Purification, Renaturation, and Reconstituted Protein Kinase Activity of the Sendai Virus Large (L) Protein: L Protein Phosphorylates the NP and P Proteins In Vitro

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Sodium dodecyl sulfate-solubilized Sendai virus large (L) protein was highly purified by a one-step procedure, using hydroxylapatite column chromatography. Monoclonal antibodies addressed to the carboxyl-terminal amino acid sequence of the L protein were used for monitoring L protein during purification. By removing sodium dodecyl sulfate from purified L protein, a protein kinase activity was successfully renatured. P and NP proteins served as its substrates. After immunoprecipitation with anti-L antibodies, the immuno-complex already showed protein kinase activity. In the presence of P protein, the NP protein was more highly phosphorylated. The results show that Sendai virus L protein possesses a protein kinase activity phosphorylating the other proteins of the viral nucleocapsid in vitro.

The transcriptive and replicative complexes of all nonsegmented negative-strand viruses contain catalytic amounts of a virus-encoded very large (L) polypeptide with a molecular weight greater than 200,000. In the case of Sendai virus, a paramyxovirus, the L protein resides together with the polymerase-associated protein P (molecular weight, 79,000) and the nucleocapsid structure unit protein NP (molecular weight, 60,000) on the viral genome, which is a single-stranded, nonsegmented, 15-kilobase-long RNA of negative polarity (17). The copies found per virion of the viral proteins L, P, and NP are 30, 300, and 2,600, respectively (21, 36). The resulting nucleocapsid is a highly ordered structure of characteristic helical appearance, rendering the genomic RNA highly resistant to digestion by RNase.

Little is known about the role of the L protein in the transcription and replication process of Sendai virus or most of the other nonsegmented negative-strand viruses. Synthesis of mRNA and genomic RNA in vitro by purified Sendai virus nucleocapsid has been described by Portner (27). Using extracts from infected cells, Carlsen et al. (3) studied the replication of Sendai viral RNA in an in vitro system. Studies of mutants and the results of in vitro reconstitution experiments implicate that the L proteins of vesicular stomatitis virus (VSV) and Newcastle disease virus are responsible for several multiple enzymatic functions exerted by the nucleocapsid complex, such as initiation, elongation, and termination of transcriptional and replicational products as well as methylation, capping, and polyadenylation of mRNAs (1, 10). Because of its large size and its presence in catalytic amounts in transcriptionally active nucleocapsids, the Sendai virus L protein is the candidate of choice for a multifunctional polypeptide. Recently, the L-gene sequence of different Sendai virus strains has been determined by different groups (24, 33; W. J. Neubert and H. E. Homann, unpublished data), demonstrating extensive homology of the whole gene, particularly with respect to the predicted carboxyl-terminal amino acid sequence of the L proteins of all three strains.

However, to elucidate the role of the Sendai virus L protein during viral transcription and replication, it is necessary to isolate it in a biologically active form to gain detailed information about its molecular functions, characteristics, and organization.

In this paper, we describe a simple one-step procedure for the purification of sodium dodecyl sulfate (SDS)-solubilized Sendai virus L protein by a hydroxylapatite column chromatography. The successful renaturation of the purified L protein can be demonstrated by reconstitution of a protein kinase function of the L protein following renaturation. For monitoring the L protein during the purification procedure and for assignment of a protein kinase activity, monoclonal antibodies (MAbs) were prepared, addressed to a synthetic peptide representing the predicted carboxyl terminus of the Sendai virus L protein.

MATERIALS AND METHODS

Virus. The Fushimi strain of Sendai virus was obtained from the American Type Culture Collection, Rockville, Md. Stock virus was grown in 11-day-old chicken embryos at 33°C and subsequently purified as described previously (26).

Synthetic peptide. The peptide corresponding to the 13 carboxyl-terminal residues of the Sendai virus L protein was synthesized by the Merrifield solid-phase method on a 430A Applied Biosystems peptide synthesizer, using the 9-fluorenylmethyloxycarbonyl procedure (23).

Conjugation of peptide to carrier protein. The synthetic peptide was linked to carrier proteins via its amino groups as described by Kagan and Glick (16). A 20-mg amount of bovine serum albumin (BSA; Sigma Chemical Co.) or keyhole limpet hemocyanin (Calbiochem-Behring) was mixed with 2 mg of peptide and dissolved in 2 ml of 0.1 M sodium phosphate, pH 7.5. Then 1 ml of glutaraldehyde (0.02 M) was added dropwise under constant stirring at room temperature.

Production of mouse hybridoma cell lines. For production of mouse hybridoma cell lines, the technique first described by Koehler and Milstein (18) was used. Three 6-week-old female BALB/c mice were immunized intraperitoneally at 2-week intervals with hemocyanin-conjugated peptide (25 μg of peptide conjugate per injection), using Freund complete

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adjuvant for the first injection and incomplete adjuvant for boostering. Sera were tested by enzyme-linked immunoassay and Western blotting (immunoblotting). The animals were then boosted five times at 24-h intervals with the same amount of antigen suspended in phosphate-buffered saline. Spleen cells were fused with myeloma cells (line Ag 8.653; kindly supplied by T. Hünig) by treatment with polyethylene glycol 1500 (Boehringer Mannheim). Supernatants of hypoxanthine-aminopterin-thymidine-selected colonies were screened for production of antipeptide antibodies by enzyme-linked immunosorbent assay , using BSA-conjugated synthetic peptide as antigen. Positive supernatants were tested for immunodetection of whole Sendai virus L protein by Western blot analysis. Colonies showing a positive reaction in the Western blot assay were cloned by limited dilution.

SDS-polyacrylamide gel electrophoresis (PAGE). All SDS-polyacrylamide gels were run as described by Laemmli (19), using 7.5% polyacrylamide resolving and 5% polyacrylamide stacking gels. Protein concentrations of analyzed samples were determined as described by Schaffner and Weissmann (31).

Western blotting. Viral proteins (20 μg per lane) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose sheets at 60 V and 0.17 A for 24 h at 4°C (38). The filters were soaked for 10 min in solution A (0.05 M Tris hydrochloride, 0.15 M NaCl, 0.25% gelatin, 3% BSA, 0.02% Nonidet P-40, pH 8.0) and then incubated for 1 h with the hybridoma supernatants (diluted 1:20 into solution A). Then the sheets were washed for 45 min in solution A containing 0.1% BSA and subsequently incubated for 1 h with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) (Dakopatts; diluted 1:500 into solution A). The filters were once washed in solution A and once in phosphate-buffered saline and then incubated with substrate (0.05% 3,3'-diaminobenzidine, 0.01% H_2O_2 in 0.1 M sodium phosphate, pH 7.2). The enzymatic reaction was stopped by washing the filter twice with 0.1 M sodium phosphate, pH 7.2.

Immunoprecipitation. For immunoprecipitation, MAbs were coupled to protein A-Sepharose as described by Schneider et al. (32). Immobilized antibodies were added to the kinase assay and incubated for 5 h at 4°C. The immune complexes coupled to the Sepharose beads were removed in an Eppendorf centrifuge.

Hydroxylapatite chromatography. Chromatography of SDS-solubilized viral proteins on hydroxylapatite was performed by using a modification of the procedure described by Moss and Rosenblum (25). Purified Sendai virus (60 mg) dissolved in 100 ml of 0.01 M sodium phosphate (pH 6.8)–3% SDS (BHD Chemicals Ltd., Poole, England)–0.01 M dithiothreitol (DTT; Boehringer) was incubated at 50 and 25°C each for 60 min. Unsolubilized material was pelleted overnight at 23,000 rpm and 25°C in a Beckman SW28 rotor; the protein sample was applied to a hydroxylapatite (Bio-Gel HTP; Bio-Rad Laboratories) column (0.9 by 20 cm). The column was washed with 2 column volumes of 0.01 M sodium phosphate, pH 6.8, containing 0.1% SDS–0.02% NaN_3–0.002 M DTT, and the proteins were eluted with a linear gradient of 0.01 to 0.5 M sodium phosphate (100 ml). Fractions, 1 ml, were collected and the linearity of the gradient was tested by measuring its conductivity. The protein concentration of each fraction was determined (31), and protein-containing fractions were dialyzed against phosphate-buffered saline.

Renaturation procedures. For renaturation, the following optimized assay was used, in principle described by Hager and Burgess (9). Four volumes of cold acetone (−20°C) were added to the SDS-protein solution, and the samples were allowed to precipitate for 30 min in a dry ice-ethanol bath. The tubes then were centrifuged for 10 min at 12,000 × g, the acetone supernatant was poured off, and finally the tubes were inverted to drain. The acetone precipitate was allowed to dry for about 10 min and completely dissolved in 20 μl of dilution buffer (8 M guanadine hydrochloride, 0.1 M Tris hydrochloride [pH 8.5], 0.04 M $MgCl_2$, 0.01 M DTT, 0.04% Nonidet P-40). For renaturation, the samples were dialyzed for 2 h at room temperature and for 12 to 16 h at 4°C against renaturation buffer (0.1 M Tris hydrochloride [pH 8.5], 0.04 M $MgCl_2$, 0.002 M DTT) and subsequently diluted with one volume of 0.02 M DTT.

Protein kinase assay. The protein kinase assay was performed following a method first described by Lamb (20). The renatured protein samples were incubated with 50 μCi of [γ-^{32}P]ATP for 2 h at 32°C. For PAGE analysis, the reaction products were precipitated with 10 μl of 60% trichloroacetic acid. After 30 min at 0°C, the precipitate was collected by centrifugation at 12,000 × g for 20 min. The precipitate was washed four times with ice-cold 80% acetone, dried in vacuo, and diluted in sample buffer. After separation of the proteins by SDS-PAGE, the radioactivity was determined by autoradiography.

RESULTS

Production of monoclonal anti-L-peptide antibodies. A prerequisite for the detection of L protein during the isolation procedure is the availability of highly specific antibodies. Since the L protein of Sendai virus is present only in very tiny amounts in virus particles and in infected cells, synthetic peptides of parts of the L protein were synthesized: recent nucleic acid sequencing data have shown that the predicted carboxyl-terminal amino acid sequence of the Sendai virus L protein is identical in three strains (24, 33; Neubert and Homann, unpublished data). Moreover, the predicted carboxyl-terminal 13 amino acids of the L protein exhibit a high probability for a beta-turn folding of the native polypeptide chain according to Chou and Fasman (5) as well as Garnier et al. (8) and a high antigenicity according to Jameson and Wolf (15).

Based on these considerations, we synthesized a peptide corresponding to the 13 carboxyl-terminal residues of the Sendai virus L protein: Asp Gly Ser Leu Gly Asp Ile Glu Pro Tyr Asp Ser Ser (AS 2216–2228).

The synthetic peptide was conjugated to keyhole limpet hemocyanin with glutaraldehyde, as described by Kagan and Glick (16), and used for immunization of BALB/c mice. Eight hybridoma clones secreting antipeptide antibodies were identified by an indirect enzyme-linked immunosorbent assay, using BSA-conjugated synthetic peptide as antigen. Seven hybridoma cell lines produced IgG antibodies; one clone produced IgM antibodies (data not shown). For further studies, supernatant from the IgG-producing hybridoma clone VII E 7 (MAb VII-E-7) was used.

Recognition of denatured and native L proteins by MAbs. As can be shown by Western blot analysis, all seven IgG class hybridoma clones secrete antibodies which specifically react with the synthetic peptide used for immunization and with SDS-denatured viral L protein (Fig. 1, lanes 1 to 7). The MAbs react exclusively with a viral polypeptide having a molecular weight of >200,000 as estimated from its migration rate relative to the major chain of myosin in the
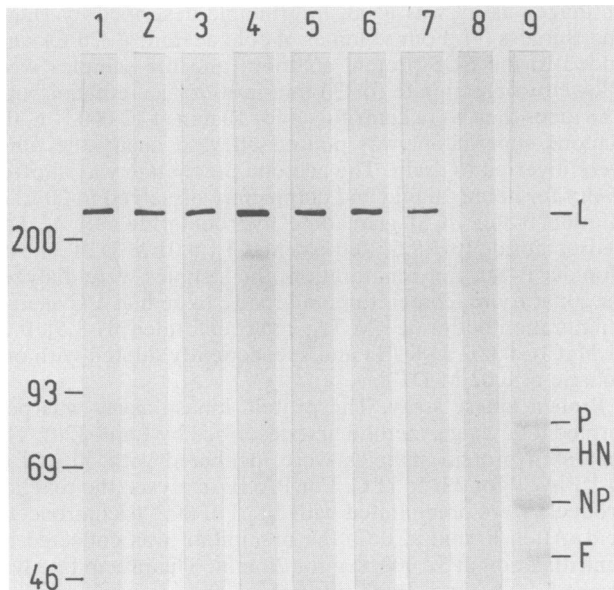
FIG. 1. Characterization of anti-L MAbs by Western blot analysis. Total proteins from purified egg-grown Sendai 6/94 virions (20 µg) were electrophoresed in 7.5% Laemmli gels, electrophoretically transferred to nitrocellulose, and reacted with the following hybridoma supernatants: II-E-10, III-C-7, IV-A-2, VI-B-5, VI-C-8, VII-E-7, VII-E-9 (lanes 1 to 7). Lane 8 shows a strip incubated with a nonproducing clone; lane 9 shows total viral proteins stained with Ponceau S. Numbers on the left-hand side represent molecular weights (10³) of marker proteins.

SDS-polyacrylamide gel. This value is in accordance with the size predicted for the Sendai virus L protein from the published nucleic acid sequences (24, 33). Calculations according to Emini et al. (7) predict a high probability for the presence of the carboxyl-terminal amino acids on the surface of the native L-polypeptide chain. By immunoelectron microscopy, using MAb VII-E-7 and anti-mouse immunoglob-

ulins conjugated to 15-nm gold particles as a second antibody, we showed that native L protein on viral nucleocapsids is detected by MAb VII-E-7 on cryosections of infected cells (13) as well as from virus particles (unpublished data). Therefore, the anti-L MAbs provided an excellent tool for the identification of Sendai virus L protein in fractions of separated viral proteins.

Isolation of the Sendai virus L protein. For the isolation of Sendai virus L protein, we tried to use a protocol used by Emerson and Yu (6) for the L protein of VSV. They isolated this protein in a highly purified form by disruption of virions with high-salt buffers containing Triton X-100 and chromatography of solubilized viral proteins on phosphocellulose. However, the solubilization of the nucleocapsid-associated proteins of egg-grown Sendai virus was incomplete with nonionic and dipolar ionic detergents, high-salt buffers, and solutions of NaSCN or urea, when checked by gel filtration (data not shown). We therefore tested several commonly used detergents and chaotropic reagents under different experimental conditions: taken together, the Sendai virus proteins NP, P, and L could only be dissociated completely to the monomeric form by high concentrations of guanidinium hydrochloride or SDS. In subsequent experiments, virions were disrupted by 3% SDS.

Attempts to elute SDS-solubilized L protein from preparative SDS-polyacrylamide gels by the methods of Hunkapiller et al. (14) and Stearne et al. (34) resulted in loss of >95% of the L protein. Gel filtration likewise is not sufficient for a final purification of Sendai virus L protein, since paramyxoviruses contain considerable amounts of cellular proteins of high molecular weight.

Finally, chromatography of SDS-solubilized viral proteins on hydroxylapatite was performed following a procedure described by Moss and Rosenblum (25). Sendai virus proteins were applied to a hydroxylapatite column and separated by a linear gradient of 0.01 to 0.5 M sodium phosphate, and the fractions obtained were analyzed by SDS-PAGE.

A complete separation of a 250-kilodalton protein from all other viral and cellular proteins was observed (Fig. 2A, lanes
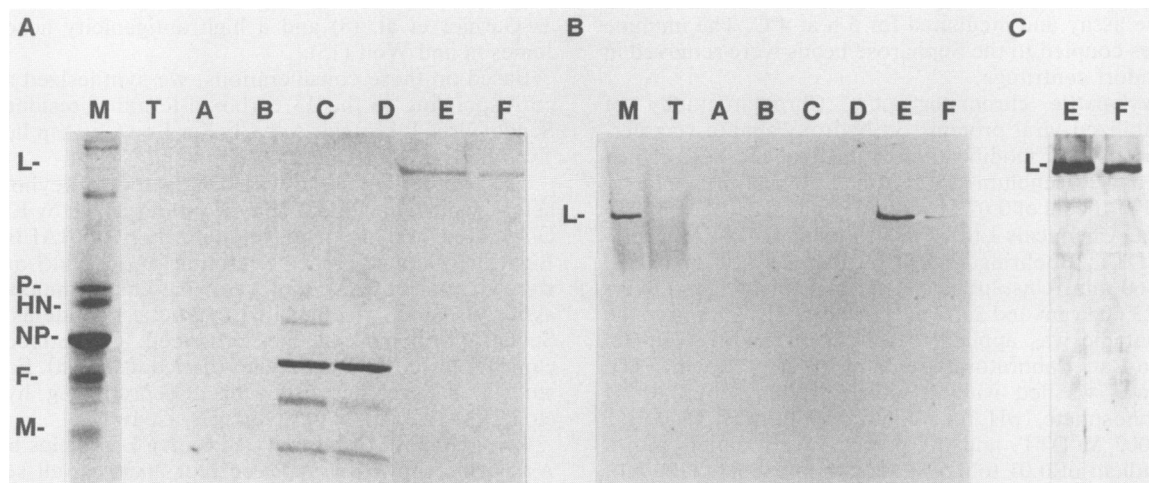

FIG. 2. Purification of the Sendai virus L protein by hydroxylapatite column chromatography. SDS-solubilized viral proteins were separated by a hydroxylapatite column, and the resulting fractions were tested for eluted proteins. (A) Detection of proteins in different fractions of the column by SDS-PAGE separation and subsequent staining with Coomassie brilliant blue. (B) Western blot analysis of the separated proteins with MAb VII-E-7. (C) Silver staining of all proteins in fractions containing Sendai virus L protein. Lane M, Proteins of egg-grown Sendai virus as a marker; lane T, flowthrough fraction; lanes A to F, selected fractions of the hydroxylapatite column with conducitivities of 24, 25, 27, 29, 31, and 32 mS/m, respectively.

E and F). This protein eluted in fractions with a conductivity of >30 mS/m. Viral proteins P, HN, NP, F, and M, as identified by size, were found in fractions with a conductivity of 26 to 30 mS/m (Fig. 2A, lanes C and D show two selected fractions).

The 250-kilodalton protein isolated by this procedure was identified as Sendai virus L protein by Western blot analysis, using MAb VII-E-7 (Fig. 2B, lanes E and F).

Purity of the isolated L protein is demonstrated by over-loading SDS-PAGE and subsequent silver staining (11). With this sensitive technique, no bands of contaminating proteins could be identified in the fraction of purified L protein (Fig. 2C, lanes E and F).

Renatured protein kinase activity within capsid proteins NP, P, and L. A protein kinase activity in Sendai virions had been reported (29). Lamb (20) extended this observation, suggesting that the enzyme is associated within the matrix protein, but not with the nucleocapsid complex. Recently, Sanchez et al. (30) reported on in vitro phosphorylation of the NS protein by the L protein of VSV. Therefore, we wondered whether a protein kinase activity is detectable when the three purified nucleocapsid proteins are renatured together.

Sendai virus L protein, purified by hydroxylapatite column chromatography, and NP and P proteins, isolated by preparative SDS-PAGE, were mixed. SDS was removed by acetone precipitation, and the proteins were dissolved in guanidine hydrochloride, renatured together, and tested for kinase activity. Indeed, a protein kinase activity was demonstrated when ^{32}P became incorporated into proteins at the positions of the NP and P proteins in SDS-PAGE.

Different degrees of renaturation of the purified viral proteins were obtained (Fig. 3, lanes 1 to 4) when modifications of a protocol described by Hager and Burgess (9) were used. The best results were observed when the excess reagents were removed by dialysis in the presence of DTT (Fig. 3, lane 4). A standard renaturation assay, tested in parallel under conditions as described by Stewart et al. (35), showed no protein kinase activity (Fig. 3, lanes 5 and 6).

Thus, a protein kinase activity within the Sendai virus capsid proteins NP, P, and L is detectable, simultaneously indicating the successful renaturation of at least one enzymatic function.

Protein kinase activity of the renatured Sendai virus L protein. To assign the protein kinase activity to one of the viral capsid proteins, we used several combinations of purified viral proteins in the kinase assay.

In the first set of experiments, identical amounts of viral proteins L and P were renatured together and tested for protein kinase activity. Two bands are visible in the autoradiography: the major one at the position of the P protein, and a second band at the position of the NP protein (Fig. 4A, lane 6); an autophosphorylation of the L protein was not detected. By removing the L protein from the assay by immunoprecipitation with MAb VII-E-7 coupled to protein A-Sepharose, the phosphorylation of P protein was nearly completely eliminated (Fig. 4A, lane 5). As a control, addition of uncoupled protein A-Sepharose to the assay had no effect (Fig. 4A, lane 3). Uncoupled MAb VII-E-7 added to the assay had no influence on the kinase activity, indicating that the MAb binds to a domain of the L protein which is not involved in the phosphorylating activity (Fig. 4A, lane 4).

The band at the position of the NP protein apparently results from contaminating NP protein in the L-protein fraction (Fig. 5, lane 1). It was not detectable by silver staining (Fig. 2B, lanes E and F) and could be removed by
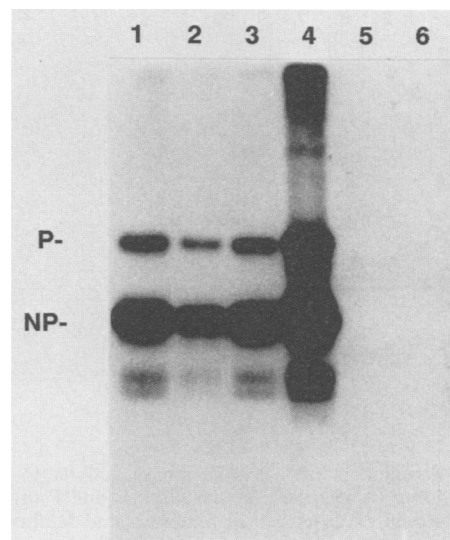

FIG. 3. Renaturation of the purified Sendai virus proteins. Sendai virus L protein, purified by hydroxylapatite column chromatography, and NP and P proteins, isolated by preparative SDS-PAGE, were mixed and tested in different assays for renaturation of a protein kinase activity. Lanes 1 to 3, Renaturation assay as described by Hager and Burgess (9) (lane 2): addition of 0.005 M glutathione and 0.0005 M glutathione disulfide (lane 1), and 0.002 M DTT (lane 3) during the denaturation. Lane 4, Optimized renaturation assay as described in Materials and Methods. Lanes 5 and 6, Renaturation assays as described by Stewart et al. (35): removal of excess reagents by dialysis (lane 5) and dilution (lane 6). After renaturation, the assays were diluted 1:1 with 0.02 M DTT and tested for protein kinase activity as described in Materials and Methods. Positions of the viral proteins NP and P are indicated.

immunoprecipitation with immobilized monoclonal anti-NP antibodies, kindly supplied by W. Willenbrink from our laboratory (Fig. 4A, lane 2).

To show clearly that a kinase activity is assigned to L protein, it was immunoprecipitated by MAb VII-E-7 and the antibody-protein complex was tested in a subsequent kinase assay after adding P protein as a substrate. About 50% of the kinase activity was lost after immunoprecipitation of the L protein (Fig. 4B, lane 3), but the immunocomplex remained stable even after additional washing steps (Fig. 4B, lane 4). When an unspecific MAb was used for immunoprecipitation, no kinase activity was detected (Fig. 4B, lane 2). From these data, we conclude that Sendai virus L protein contains a protein kinase activity which phosphorylates the P protein.

Subsequent analysis indicated that phosphorylation by the renatured L protein occurred at the threonine residues of the NP and P proteins. A second kinase activity of the renatured L protein was not detected. Exogenous phosphate acceptors such as phosvitin and lipovitellin-related proteins, but not BSA, also served as substrates for the kinase. The intensity of the phosphorylation signal correlated with the content of threonine residues in the proteins (data not shown).

In a second set of experiments, viral proteins L, NP, P, and M were mixed in different combinations, renatured together, and tested in the kinase assay (Fig. 5). L protein alone showed no autophosphorylation, but a weak band at the position of NP protein could be detected (Fig. 5, lane 1). When NP protein was added, the signal at the position of NP protein only became two to four times stronger (Fig. 5, lane 3), although the amount of NP protein increased from subnanogram contamination of 4.0 μg. Addition of P protein
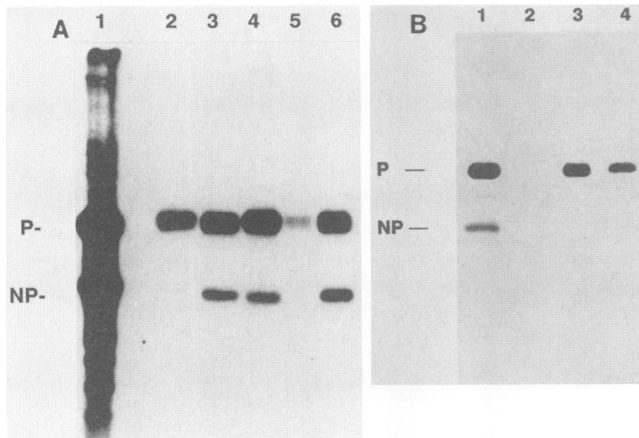
FIG. 4. Assignment of a protein kinase activity to the Sendai virus L protein. (A) Purified Sendai virus L and P proteins were mixed, renatured as described in Materials and Methods, divided into aliquots containing 0.4 μg of L protein and 4.0 μg of P protein, and tested for protein kinase activity. Lane 1, Protein kinase assay of detergent-disrupted Sendai virus as positive control (20); lane 2, removal of traces of NP protein before the kinase reaction by immunoprecipitation with protein A-Sepharose-coupled anti-NP MAbs; lane 3, addition of protein A-Sepharose as a control; lane 4, addition of MAb VII-E-7 to the kinase reaction; lane 5, removal of L protein before the kinase reaction by immunoprecipitation with protein A-Sepharose-coupled MAb VII-E-7; lane 6, optimized in vitro protein kinase assay as a positive control. (B) Renatured Sendai virus L protein (0.4 μg) was further purified by immunoprecipitation with protein A-Sepharose-coupled MAb VII-E-7, and the antibody-protein complex was tested for protein kinase activity, using P protein (4.0 μg) as substrate. Lane 1, L protein without immunoprecipitation; lane 2, immunoprecipitation with the unspecific anti-NP MAb; lane 3, immunoprecipitated L protein; lane 4, immunoprecipitated L protein immunocomplex washed five times more.

FIG. 5. Purified Sendai virus proteins L (0.4 μg) and NP, P, and M (each 4.0 μg) were mixed in different combinations, renatured together, and tested for protein kinase activity. Lane 1, L protein; lane 2, L and P protein; lane 3, L and NP protein; lane 4, L and M protein; lane 5, P protein; lane 6, P and NP protein; lane 7, P and M protein; lane 8, NP protein; lane 9, kinase assay of detergent-disrupted Sendai virus as a positive control (20). Positions of the viral proteins NP and P are indicated.

led to its strong phosphorylation (Fig. 5, lane 2). Also, in this experiment the signal of the contaminating NP protein became much more intense, although only traces of NP protein were present in the assay. The phosphorylation detected thus resulted from a protein kinase activity and not from an exchange of ^{32}P with unlabeled amino acid phosphates. When M protein was added (Fig. 5, lane 4), only the contaminating NP protein was phosphorylated, but weaker than in lane 1. Neither P (Fig. 5, lane 5) or NP (Fig. 5, lane 8) protein alone nor combinations of P and NP protein (Fig. 5, lane 6) and P and M protein (Fig. 5, lane 7) showed any phosphorylating activity in the absence of L protein. Summarizing, we can assign a protein kinase activity to the Sendai virus L protein, which phosphorylates the P and NP proteins.

DISCUSSION

The L protein of paramyxoviruses, an important component of the viral replication complex, so far could not be isolated in a functional active state. Therefore, only little information about the role of this protein during transcription and replication of the viral RNA is available. To gain insight into its functions, we established a highly effective one-step procedure for the isolation of Sendai virus L protein. This large protein was purified in a denatured form, but could be renatured to render a protein kinase activity phosphorylating the viral P and NP proteins.
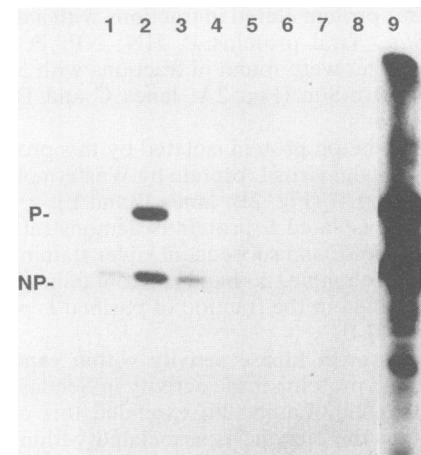
For monitoring the L protein during the purification steps, specific antibodies were essential. Due to its occurrence in very small amounts in virions and in infected cells (36), we used synthetic peptides as antigens. In rabbits, we obtained high antibody titers against peptides representing amino acids 159 to 171 and 1620 to 1631 of the L polypeptide. Although these monospecific antisera recognized their respective peptides, they failed to recognize viral L protein (data not shown). It is likely that the corresponding epitopes are inaccessible on the full-length polypeptide chain due to its three-dimensional folding.

After immunizing BALB/c mice with a synthetic peptide corresponding to the carboxyl-terminal amino acid sequence of the L protein, we obtained MAbs which react to the viral L protein in the native, nucleocapsid-associated state as well as in the SDS-denatured state. The results are in accordance with recent data reported by Portner et al. (28). They obtained a monospecific antiserum only against one of four synthetic peptides. This one also represents a part of the carboxyl terminus.

Our attempts to purify the Sendai virus L protein under native conditions failed because of the high affinity of L protein for other viral proteins. Dissociation of proteins NP, P, and L to monomeric form was obtained only by use of guanidinium hydrochloride or SDS. Under many methods tested, only chromatography of SDS-solubilized viral proteins on hydroxylapatite was successful. This technique had been used by Moss and Rosenblum (25) to separate vaccinia virus structural proteins with high resolution. The resolution of Sendai virus proteins was excellent, and a protein with a molecular weight of >200,000 eluted separately from all other proteins. This protein was identified as Sendai virus L protein by Western blot analysis, using our MAb VII-E-7.

Since purification of the L protein was possible only under denaturing conditions, a subsequent renaturation was essential. Recently, Szewczyk et al. (37) renatured the three subunits of influenza A virus RNA polymerase by using bacterial thioredoxin. However, Sendai virus L protein has a molecular weight of >200,000 and so we tried modifications

of a protocol described by Hager and Burgess (9), who renatured enzymes of >100,000 molecular weight. We chose a protein kinase activity assay to test the efficiency of our renaturation conditions. The presence of a protein kinase activity in Sendai virus particles has been known for some time (29), and this activity was ascribed to the M protein and not to the nucleocapsid complex (20). However, recently, Sanchez et al. (30) found for VSV a protein kinase activity associated with purified L protein. To reconstitute a Sendai virus protein kinase activity, we mixed purified L protein with NP and P and then renatured the proteins. Since we are able to restore a Sendai virus protein kinase activity within these three proteins, this activity has to be assigned to one of the proteins of the viral nucleocapsid.

None of the proteins NP, P, and L alone showed autophosphorylation, while P protein was highly phosphorylated when added to the L-protein fraction. Immunoprecipitation with MAb VII-E-7, preceding the kinase activity assay, almost completely abolished phophorylation of P protein. On the other hand, after immunoprecipitation of L protein, a kinase activity remained restored as tested in a subsequent assay. These results provide strong evidence that Sendai virus L protein contains a protein kinase activity, whereas P and NP proteins are its substrates. By using the renatured L protein, the capsid proteins NP and P are phosphorylated on threonine residues. These findings are in agreement with Lamb (20), who described earlier a threonine kinase within disrupted Sendai virions.

Regarding VSV, there is certainly some confusion as to whether the L-protein-associated kinase activity is of viral or cellular origin (22, 30). For the Sendai virus, however, we can assign a protein kinase activity to the L protein and we can also exclude that the activity measured here is of cellular origin.

In Sendai virions P protein represents only about 10% of the viral protein mass, but contains about 40% of the virion-bound phosphate (12). The analogous protein species of VSV, NS, also is the most highly phosphorylated viral protein. Its specific phosphorylation by L protein at serine residues 236 and 242 is essential for its binding to the other components of the nucleocapsid and seems to play a key role in initiation of RNA synthesis (4, 30). Phosphorylation of Sendai virus P protein by the L-protein-associated kinase may have the same effect.

The affinity of the capsid proteins to each other is very high. With VSV, small amounts of N and NS protein were copurified with L protein. They were removable only after repeated chromatography (30). We found trace amounts of NP protein in our L-protein fractions, detectable only in the protein kinase assay. On the other hand, when 4 μg of NP protein eluted from SDS-PAGE was added to the L-protein fraction, the phosphorylation increased only slightly. Thus, the extent of phosphorylation of the NP protein does not correlate with the amount of NP protein in the assay.

There might be two populations of NP protein, a small one strongly associated with L protein and a larger one dissociated from the L protein. The L-associated NP protein fraction, even if copurified with L protein under strongly denaturing conditions, may be phosphorylated to a high extent. The second, "dissociated" fraction may be a poor substrate for phosphorylation by L protein. In any case, a different degree of renaturation of the two postulated NP protein populations cannot be responsible for the differences in phosphorylation since renaturation was always performed under identical conditions.

The degree of phosphorylation of the NP contamination

became much higher when P protein was added. Eventually, complexes of the proteins NP, P, and L may be formed, leading to a higher phosphorylation of NP protein. From our data we cannot draw any conclusion with regard to a possible existence and/or activity of these complexes. Portner et al. (28) reported that, for Sendai virus, L and P molecules are distributed in clusters along the viral nucleocapsids. They presumed that the clustering permits cooperative interactions within the proteins possibly essential for the function of these proteins in RNA synthesis. Extending this model, we can interpret our findings that a cooperation between L and P protein leads to a higher phosphorylation of NP protein. This higher degree of phosphorylation may be important for the movement of the transcriptive complex along the nucleocapsid. Alternatively, the higher phosphorylation of NP protein in the presence of P protein could result from (i) a phosphatase activity within the P protein or (ii) a cascade mechanism, in which L phosphorylates P and P^P phosphorylates NP to a higher extent. These explanations are less probable, since Lamb (20) could not detect a phosphoprotein phosphatase activity in disrupted virions. Furthermore, the cascade model seems to be unlikely since the P protein is purified from virions, i.e., phosphorylated in vivo, but does not show any protein kinase activity in the absence of the L fraction.

Sendai virus M protein is phosphorylated in vivo, but in our assay it does not work as a substrate for the L-associated protein kinase. This may depend on insufficient renaturation conditions for this protein. On the other hand, with VSV a second virion-associated protein kinase activity, specific for M protein, has been suggested (2). Thus, except for the possibility that Sendai virus M protein is phosphorylated exclusively by cellular protein kinases, there may also exist a second viral protein kinase, specific for M protein.

The purification of Sendai virus L protein described in this paper has led to the identification of an associated protein kinase activity, although the biological role of the protein kinase still remains unknown. It might now be possible to investigate other functions of the L protein in vitro and to elucidate regulatory events during the replicative process of the virus.

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