Sendai Virus Protein-Protein Interactions Studied by a Protein-Blotting Protein-Overlay Technique: Mapping of Domains on NP Protein Required for Binding to P Protein

H. E. HOMANN,* W. WILLENBRINK, C. J. BUCHHOLZ, AND W. J. NEUBERT
Abteilung für Virusforschung, Max-Planck-Institut für Biochemie, 8033 Martinsried, Federal Republic of Germany

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Proteins from Sendai virus particles and from infected cells were analyzed in a protein-blotting protein-overlay assay for their interaction with [35S]methionine-labeled viral proteins NP, P, and M. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transfer onto polyvinylidene difluoride membranes, and renaturation, the immobilized proteins were found to interact specifically with radiolabeled proteins. NP proteins from virus particles and from infected cells retained [35S]-P protein equally well. Conversely, P protein from virus particles and from infected cells retained [35S]-NP protein. [35S]-M protein was retained mainly by NP protein but also by several cellular proteins. To determine the domains on NP protein required for binding to immobilized P protein, a series of truncated and internally deleted [35S]-NP proteins was constructed. The only deletion that did not affect binding resides between residues 426 and 497. The carboxyl-terminal 27 residues (positions 498 to 524) contribute significantly to the binding affinity. Removal of 20 residues (positions 225 to 244) in the hydrophobic middle part of NP protein completely abolished its binding to P protein.

Viral transcription and replication take place in the cytoplasm of the infected cell. During transcription, monocistronic mRNAs are synthesized. During replication, full-size antigenome RNA is produced, which concurrently becomes encapsidated by NP protein. It is not completely understood how the switch between transcription and replication is achieved. This derives mainly from the lack of knowledge about how proteins of the polymerase complex interact with each other, with the nucleocapsid template, and possibly with tubulin and microtubule-associated proteins, which have been found to stimulate transcription in vitro (19, 30). Nucleocapsids isolated from infected cells were shown previously to bind in vitro-synthesized P protein (36), but it is still not exactly known which component of the nucleocapsid mediates this association. Furthermore, M protein also binds to nucleocapsids (27) and is regarded as a negative regulator for transcription. It is therefore of importance to learn which factors affect and modulate the interaction between the nucleocapsid (i.e., NP protein) and P protein as well as M protein.

We analyzed the interactions between these proteins by using renaturing protein blots. We applied cDNA-derived protein NP, P, or M, labeled with [35S]methionine during in vitro translation, to blots containing proteins from egg-grown Sendai virus particles and from infected cells separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All three in vitro-synthesized proteins were specifically retained by immobilized viral proteins. We combined this protein-binding assay and mutagenesis to map domains on NP protein responsible for its binding to P protein.

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MATERIALS AND METHODS

Virus, cell cultures, infection, and preparation of cellular extracts. Seed stocks of the Fushimi (DS2) strain of Sendai virus were grown in embryonated eggs, and virus purification, Vero cell culture, and cell infection (0.1 infectious unit per cell) were performed as previously described (20). Extracts were prepared 12 h after infection. Cells were detached from culture dishes by treatment with 20 mM EDTA in phosphate-buffered saline (PBS). They were pelleted for 5 min at 2,000 × g and resuspended in lysis buffer (50 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 1.5 mM MgCl2, 1% Nonidet P-40, 0.1% SDS [BDH], 1 μg of aprotinin per ml, 200 μg of phenylmethylsulfonyl fluoride per ml). Nuclei were removed by centrifugation at 12,000 × g for 2 min. The supernatant was stored at −20°C.

Synthesis of cDNA and cloning of the NP, P, and M genes. Sendai virus Fushimi strain genomic RNA, purified by the guanidinium-isothiocyanate lysis procedure (4), was transcribed into cDNA by using avian myeloblastosis virus
reverse transcriptase (Beard, Life Sciences) and synthetic oligonucleotides as gene-specific primers, as previously described (20). Primers used for first-strand cDNA synthesis were NP_{138-157}, P_{335-353}, and M_{4748-4730}. The cDNA was amplified by 30 cycles of polymerase chain reaction using oligonucleotides NP_{54-75}, NP_{1570-1729}, and P_{1720-3139} as second-strand primers. The polymerase chain reaction-amplified M gene cDNA was cut with C_{9}OII. Thus, the NP gene cDNA ranges from positions 54 to 1728, the P/C gene from positions 1705 to 3555, and the M gene cDNA ranges from positions 3666 to 4748. DNA was filled in with Klengow polymerase, phosphorylated, and ligated into Smal cut, dephosphorylated plasmids pBluescribe M13+ or pBluescriptII SK+ bearing promoters for T3 or T7 RNA polymerase on both sides of the polylinker. The DNA was transformed into XLI-Blue cells (Stratagene). All clones were sequenced (32, 33, 45). All numbering refers to the Sendai virus Z strain sequence (39, 40), irrespective of a nucleotide insertion detected at position 1598 in the NP gene (33).

Synthesis of NP gene deletion clones and in vitro transcription and translation. Constructs 1, 2, and 9 (see Fig. 3A) were generated from pBS-NP_{rev}, which carries the NP gene start codon next to the T7 promoter. Internal deletion constructs 3 to 8 were generated from clone pBS-NP, which carries the insert in the opposite orientation, by using the restriction enzymes depicted in Fig. 3. When necessary, we filled in the ends and subsequently cut at the single NdeI site in the vector. The resulting fragments were purified from agarose gels by electroelution. Construct 9 was generated from pBS-NP_{rev}. The identity of the every recombinant was verified by nucleotide sequencing to ensure that all deletions were in frame. Plasmid pBS-NP and NP gene deletion clones, linearized with PvuII, and plasmids pBS-P and pBS-M, linearized with HindIII, were transcribed with T3 RNA polymerase (Boehringer Mannheim Biochemicals). Truncated NP gene RNA was generated from pBS-NP_{rev}, and linearized with BalI or XbaI by transcription with T7 RNA polymerase in a 20-μl reaction (28). After removal of template DNA by treatment with DNase I, 3 μl of the reaction mix was translated in vitro by using 35 μl of rabbit reticulocyte lysate (Promega) and 50 μCi of [35S]methionine (1,000 Ci/mmol; Amersham) in a volume of 50 μl. The incorporation of [35S]methionine was determined by trichloroacetic acid precipitation. A 2.5-μl sample of the translation reaction was analyzed by SDS-PAGE. For detection of radiolabeled proteins, gels were acid fixed, dried, and placed on X-ray film.

Protein binding assay. Purified virus particles and extracts from infected or mock-infected cells were denatured with SDS (BDH) in the presence of β-mercaptoethanol at room temperature. Protein (1.2 mg) was applied to a 12-cm slot of a preparative SDS-polyacrylamide gel (24). After electrophoresis, the proteins were blotted to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore) (42). Transfer buffer was 25 mM Tris–192 mM glycine (pH 8.3). Blots were then incubated in standard binding buffer (SB) (10 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid] [pH 7.4], 10 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) for 12 h at 4°C. They were cut into 5-mm strips, which were then incubated for 3 h in SB-5% bovine serum albumin (BSA) to saturate free binding sites. For binding of [35S]methionine-labeled proteins, individual strips were incubated for 24 h at 4°C with 1.4 ml of SB-5% BSA containing 35,000 cpm of trichloroacetic acid-precipitable activity of in vitro translation products per ml. Subsequently, the strips were washed five to six times for 30 min each in SB-0.25% BSA, dried, treated with a fluorographic reagent (Amplify-Spray; Amersham), and exposed to Kodak X-Omat R films. When signals were to be quantitated by liquid scintillation counting, fluorography reagent was omitted.

RESULTS

In vitro-synthesized P protein is retained by immobilized Sendai virus NP protein. P protein was shown earlier to attach to purified nucleocapsids, and the P protein domains required for attachment were thoroughly mapped (36, 37). In an attempt to understand which component of the nucleocapsid mediates binding of P protein, we separated Sendai virus proteins by SDS-PAGE, blotted them on PVDF membranes, renatured the immobilized proteins, and then tested which of them would bind in vitro-synthesized P protein. However, P RNA also encodes the nonstructural proteins C′, C, Y, and X. The X protein, which is identical to the carboxyl-terminal 95 amino acids of P protein (5, 11), was previously shown to be unable to attach to nucleocapsids (37). Therefore, X protein should have no effect in subsequent binding assays. We chose to study proteins C′, C, and Y, which are encoded in a different reading frame, would also bind to viral proteins remains to be investigated.

The P/C gene cDNA was transcribed in vitro, and the RNA obtained was subsequently translated in a rabbit reticulocyte lysate in the presence of [35S]methionine. Separation of the translation products by SDS-PAGE revealed that in vitro-synthesized 35S-P protein comigrated exactly with P protein from in vivo-labeled virus particles (Fig. 1A, lane P). The products of low molecular weight represent the nonstructural proteins C′, C, Y, and X. In the unprogrammed control translation reaction no proteins were detected (lane —). Blots were incubated with the radiolabeled in vitro translation products in a buffer solution containing BSA as a nonspecific competitor (see Materials and Methods for details). The negative control contained unprogrammed translation reaction. Translation products of P RNA were retained by immobilized NP protein (Fig. 1B, lane P). The identity of NP protein was proven by immunostaining with a monoclonal anti-NP antibody (data not shown). To analyze which of the translation products of P RNA were retained by immobilized NP protein, we generated truncated RNA lacking the last 30 carboxyl-terminal codons from NdeI-cleaved Sendai virus P/C gene cDNA. The resulting 35S-P protein moved in SDS-polyacrylamide gels slightly faster than the 568-amino-acid full-size P protein. The bands representing the proteins C′, C, Y remained unchanged, and the X protein band disappeared (Fig. 1A, lane Pp). When we applied the truncated translation products to a blot containing immobilized Sendai virus protein, no radioactivity was retained (Fig. 1B, lane Pp). This result demonstrates that 35S-P protein is the only translation product of P/C gene RNA that binds to NP protein. Upon removal of the carboxyl-terminal 30 amino acids, the binding is abolished.

The carboxyl-terminal 30 amino acids of P protein were found to be required for binding of P protein to nucleocapsids (36). As the same region of P protein is required for binding to immobilized NP protein, this NP protein seems to behave identically to NP protein assembled within a nucleocapsid. Our results therefore show that the method applied is well suited to analyze interactions between proteins. The protein binding assay we performed not only confirms the earlier results of Ryan and Kingsbury (36) but beyond that shows that NP protein is the component of the nucleocapsid
to which P protein binds and that NP protein interacts directly with P protein.

Analysis of interactions of in vitro-synthesized proteins NP, P, and M with viral and cellular proteins. To test for the specificity of the viral protein-protein interaction and to reveal whether viral proteins would also bind to cellular proteins, we performed comparative binding assays. Proteins from egg-grown virus particles and from infected and mock-infected cells were separated by SDS-PAGE and blotted to PVDF membranes. The blotted proteins were then tested for their ability to retain 35S-P protein, 35S-NP protein, or 35S-M protein.

All radiolabeled in vitro translation products (Fig. 2A, lanes NP, P, and M) in SDS-polyacrylamide gels move identically to in vivo-labeled virus particle proteins (lane V). 35S-M protein, like virus-particle M protein, separates into two bands which have been described previously as representing different phosphorylated forms (25).

In the protein-binding assay with blots containing proteins from egg-grown virus particles, 35S-NP was predominantly retained by P protein, and conversely, 35S-P was predominantly retained by NP protein (Fig. 2B). The bands at the position of NP and P protein appear broader than those seen in the aniline blue stain (lane V) because the film was overexposed to show that a subset of additional light bands appears. Neither 35S-NP nor 35S-P protein is retained by mock-infected cell proteins (Fig. 2B, middle panel). Therefore, the additional bands probably represent degradation products of viral proteins or egg proteins that have been packaged into virions. 35S-M was predominantly retained by NP protein. This is consistent with results of cross-linking experiments performed by Markwell and Fox (27). With blots containing immobilized infected cell proteins, 35S-NP and 35S-P were also retained by P and NP proteins, respectively (Fig. 2B, lanes NP and P). The signal intensity with 35S-NP, relative to that of 35S-P protein, was weaker than on blots containing virus-particle proteins. This was due to the fact that Vero cells contain only small amounts of P protein, as revealed by immunostaining (data not shown). 35S-M protein binding was less specific than binding of 35S-NP and 35S-P proteins. Although 35S-M was predominantly retained by NP protein, it also bound to several nonviral proteins (Fig. 2, lanes M). However, the identity of these proteins and their effect on virus multiplication remain to be clarified.

Characterization of domains on NP protein responsible for binding to immobilized renatured Sendai virus P protein. Our assay system allowed mapping of functional domains on NP protein, which so far had not been possible. To identify the domains on NP protein that were required for binding to P protein, a series of nine truncated or internally deleted 35S-NP proteins was synthesized. The mutated regions cover about 80% of the coding region. Restriction enzyme sites used, positions of the deleted amino acids, and the deduced
molecular masses of translation products are depicted in Fig. 3A. The deletion junctions were sequenced to confirm that the deletions were in frame. The migration of truncated and internally deleted [35S]methionine-labeled in vitro translation products in SDS-polyacrylamide gels corresponded to the reduction in their sizes, with the exception of construct 8 which has a higher apparent molecular mass than that predicted from the sequence (Fig. 3B). Minor amounts of smaller radiolabeled products presumably represent peptides whose synthesis has started at internal AUG codons. This has also been observed upon cell-free synthesis of measles virus NP protein (16).

We tested these mutated [35S]-NP proteins for their ability to bind to P protein. The mutated proteins contain 7 to 28% (i.e., 1 to 4) fewer methionine residues than the full-size [35S]-NP protein. By using 7% less trichloroacetic acid-precipitable activity for every missing methionine, we applied equimolar amounts of the mutated [35S]-NP proteins to individual strips of one preparative blot containing Sendai virus particle proteins. The signals obtained in the binding assay (Fig. 3C) were quantified by cutting the respective bands from the blot and by subsequent liquid scintillation counting. The radioactivity retained by adjacent pieces of equal size was subtracted as background. The percentage of activity retained by immobilized P protein versus the total radioactivity employed was determined. The values obtained for mutated NP proteins were set in relation to that of full-size [35S]-NP protein, which was considered 100%. The results are depicted in Fig. 3A as relative binding affinity.

The NP gene of Sendai virus Fushimi strain contains 524 amino acids (34). Deletion of amino acids 426 to 497 (construct 3) did not affect binding of NP to immobilized P protein. Removal of carboxyl-terminal amino acids 456 to 524 (construct 1) or 421 to 524 (construct 2) reduced the binding affinity to about 50%. With constructs 4, 8, and 9, in which the deletions reside between amino acid positions 319 and 425, 82 and 187, and 80 and 134, respectively, the affinity was even lower. In repeated experiments, the least binding was observed with constructs 5, 6, and 7, lacking 67, 20, and 55 overlapping amino acids between positions 226 and 293, 225 and 244, and 189 and 243, respectively, in a hydrophobic domain of the NP protein. Our data thus indicate that large parts of NP protein seem to be involved in binding of NP to P protein. The hydrophilic carboxyl terminus (positions 498 to 524) plays an important role, as comparison of constructs 1 and 2 to construct 3 shows, but the hydrophobic middle part of NP protein proved to be more important. The small region between positions 225 and 244 is essential for binding.
DISCUSSION

This report demonstrates that Sendai virus proteins, separated by SDS-PAGE and blotted to immobilizing membranes, can be used to study their interactions with proteins synthesized in vitro from recombinant cDNA clones. Immobilizing electrophoretically separated proteins not only uses the high resolving power of SDS-PAGE but also allows renaturation of enzymatic activity through removal of the detergent (14, 23, 41, 44). Renaturation of proteins blotted to an immobilizing matrix (42) proved to be useful for studying their interactions with DNA and RNA (2, 3, 12, 21, 35, 38), with virus particles (6, 29), with growth factors (31), and with iodinated purified proteins (2, 26).

In combination with in vitro systems for the synthesis of proteins from cloned cDNA, these renaturing blots represent a powerful tool for studying protein-protein interactions. We applied in vitro-synthesized radiolabeled Sendai virus proteins NP, P, and M to renaturing blots and observed specific retention by immobilized viral proteins in the absence of RNA. The sensitivity of the method probably derives from the high specific activity of the protein probes that will be retained, even if only a small percentage of immobilized proteins on the membranes is renatured. The facts that 35S-NP is retained by immobilized P protein and, conversely, that 35S-P is retained by immobilized NP protein indicate that the interaction detected with our assay system is indeed specific. Neither 35S-NP nor 35S-P protein is retained by nonviral proteins. Additionally, the domain responsible for P protein binding to nucleocapsids (37) is identical to that required for binding to immobilized renatured NP protein. Our results therefore confirm that NP protein is the component of the nucleocapsid to which P protein binds.

We observed that NP proteins from virus particles and from infected cells retain 35S-P protein equally well. However, the signals obtained could very well be caused by only a small subpopulation of the immobilized proteins, since the molar amounts of radiolabeled proteins applied in the binding assay are less than 0.1% of those blotted to the PVDF matrix. NP and P are both phosphoproteins (25, 43) which are phosphorylated, at least in vitro, by L protein (9). Phosphorylation may define different NP or P protein populations between which we could not discriminate in our assay system. Further work is now in progress to analyze isoforms of NP and P proteins, separated by two-dimensional electrophoresis, for their binding affinities.

The assay system developed now allows definition of domains on NP protein required for interaction with other proteins. This will help elucidate the role(s) of NP protein, the most abundant viral protein, in virus multiplication. The deletion mutant analysis with NP protein revealed that very large parts of this protein are involved in the binding to P protein. From the seven internally deleted NP proteins tested, only the deletion of amino acids 426 to 497 did not affect the affinity to P protein. These results do not necessarily show that all of the residues from positions 80 to 426 are directly involved in complex formation, but they may rather reflect that the NP–P protein interaction is very sensitive to conformational changes in NP protein.

However, the effect of internal deletions does not correlate with the size of the region removed. With the exception of amino acids 80 and 81, the deletion of construct 9 is included in the deletion of construct 8. Although the deletion in construct 8 is nearly twice that in construct 9, its effect on the binding affinity is weaker than that observed with construct 9. Therefore, amino acids 80 and 81 may indeed be involved in binding to P protein. Additionally, the smallest deletion (construct 6) causes the most pronounced reduction in binding to P protein. The carboxyl-terminal 27 amino acids of NP protein also contribute significantly to its affinity to P protein, but binding is still observed when this region is removed. The carboxyl terminus does not represent an independent binding domain for P protein, since most internal deletions abolish binding to P protein even though the carboxyl terminus is present.

The resolution of our deletion analysis may be too low to state that amino acids 80 and 81, 225 to 244, and 498 to 524 indeed cooperate to form the binding site for P protein, but, even if these amino acids may not be in physical contact with P protein, our results show that these regions are important for binding. With NP protein assembled into a nucleocapsid, only its carboxyl terminus is exposed to the surface (17). But internal regions of NP protein are also likely to bind directly to P protein. Transcriptionally active nucleocapsids are resistant to RNAs, and additionally, P protein was shown by cross-linking experiments to contact the RNA (34). Therefore, it seems that NP proteins are not removed at the sites of polymerase action but that the polymerase complex stays in close contact to NP proteins. The hydrophobic middle half of paramyxovirus NP proteins, unlike the hydrophilic and highly charged carboxyl termini, is highly conserved (33). This also indicates that regions required for essential functions of NP protein reside in its middle half. This is further supported by the finding that a monoclonal antibody which recognizes a determinant between positions 290 and 295 most effectively inhibits in vitro transcription (7, 10).

More elaborate analysis of NP protein by linker-insertion and site-directed mutagenesis will provide further information about which residues are required for interaction with viral polymerase proteins.

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