed distinctly, that is, they were organized in numerous granules that were similar in appearance to those found in virus-infected cells (Fig. 1).

Subsequently, we determined the ability of deletion mutants of P proteins to associate with NP proteins in such clusters. Cell lines which constitutively expressed NP and PAN47 or PAC118 proteins were obtained by transfecting the relevant plasmids into HeLa–NP cells. These cell lines showed different immunofluorescence staining patterns. In the case of PAN47, staining for NP and P proteins showed almost the same granular distribution as for the wild-type proteins and in virus-infected cells (Fig. 8a, b). In contrast, staining patterns were diffuse when the other deleted protein, PAC118, was co-expressed with NP protein. These staining patterns are similar to those in HeLa–NP or HeLa–P cells which expressed each protein independently (Fig. 8c, d). These results suggest that...
Fig. 8. Immunofluorescent staining of cell lines that constitutively express wild-type NP and truncated P proteins. HeLa-NP+PAc118 (c, d) were fixed with 10% paraformaldehyde for 15 min and permeabilized for 20 min with 0.05% Triton X-100 in PBS. Viral proteins were stained with anti-NP MAb (28A, 3851, 159-1A) (a, c) or anti-P MAb (85A) (b, d) for 1 h, and then reacted with FITC-conjugated anti-mouse IgG goat serum.

the interaction of NP protein with the N-terminal region of P protein is different from that with the C-terminal region of P protein, and that the C-terminal region of P protein is required for the NP-P complex to form granules.

Discussion

In this study, we investigated the interaction between hPIV-2 NP and P proteins using HeLa cell lines constitutively expressing virus-specific protein(s). Interactions were analysed by co-immunoprecipitation of both proteins with an antibody specific for one component followed by Western blotting and by immunofluorescence experiments. We could show the physical interaction between NP and P proteins in vivo and in vitro. Furthermore, we identified the domains on P protein responsible for binding to NP protein by using deletion mutants. Both PAc118 and PAN47 interacted with NP protein, while a mutant P protein deleted at both the N-terminal and C-terminal regions could not interact with NP protein. Therefore, P protein carries two binding sites; one is located in the N-terminal region, aa 1–47 and another in the C-terminal region, aa 357–395. To further characterize the binding regions, we tested the ability of anti-P antibodies to block interaction with NP protein. MAb 57-1A, which recognizes the C-terminal region, blocked the interaction between the P and NP proteins, indicating that the C-terminal epitope recognized by MAb 57-1A is related to NP-P complex formation. In addition, binding of the MAb to P protein is considered to induce a conformational change of the protein, resulting in abolition of the ability of the N-terminal region to bind to NP protein.

In rabies virus (Fu et al., 1994; Chenik et al., 1994) and hPIV-3 (Zhao et al., 1995), both the N- and C-terminal domains of P protein seem to be required for its interaction with N protein. Curran et al. (1994, 1995) have recently shown that aa 33–41 are necessary for a stable interaction of P with the free NP protein of Sendai virus. Furthermore, these nine amino acids are required to form a stable complex with unassembled NP (NP⁰) and to prevent NP from assembling illegitimately, but are not required for the stable interaction of P with the assembled NP (NP⁵⁰) of the nucleocapsid. Ryan & Portner (1990) have shown that aa 344–411 and aa 479–568 are necessary and sufficient for binding of P to nucleocapsids, presumably to NP⁵⁰. Since, the C-terminal region, aa 479–568, is also required for binding to NP⁰, it would presumably be binding to a region or surface that is present on both NP⁰ and NP⁵⁰. The C-terminal end of respiratory syncytial virus P protein has been reported to be essential for interacting with the N protein (co-immunoprecipitation and cytoplasmic co-aggregation of the P and N proteins) (Garcia-
Barreno et al., 1996). The exact roles of the C-terminal and N-terminal NP binding sites of hPIV-2 in the NP–P interaction will be further studied in our laboratory.

In hPIV-2-infected cells, the NP and P proteins form granules in inclusion bodies. Co-expression of the NP and P proteins is essential for granule formation because NP and P proteins expressed alone in cells give a diffuse immunostaining pattern. In HeLa cells co-expressing NP protein and a mutant P protein with 118 aa deleted from the C terminus, granule formation by the complex was not found although the mutant P protein did bind to NP protein. In contrast, in HeLa cells co-expressing NP protein and a mutant P protein with 47 aa deleted from the N terminus, granule formation occurred in inclusion bodies, indicating that the C-terminal region is required for the NP–P complex to form granules. For Sendai virus and measles virus, expression of the NP protein alone is sufficient to induce the formation of nucleocapsid-like structures which aggregate within the cytoplasm (Buchholz et al., 1993; Fooks et al., 1993; Spehner et al., 1991). In contrast, granule formation in rabies virus-infected cells requires co-expression of the N and P proteins (Chenik et al., 1994). Similar to hPIV-2, at least two independent N protein-binding sites exist on the P protein of rabies virus: one is located in the C-terminal region and another between aa 69–177 (Chenik et al., 1994). However, both sites on the P protein of rabies virus are required for granule formation (Chenik et al., 1994).

## References


Received 9 February 1996; Accepted 6 June 1996