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NP:P and NP:V Interactions of the Paramyxovirus Simian Virus 5 Examined Using a Novel Protein:Protein Capture Assay

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Using recombinant proteins extracted from mammalian cells, in a novel protein:protein binding assay, direct interaction of the nucleoprotein (NP) of simian virus 5 with the phosphoprotein (P) and V protein (V) was demonstrated. The amount of NP bound by V was found to be significantly less than that bound by P. Furthermore, preabsorption of NP with P removed the fraction of NP that could be bound by V, but preabsorption of NP with V did not remove all the NP that could be bound by P. These results suggested that V bound a subpopulation of the NP recognised by P. Further analysis revealed that P bound both soluble and homopolymeric forms of NP, while V bound only the soluble form; thus demonstrating that the binding sites on P and V, for soluble NP, are located within the N-terminal domain common to both P and V proteins. A monoclonal antibody, which recognised an epitope in the unique C-terminus of P, blocked the binding of P to polymeric NP but not to soluble NP. These results also suggest that there are two binding sites on NP for P; the site that interacts with the P/V common domain being either hidden or conformationally altered in polymeric NP.

INTRODUCTION

The paramyxovirus simian virus 5 (SV5) has a nonsegmented, negative-stranded RNA genome (vRNA). During transcription the virus-encoded RNA polymerase binds to the 3′ end of the genome and sequentially transcribes mRNAs that encode eight polypeptides; namely, and in the order, nucleocapsid protein (NP), V protein (V) and phosphoprotein (P), matrix protein, fusion protein, small hydrophobic protein, haemagglutinin–neuraminidase protein, and large polymerase protein (L; for review of paramyxoviruses and their replication see Lamb and Kolakofsky, 1996). The P and V proteins are encoded by a single gene, and share the same 164 N-terminal amino acids, but have unique C-termini. The unique C-terminus of V is cysteine rich, binds two atoms of zinc per molecule (Paterson *et al.,* 1995), and is highly conserved amongst paramyxoviruses. The V mRNA transcript is a faithful copy of the P/V gene, but the P mRNA transcript has two additional nontemplated G residues (Thomas *et al.,* 1988), inserted by a specific polymerase stuttering mechanism during transcription of the gene (Vidal *et al.,* 1990), which alters the reading frame of the mRNA.

The virus-encoded polymerase complex does not recognise naked vRNA as a template for transcription or replication but uses vRNA that has been encapsidated by NP into a nucleocapsid structure. In the virion, the polymerase complex (which consists of the L and P proteins; Buetti and Choppin, 1977; Hamaguchi *et al.,* 1983) is associated directly with the nucleocapsid. It has been reported recently that the V protein is also closely associated with the nucleocapsid structure (Paterson *et al.,* 1995). During replication newly synthesised vRNA and complementary RNA are both encapsidated by NP. It has been shown that expression of recombinant NP alone results in the formation of homopolymers of NP in nucleocapsid-like structures (Spehner *et al.,* 1991; Fooks *et al.,* 1993; Buchholz *et al.,* 1993; Warnes *et al.,* 1995). For this reason, it has been suggested that a virus-encoded protein may interact with NP, keeping it soluble prior to encapsidation. In Sendai virus, the P protein has been ascribed this chaperone function (Curran *et al.,* 1995b); while for SV5, we have suggested that there might be a role for the V protein in keeping NP soluble (Precious *et al.,* 1995).

The suggested role for V, in keeping NP soluble in the SV5 system, comes from observations on the associations of NP with P or V in cell lines that coexpress these proteins. Immunofluorescence data demonstrated that, when expressed alone, the NP protein had a granular cytoplasmic distribution, the P protein had a diffuse cytoplasmic distribution, and the V protein had a diffuse nuclear and cytoplasmic distribution. Coexpression of the NP and P proteins resulted in the accumulation of large cytoplasmic aggregates containing both proteins, similar to those visualised at late times in virus-infected cells (Fearns *et al.,* 1994). In contrast, coexpression of the V and NP proteins led to a redistribution of NP in that NP colocalised with V, having a diffuse distribution which

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was both nuclear and cytoplasmic. Furthermore, binding studies, as visualised by immunofluorescence, revealed that NP bound to both P and V proteins (Precious *et al.,* 1995).

Here we report on the development of a novel capture assay for studying protein:protein interactions and report on the properties of NP:P and NP:V interactions.

MATERIALS AND METHODS

Cells and preparation of antigen fractions for use in the protein capture assays

Plasmids were constructed that allowed inducible expression of the NP, P, and V proteins, under the control of the tTA-responsive promoter (Precious *et al.,* 1995). Stably transformed Balb/c fibroblasts (BF), expressing the NP, P, or V protein, were grown as monolayers in 25-, 75-, or 150-cm^2 flasks in Dulbecco's modification of Eagle's tissue culture medium supplemented with 10% newborn calf serum, tetracyclin (2 μg/ml), and Geneticin (200 μg/ml). These cells were negative for mycoplasmas as screened by DAPI staining. To induce expression of the tTA-responsive genes, the cell monolayers were washed four times with tissue culture medium without tetracycline or Geneticin (Precious *et al.,* 1995) and reincubated in the same medium for either 24–30 hr for cells expressing the P or V proteins or 48–72 hr for cells expressing the NP protein. The cells were harvested by scraping them into phosphate-buffered saline (PBS, 3 ml per 10^8 cells), and 1-ml aliquots were stored at −70° until required. Antigen extracts were made by disrupting the cells in PBS (3 ml per 10^8 cells) with an ultrasonic probe (MSE Soniprep 150). Large particulate material was pelleted by centrifugation at 6500 rpm for 10 min in an MSE MicroCentuar microfuge and the "low-speed supernatant" was collected. To pellet smaller complexes, the low-speed supernatant was centrifuged, through a 5% sucrose cushion, for 1 hr at 400,000 *g* in a Beckman TL benchtop ultracentrifuge and the "high-speed supernatant" was collected. The pelleted material was resuspended by sonication with an ultrasonic probe in the original volume of PBS and was termed the high-speed pellet.

Antibodies

A detailed description of the monoclonal antibodies (mAbs) to the SV5 NP, P, and V proteins and their nomenclature have been given elsewhere (Randall *et al.,* 1987). The mAb SV5-P-k reacts with a short, 9-amino-acid epitope located within the N-terminal domain common to both P and V proteins (Southern *et al.,* 1991).

Preparation of *Staphylococcus aureus* Cowan strain A

Heat-inactivated and formalin-fixed preparations of *Staphylococcus aureus* Cowan strain A were prepared by the method of Kessler (1975) and stored as a 10% (w/v) suspension in phosphate-buffered saline at −70° before use.

Protein:protein capture assay

It has been previously shown that fixed and killed *S. aureus* can bind strongly to certain types of plastic (Randall, 1983). In the protein capture assay, 96-well microtitre plates were flooded with a 0.2% suspension of fixed and killed *S. aureus* in PBS and left for a minimum of 4 hr at 4°. Unbound material was removed by washing the plates with PBS, leaving a confluent monolayer of *S. aureus* bound to the plastic surface. Monoclonal antibodies were bound to the monolayers by adding, for 2 hr at 4°, 50 μl of serum-free tissue culture fluid harvested from hybridomas secreting the appropriate monoclonal antibody. Unbound antibody was removed by washing the wells with PBS. Any nonspecific protein binding sites were blocked by the addition of 10% bovine serum albumin for 1 hr at room temperature. The P or V proteins were captured by the mAbs by adding, to the appropriate wells for 2 hr at 4° with continuous agitation, 200 μl of low-speed supernatant extracts of cells expressing these proteins. Unbound material was removed by washing the wells (four times) with PBS. To examine the binding of the NP protein to captured P or V proteins, 200 μl of various extracts (as described in the text) of cells expressing the NP protein were incubated with the appropriate wells, for 2 hr at +4° with continuous agitation. Unbound material was removed by washing the monolayers four times with PBS. Thirty microliters of gel electrophoresis sample buffer (0.05 *M* Tris–HCl, pH 7.0, 0.2% SDS, 5% 2-mercaptoethanol, and 5% glycerol) was added to the wells, the plate was heated to 100° for 5 min, and samples were electrophoresed through SDS–polyacrylamide gels. The NP, P, and V proteins were detected either by direct visualisation of Coomassie brilliant blue-stained gels or by Western blot analysis.

Western blot analysis

Polypeptides that had been separated through SDS–polyacrylamide gels were transferred to nitrocellulose using a semidry gel electroblotter (LKB). Before the nitrocellulose filters were incubated with the appropriate mAbs, unoccupied protein binding sites on the nitrocellulose were blocked with PBS containing 10% dried milk (Marvel) and 0.1% polyoxyethylenesorbitan monolaurate (Tween 20). Bound antibody was detected with horseradish peroxidase-conjugated sheep anti-mouse Ig and ECL detection (Amersham International, Ltd.).

Immunofluorescence

BF cells to be stained for immunofluorescence were grown on multispot microscope slides (C. A. Hendley,

Ltd., Essex, UK). The cells were treated and stained with specific mAbs as has been described in detail elsewhere (Randall and Dinwoodie, 1986). Briefly, monolayers were fixed with 5% formaldehyde, 2% sucrose in PBS for 10 min at 20°, permeabilized with 0.5% Nonidet-P40, 10% sucrose in PBS for 5 min at 20° and washed three times in PBS containing 1% calf serum. For detection of the P or V proteins, cells were stained by direct immunofluorescence using SV5-P-k that had been conjugated with fluorescein isothiocyanate; to detect the NP protein cells were stained with the mAb SV5-NP-a that had been conjugated with rhodamine. In addition, cells were also stained with the DNA binding fluorochrome, DAPI. Following staining for immunofluorescence the monolayers of cells were examined using a Nikon Microphot-FXA immunofluorescence microscope.

Direct visualisation of NP:P and NP:V interactions by immunofluorescence

Cells expressing the P or V proteins were grown on multispot microscope slides and, following induction of expression for 24 hr, were fixed and permeabilized as described for immunofluorescence (above). The slides were thoroughly washed with PBS to remove any traces of detergent. Various combinations of low-speed supernatant extracts of BF cells (that had been induced for expression of the NP, P, or V proteins) were mixed for 2 hr at +4°, then reacted with these fixed cells for 1–2 hr at 4°. The slides were washed four times with PBS and fixed with 5% formaldehyde, 2% sucrose for 5 min. After washing four more times with PBS, the presence of the NP and P or V proteins was detected by immunofluorescence.

RESULTS

Competitive binding of P and V to NP, as visualised by immunofluorescence

We previously demonstrated that NP bound to both P and V using immunofluorescence assays in which extracts of NP-expressing cells were incubated with fixed and permeabilised cells that expressed either P or V (Precious *et al.,* 1995). To ascertain whether P and V bound to the same or different sites on NP, this assay was used in competitive binding experiments. Low-speed supernatant extracts of NP-expressing cells were preabsorbed with varying amounts of low-speed supernatant extracts of cells expressing either the P or the V protein for 2 hr at +4°. These mixtures were then incubated with fixed and permeabilised cells expressing either P (Fig. 1a) or V (Fig. 1b) and the binding of NP to these cells was visualised by immunofluorescence. These results confirmed that cells expressing either the P or the V protein bound NP (column 1 of Figs. 1a and 1b, respectively). Preabsorption of NP with V prevented the subsequent binding of NP to V-expressing cells (Fig. 1b, column 2), but V did not appear to reduce the binding of NP to P-expressing cells (Fig. 1a, column 2). In contrast, preabsorption of NP with P prevented the binding of NP to both P- and V-expressing cells (column 3 of Figs. 1a and 1b, respectively). However, the amount of P extract required to block the binding of NP to P-expressing cells was significantly greater than the amount of P required to block the binding of NP to V-expressing cells (compare columns 3 and 4 of Figs. 1a and 1b). These results suggested: (i) since P inhibited the binding of NP to V-expressing cells, there may be a common NP binding site on P and V, (ii) as V failed to prevent the binding of NP to P-expressing cells, either there must be a second binding site on (and unique to) P for NP or P must have a higher affinity for NP than V, and (iii) since less P was required to inhibit the binding of NP to V-expressing cells than to P-expressing cells, either P must have a higher affinity for NP than V or V must only recognise a subpopulation of NP present in the low-speed supernatant extract from NP-expressing cells.

Development of a protein:protein capture assay for examining NP:P and NP:V interactions

To address these questions and to further investigate NP:P and NP:V interactions, we developed an assay for examining protein:protein interactions in 96-well microtitre plates. Two considerations had to be taken into account when developing this assay. First, in NP-expressing cells the bulk of NP was found assembled in polymers that were pelleted by high-speed centrifugation (below) and second, results using the immunofluorescence binding assay described above showed that NP:V interactions were sensitive to the presence of ionic and nonionic detergents (Precious *et al.,* 1995). Consequently, simple coimmune precipitation of mixed low-speed supernatant extracts of cells expressing the NP, P, or V proteins were not successful because of the high background levels of proteins pelleted upon centrifugation (data not shown). To overcome these problems, a solid-phase panning assay was developed in 96-well microtitre plates. It had been previously shown that fixed and killed suspensions of *S. aureus* Cowan strain A could simply and easily be bound to certain plastics (Randall, 1983). Using this procedure, confluent monolayers of *S. aureus* were formed in the wells of 96-well microtitre plates. The mAb SV5-P-k, which recognises an epitope common to P and V, was bound to these monolayers (Fig. 2, lanes 1 to 6). [Approximately 0.5 μg of antibody was bound per monolayer of *S. aureus* as opposed to $<$0.05 μg of antibody bound directly onto the plastic surface (data not shown).] The antibody was then used to capture either P or V from extracts of cells expressing

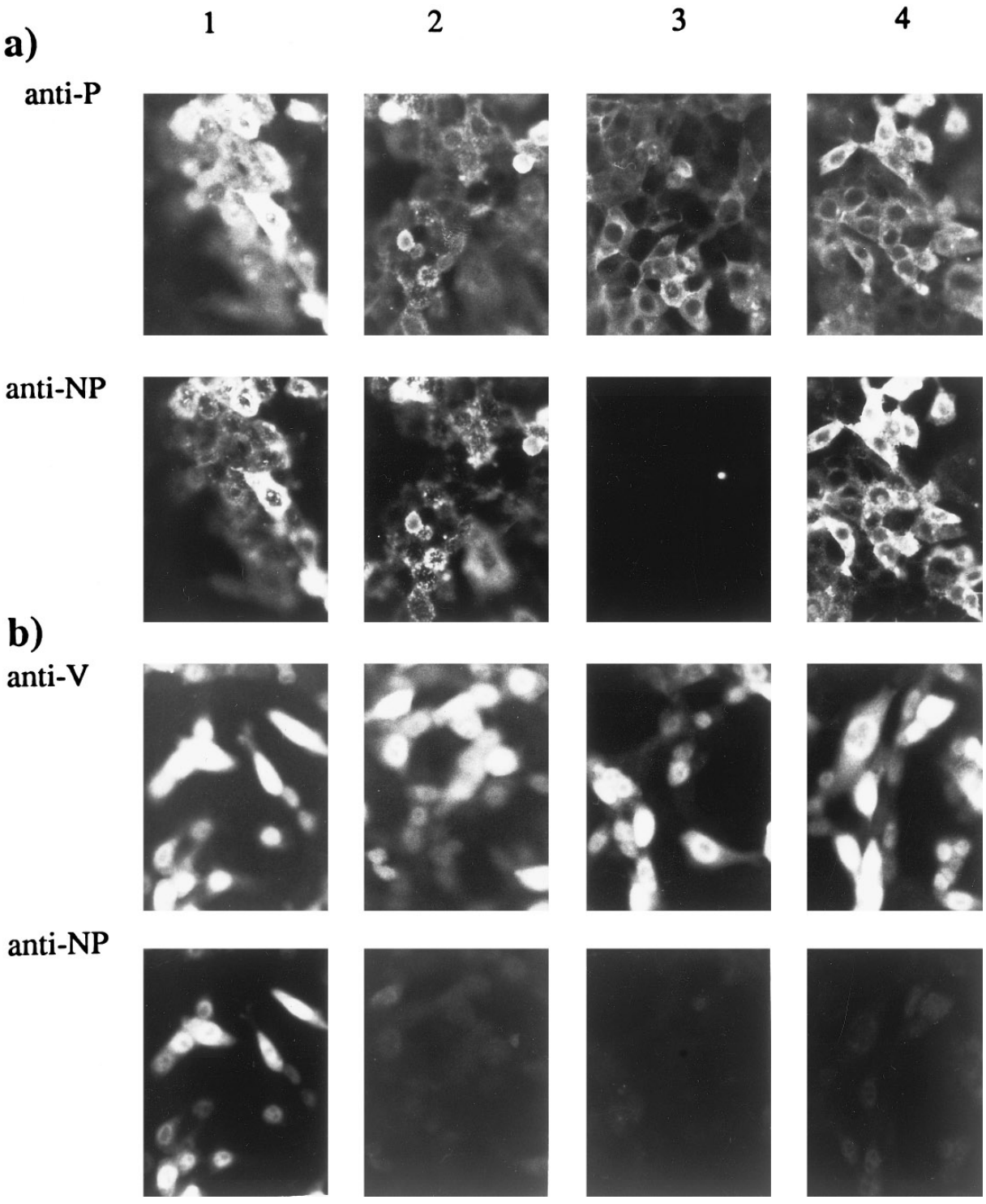

FIG. 1. Photographs illustrating the binding of NP to cells expressing P or V, both before and after preabsorption of NP with P or V. Low-speed supernatant extracts were made from cells expressing the P, V, or NP protein. In column 1, extracts from cells that expressed NP were incubated with fixed and permeabilised cells that expressed P (a) or V (b). In column 2, extracts from cells expressing NP and V were mixed at a ratio of 1:1 prior to incubation with the fixed and permeabilised cells. In columns 3 and 4 extracts from cells expressing NP and P were mixed at ratios of 1:1 and 4:1, respectively, prior to incubation with the fixed and permeabilised cells. Note that at a ratio of 4:1, preincubation of NP with P failed to prevent the binding of NP to P-expressing cells, whereas NP from the same mixture did not bind to V-expressing cells. Cells were stained with FITC-labelled SV5-P-k (anti-P and anti-V) and rhodamine-labelled SV5-NP-a (anti-NP).

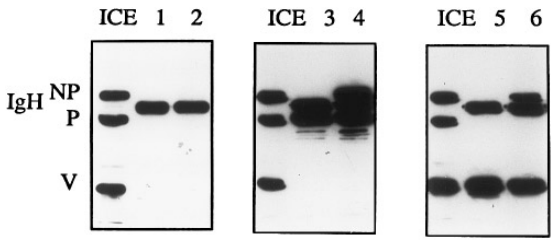

FIG. 2. Protein:protein capture assay demonstrating NP:P and NP:V interactions. The mAb SV5-P-k was bound to wells of a *S. aureus* microtitre plate (lanes 1 to 6). Low-speed supernatant extracts of cells expressing P, V, or NP were made. The P extract was added to wells 3 and 4, the V extract to wells 5 and 6, and PBS to wells 1 and 2. Following binding of P and V to the mAb SV5-P-k, the wells were washed thoroughly and the NP extract was added to wells 2, 4, and 6. After incubation at +4° for 2 hr, the wells were washed and polypeptides present were separated by electrophoresis through a 12% SDS–polyacrylamide slab gel and visualised by Western blot analysis. P and V were detected with the mAb SV5-P-k and NP with the mAb SV5-NP-d. Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig. The anti-mouse Ig also detected the heavy chain (IgH) of the original SV5-P-k mAb capture by *S. aureus* and was thus visualised in the Western blot. An infected cell extract (ICE) was also Western blotted to show the positions to which the NP, P, and V polypeptides migrated in the polyacrylamide gel.

these proteins (Fig. 2, lanes 3, 4, and 5, 6, respectively). Following removal of unbound material a low-speed supernatant extract prepared from cells expressing NP was added to the wells, and the proteins captured in the wells were analysed by SDS–polyacrylamide gel electrophoresis and Western blotting. These results demonstrated that NP bound specifically to those wells in which either P or V had been captured (Fig. 2, lanes 4 and 6, respectively) but not to control wells (Fig. 2, lane 2). A shorter exposure of the ECL Western blot also showed that the amount of NP captured by V was significantly less than the amount of NP captured by P (data not shown; see also Figs. 3 and 5).

This latter result suggested that V may only bind a subpopulation of the NP recognised by P. To ascertain whether this was the case, all the NP that could be bound by V was removed by absorption with V and the residual fraction was tested for its ability to bind to P. In these experiments, a low-speed supernatant extract of cells expressing NP was incubated with a well containing V. After 1-hr absorption, the supernatant was collected and reacted in a similar manner with another well containing V. The bound proteins from the first well were eluted for Western blot analysis (Fig. 3b, lane 10). This process was repeated a further five times (Fig. 3b, lanes 11–15). After the final absorption, the supernatant from well 15 was collected and reacted with a well containing the P protein. Clearly, V only bound a subpopulation of NP present in the extract as the majority of NP was not removed by absorption with V and this unabsorbed NP was bound by P (Fig. 3a, lane 8). In contrast, incubation of an identical low-speed supernatant extract of cells expressing NP sequentially with multiple wells containing P (Fig. 3a, lanes 2 to 7) resulted in the absorption of all the NP that could be bound by V (Fig. 3b, lane 16).

P and V bind soluble NP, but only P binds polymeric NP

In the protein:protein capture assays described above, sufficiently large amounts of NP were captured by P (in contrast to V) to be visualised by Coomassie brilliant blue staining of the captured proteins. Furthermore, the amount of NP bound was, under certain circumstances, in molar excess over the amount of P present in the assay. To confirm these latter results, doubling dilutions of a low-speed supernantant extract of P-expressing cells were captured by the mAb SV5-P-k. The same amount of a low-speed supernatant extract of NP-expressing cells was then added to each well and the relative amounts of NP captured by P were estimated by Coomassie brilliant blue analysis of the polypeptides

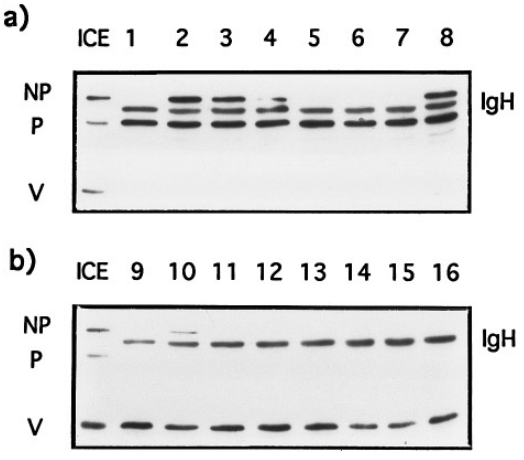

FIG. 3. Western blot analysis demonstrating that preabsorption of NP with P removed the fraction of NP that bound V but that preabsorption of NP with V did not remove all the NP that bound to P. The mAb SV5-P-k, attached to *S. aureus* microtitre plates, was used to capture P (a) or V (b) from low-speed supernatant extracts of cells expressing these proteins. A low-speed supernatant extract of NP-expressing cells was added, for 2 hr at 4°, to wells 2 and 10. The unbound material from these wells was collected and incubated with P and V present in wells 3 and 11, respectively. Similar absorptions of the NP protein continued until wells 7 and 15, respectively. Following this final absorption, the unbound material that had been incubated with P was added to a well containing V (well 16) and the unbound material that had been incubated with V was added to a well containing P (well 8). Polypeptides present in all the wells were separated by electrophoresis through a 12% SDS–polyacrylamide slab gel and subjected to Western blot analysis. The P and V proteins were detected with the mAb SV5-P-k and the NP protein with the mAb SV5-NP-d. Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig. An infected cell extract (ICE) was also Western blotted to show the positions to which the NP, P, and V polypeptides migrated in the polyacrylamide gel.

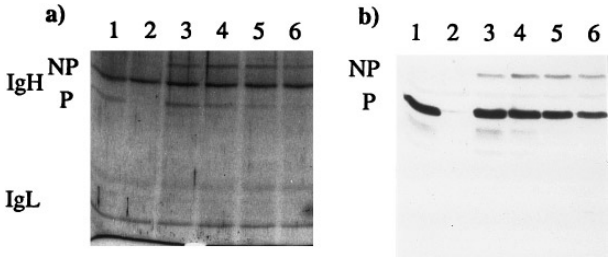

FIG. 4. Coomassie brilliant blue staining and Western blot analysis of a protein:protein capture assay demonstrating that a molar excess of NP could be bound by P. The mAb SV5-P-k was bound to wells of a *S. aureus* microtitre plate. Low-speed supernatant extracts of cells expressing P or NP were made. The P extract was added to wells 1 and 3, and doubling dilutions of P extract was added to wells 4 to 6. Following removal of unbound proteins by washing with PBS, the NP extract was added to all the wells except well 1 (to which PBS was added). Following incubation for 2 hr at 4°C, the plates were washed and the bound polypeptides separated by electrophoresis through a 12% SDS–polyacrylamide gel. (a) Coomassie brilliant blue-stained profile of these samples. Duplicate samples were analysed by Western blotting using the mAbs SV5-P-k and SV5-NP-d to detect P and NP, respectively (b). Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig. This reagent also detected the heavy (IgH) chain of the SV5-P-k antibody originally captured on the *S. aureus* plate. Note that in these Western blots, NP is not detected as efficiently as P (compare a and b).

after they had been separated by SDS–PAGE (Fig. 4a, lanes 3 to 6). At lower concentrations of P, it was clear that relatively more NP was bound than there was P present (Fig. 4a, lanes 5 and 6).

These observations suggested that P may bind polymeric NP. To address this question, polymeric NP was pelleted by high-speed centrifugation from low-speed supernatant extracts of cells expressing NP. Following centrifugation, the high-speed supernatant NP fraction was collected and the high-speed pellet, containing polymeric NP, was resuspended by sonication in the original volume of PBS. Western blot analysis of these fractions demonstrated that the majority of the NP present in the low-speed supernatant (Fig. 5a, lane 1) was polymeric and could be pelleted by centrifugation into the high-speed pellet (Fig. 5a, lane 3). However, a small but significant fraction of NP remained soluble and could be detected in the high-speed supernantant (Fig. 5a, lane 2). The ability of P and V to bind NP present in these fractions was examined in the protein capture assay described above. This analysis revealed that P bound NP present in all three fractions (Fig. 5c). As previously shown, V bound only small amounts of NP present in the low-speed supernantant (Fig. 5d, lane 1). Furthermore, although V bound soluble NP in the high-speed supernantant (Fig. 5d, lane 2), it failed to bind polymeric NP present in the high-speed pellet (Fig. 5d, lane 3). The small amount of NP bound by V in the low-speed supernatant thus presumably represents the soluble NP present in this fraction.

The binding site on P which recognises polymeric NP is located at its unique carboxy terminus

These observations suggested that there may be a unique site on P that interacts with polymeric NP. Since we had previously isolated a panel of mAbs that mapped to the unique carboxy terminus of P (Randall *et al.,* 1987), it was of interest to determine whether any of these mAbs could inhibit the binding of P to polymeric NP. These mAbs were used to capture P from low-speed supernatant extracts of P-expressing cells in a protein:protein capture assay. In the majority of cases, P captured with these mAbs retained the capacity to bind NP present in low-speed supernatants, high-speed supernatants, and high-speed pellets and showed a profile similar to that obtained with the mAb SV5-P-k (Fig. 6a, lanes 2 to 4, respectively). However, P that was captured by the mAb SV5-P-e bound relatively small amounts of NP present in the low-speed supernatant (Fig. 6b, lane 2) and, in a similar fashion to V, bound soluble NP (Fig. 6b, lane 3) but failed to bind polymeric NP (Fig. 6b, lane 4).

DISCUSSION

In this paper, we describe a novel assay for examining protein:protein interactions. The assay was extremely easy to perform on numerous samples and could be used to examine the interaction of large complexes of proteins (in this case polymeric NP) in cell extracts, prepared without the use of detergents, and from which only extremely large particulate material had been removed by low-speed centrifugation. The technique can also be adapted for performing simple immune precipitations (data not shown). Furthermore, recent results have shown that, if required, it is possible to simply and easily cross-link the antibody to the *S. aureus,* without loss of biological activity, with dimethylpimelimidate (as described in Harlow and Lane, 1988; A. Bermingham, unpublished observations).

Using this assay, we examined the nature of NP:P and NP:V interactions. These data demonstrated that there were at least two binding sites for NP on the P protein of SV5. One of these sites was common to an NP-binding site on the V protein. Studies with a number of other paramyxoviruses and rhabdoviruses, including VSV, rabies, Sendai, measles, respiratory syncytial virus, and pneumovirus of mice, have also shown that there are two binding sites on P for NP; one at the N-terminus, the other at the C-terminus (Chenik *et al.,* 1994; Takacs *et al.,* 1993; Fu *et al.,* 1994; Harty and Palese, 1995; Barr and Easton, 1995). These binding sites may have very different functions in that, at least for SV5, the unique C-terminal site on P bound polymeric NP, while the P/V-common N-terminal site bound soluble NP. The data also suggest that there may be two binding sites on NP for P; one of which binds to the P/V-common site, the other

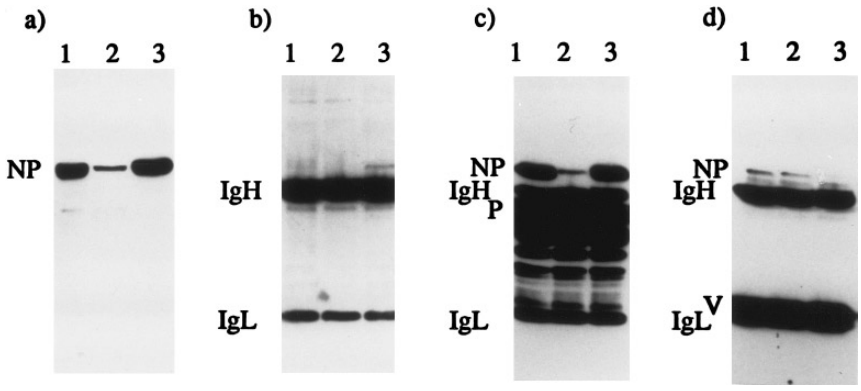

FIG. 5. Western blot analysis demonstrating the interaction of the P or V proteins with soluble and polymeric forms of NP. The mAb SV5-P-k was bound to all wells of a *S. aureus* microtitre plate (b, c, and d). Phosphate-buffered saline or P or V proteins were incubated with all wells of b, c, and d, respectively. Low-speed supernatant, high-speed supernatant, and high-speed pellet extracts from cells expressing the NP protein (a, wells 1, 2, and 3, respectively) were then added to wells 1, 2, and 3, respectively, in each of b, c, and d. Following removal of unbound material, the polypeptides present in the wells were separated by electrophoresis through a 12% SDS–polyacrylamide slab gel. The P and V proteins were detected with the mAb SV5-P-k and the NP protein with the mAb SV5-NP-d. Bound antibody was detected in an ECL reaction using HRP-conjugated sheep anti-mouse Ig. Note that the multiple bands visualised in c with molecular weights lower than P but higher than V were degradation products of P.

to the P-unique site. Furthermore, since V (or P captured with the mAb P-e) bound only soluble but not polymeric NP, the binding site on NP that recognises the P/V-common site must be either masked or conformationally altered in polymeric NP. Given that in polymeric NP it might be expected that there would be at least one exposed P/V-common binding site per polymeric particle, it seems more likely that this site would be conformationally altered, rather than masked. Changes in the conformation of NP in nucleocapsids of Sendai virus have also been invoked to explain the finding that monoclonal antibodies to the C-terminal portion of NP release P from nucleocapsids (Ryan *et al.,* 1993).

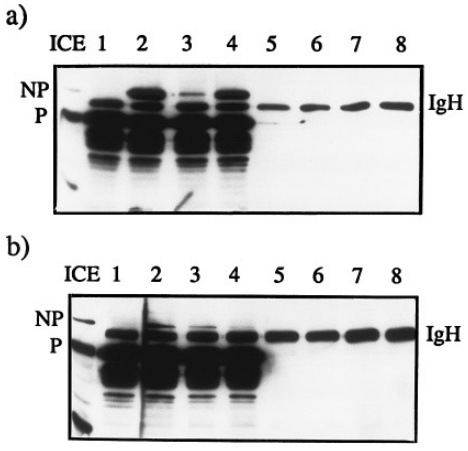

FIG. 6. Western blot analysis demonstrating the ability of the mAb SV5-P-e to prevent binding of P to the polymeric form of NP. The mAbs SV5-P-k (a) or SV5-P-e (b) were captured on a *S. aureus* plate. A low-speed supernatant extract of cells expressing the P protein or PBS was added to the plate (wells 1 to 4 and 5 to 8, respectively). Unbound material was removed by washing with PBS. Low-speed supernatant, high-speed supernatant, and high-speed pellet extracts of cells expressing NP were prepared. The low-speed supernatant was added to wells 2 and 6, the high-speed supernatant to wells 3 and 7, and the high-speed pellet to wells 4 and 8. PBS was added to wells 1 and 5. Polypeptides, including those from an SV5-infected cell extracted (ICE), were separated by electrophoresis through a 12% SDS–polyacrylamide slab gel. Using Western blot analysis, P was detected with the mAb SV5-P-k and the NP protein with the mAb SV5-NP-d. Note that the multiple bands visualised with molecular weights lower than P were degradation products of P.

The observation that V did not bind polymeric NP suggests that V might be associated with nucleocapsids (Paterson *et al.,* 1995) either through its interaction with virus proteins other than NP or via a direct interaction with vRNA. In human parainfluenza virus type 2, the site on V that binds NP and that, in the absence of virus replication, transports NP to the nucleus has been mapped to amino acids 1–46 (Watanabe *et al.,* 1996). By analogy with SV5, this site would represent the P/V-common domain which binds soluble NP.

The finding that coexpression of recombinant NP and P results in the formation of large cytoplasmic aggregates (Precious *et al.,* 1995; Chenik *et al.,* 1994; Garcia *et al.,* 1993; Garcia-Barreno *et al.,* 1996), similar to those observed in virus-infected cells (Fearns *et al.,* 1994), seems, at first sight, incompatible with the suggestion (from work on Sendai virus) that P acts as a chaperone for NP keeping it soluble prior to encapsidation (Curran *et al.,* 1995b). However, these observations are not necessarily irreconcilable. Thus, it is not known whether the large NP:P cytoplasmic aggregates are formed by P cross-linking polymeric NP, soluble NP, or a combination of both, or whether any given molecule of P (or homotrimer of P; Markwell and Fox, 1980; Curran *et al.,* 1995a) can bind both soluble and polymeric forms of NP at the same time. Indeed in Sendai virus, for functional NP:P

interactions, P must interact with newly synthesised, and thus presumably soluble, NP (Horikami *et al.,* 1992). Also in infected cells, different phosphorylation states of P and NP, and the ongoing process of virus replication, may influence their interactions and thus the formation of cytoplasmic aggregates. Indeed, since V interacts with soluble NP and may prevent NP from polymerising (Precious *et al.,* 1995), it might be expected that the binding of P to the P/V-common site on soluble NP might also inhibit the self-assembly of NP.

In Sendai virus, the N-terminus of P contains a region necessary for the encapsidation step during RNA replication (Curran *et al.,* 1994). Furthermore, the Sendai virus V protein, presumably by interfering with the RNA encapsidation step, was shown to inhibit genome replication in a dose-dependent fashion, but had little or no effect on mRNA synthesis (Curran *et al.,* 1991, 1994). Although we have demonstrated a clear interaction between V and soluble NP in the SV5 system, it is not clear what the functional significance of this interaction is and whether this differs from Sendai virus. On one hand, and as we have previously suggested (Precious *et al.,* 1995), V may help keep NP soluble prior to encapsidation. Alternatively, V may also compete with P for soluble NP, delaying encapsidation and thus being part of a controlling mechanism which switches from virus transcription to replication.

Thus, it is clear that, during virus replication, there must be certain rules that govern the interactions of NP with P and with V which still have to be elucidated, and that the exact nature of these rules may differ between paramyxoviruses. In addition, since the N-terminal P/V-common domain of V interacts with soluble NP, it may be that V is a multifunctional protein with a separate function being ascribed to its unique and highly conserved C-terminal cysteine-rich domain.

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