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Serine Protein Kinase Activity Associated with Rotavirus Phosphoprotein NSP5

J. BLACKHALL,[1,2] A. FUENTES,[1] K. HANSEN,[3]† AND G. MAGNUSSON[1]*

Department of Medical Immunology and Microbiology, Uppsala University, S-751 23 Uppsala,[1] and Ludwig Institute for Cancer Research, S-751 24 Uppsala,[3] Sweden, and Centro de Virología Animal, CONICET, Serrano 669, 1414 Capital Federal, Argentina[2]

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The rotavirus nonstructural protein NSP5, a product of the smallest genomic RNA segment, is a phosphoprotein containing O-linked *N*-acetylglucosamine. We investigated the phosphorylation of NSP5 in monkey MA104 cells infected with simian rotavirus SA11. Immunoprecipitated NSP5 was analyzed with respect to phosphorylation and protein kinase activity. After metabolic labeling of NSP5 with $^{32}P_i$, only serine residues were phosphorylated. Separation of tryptic peptides revealed four to six strongly labeled products and several weakly labeled products. Phosphorylation at multiple sites was also shown by two-dimensional polyacrylamide gel electrophoresis (PAGE), where several isoforms of NSP5 with different pIs were identified. Analysis by PAGE of protein reacting with an NSP5-specific antiserum showed major forms at 26 to 28 and 35 kDa. Moreover, there were polypeptides migrating between 28 and 35 kDa. Treatment of the immunoprecipitated material with protein phosphatase 2A shifted the mobilities of the 28- to 35-kDa polypeptides to the 26-kDa position, suggesting that the slower electrophoretic mobility was caused by phosphorylation. Radioactive labeling showed that the 26-kDa form contained additional phosphate groups that were not removed by protein phosphatase 2A. The immunoprecipitated NSP5 possessed protein kinase activity. Incubation with [γ-^{32}P]ATP resulted in ^{32}P labeling of 28- to 35-kDa NSP5. The distribution of ^{32}P radioactivity between the components of the complex was similar to the phosphorylation in vivo. Assays of the protein kinase activity of a glutathione *S*-transferase–NSP5 fusion polypeptide expressed in *Escherichia coli* demonstrated autophosphorylation, suggesting that NSP5 was the active component in the material isolated from infected cells.

Rotavirus has been identified as a major cause of gastroenteritis in the young of humans and many animal species including birds (18). The rotavirus genome consists of 11 segments of double-stranded RNA. Six of them contain genes coding for proteins present in the virus particles (VP1 to -7), while the remaining five RNA segments encode nonstructural proteins (NSP1 to -5). The structural proteins form a core containing the double-stranded RNA segments and surrounding inner and outer shells. Assortment and replication of viral RNA segments take place in viroplasms, cytoplasmic inclusion bodies present in rotavirus-infected cells. Isolation of replicative intermediates from this material showed that the smallest contain VP1 and VP3 together with NSP1, NSP2, NSP3, and NSP5 (11, 27, 28). The addition of VP2 and VP6 and gradual removal of the nonstructural proteins were observed during maturation of the replicative intermediates. NSP1 (16) and NSP3 (29) bind specifically to viral RNA and might participate in assortment of viral RNA, but in general the function of the nonstructural proteins in assortment and replication of RNA is poorly understood.

The *NSP5* gene is carried by the smallest RNA segment of group A rotaviruses (22). To date, cDNAs corresponding to the *NSP5* genes of 15 virus strains have been sequenced. The protein is 197 to 198 amino acids in length, and a striking feature is its high-level contents of serine (20%), lysine (10%), and aspartate (10%). The amino acid sequence conservation of the known NSP5 exceeds 78%, with a more variable region present between residues 102 and 156 (20). All the polypeptides show two clusters of basic amino acids and one acid region at the C termini. NSP5 has a predicted size of 22 kDa. However, in analysis by polyacrylamide gel electrophoresis (PAGE), its size corresponds to 26- and 28-kDa products. The shift has been attributed to posttranslational addition of O-linked *N*-acetylglucosamine (13) and phosphorylation (34). Two smaller polypeptides of 20 and 22 kDa are present in infected cells. They probably represent a translation product initiated at an AUG codon corresponding to position 52 of the full-length polypeptide. This truncated form of NSP5 is also phosphorylated (23).

We investigated the phosphorylation of NSP5 protein during rotavirus infection in MA104 cells and a protein kinase activity associated with NSP5 isolated from infected cells. In addition, we studied the protein kinase activity of purified NSP5 expressed in bacteria and observed that it is capable of autophosphorylation.

MATERIALS AND METHODS

Cell culture and viruses. Rhesus monkey fetal kidney (MA104) cells were maintained in Dulbecco modified Eagle medium (DMEM; HyClone) supplemented with 5% fetal calf serum (HyClone), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Simian rotavirus strain SA11 clone 3 (10) was obtained from R. F. Ramig (Baylor College of Medicine, Houston, Tex.). Propagation of virus in MA104 cells was carried out as previously described (21). Virus was titrated on MA104 cells. Cells expressing capsid antigen were stained by the immune peroxidase method (3), and titers were expressed in focus-forming units.

Rotavirus infection and radiolabeling of proteins. Virus was treated with 20 μg of trypsin (1:250; Difco) per ml for 30 min at 37°C. Then the activated virus was diluted and added to cell monolayers at a multiplicity of infection of 10 focus-forming units/cell. After 1 h of adsorption, the inoculum was replaced by DMEM without serum. At 4 h postinfection, cells were incubated with either 50 μCi of [^{35}S]methionine per ml in methionine-free DMEM (Sigma) or with 0.2

* Corresponding author. Mailing address: Department of Medical Immunology and Microbiology, Uppsala University, Biomedical Centre, Box 582, S-751 23 Uppsala, Sweden. Phone: 46-18-174560. Fax: 46-18-509876. E-mail: mago@bio.embnet.se.

† Present address: Danish Cancer Society, Division for Cancer Biology, DK-2100 Copenhagen Ø, Denmark.

mCi of carrier-free $^{32}P_i$ per ml in phosphate-free DMEM (Sigma). Cells were labeled for 2 h at 37°C before harvest.

Immunoprecipitation of NSP5 protein. A mouse NSP5 antiserum was raised against a glutathione *S*-transferase (GST)–NSP5 fusion protein (4). The antibodies were specific for the NSP5 moiety of the fusion protein in immunoblot analysis at a dilution of ≥1:1,000. To prepare protein extracts from infected cells, the culture medium from each 60-mm-diameter petri dish was aspirated and the cells were scraped off the plastic surface. They were then suspended in 1 ml of Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1.0 mM dithiothreitol [DTT] containing 50 mM NaF and 0.10 mM Na_3VO_4. After sedimentation for 5 min at 5,000 × *g*, cells were resuspended in 100 μl of TX lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1.0 mM DTT, 50 mM NaF, 0.10 mM Na_3VO_4, 30 mM $NaPP_i$, 1.0% Triton X-100, 10% glycerol, 1.0 μg of aprotinin per ml, and 50 μg of phenylmethylsulfonyl fluoride per ml) and then incubated for 10 min at room temperature. Cell nuclei and debris were removed by centrifugation for 15 min at 20,000 × *g*, and the supernatant was collected. Ten microliters of undiluted NSP5 antiserum was added, and after 1 h at 4°C, antibody-antigen complexes were adsorbed to protein G-Sepharose (Pharmacia). Fifteen microliters of a 50% (vol/vol) protein G-Sepharose slurry was used. After 1 h at 4°C, the beads were sedimented at 2,000 × *g* for 2 min and washed twice with TX lysis buffer, once with TX lysis buffer containing 400 mM NaCl, and finally twice with TBS buffer.

Dephosphorylation of NSP5 polypeptides in vitro. Proteins of SA11-infected MA104 cells were labeled at 4 to 6 h postinfection with ^{35}S or ^{32}P as described above. NSP5 was isolated by immunoprecipitation. Phosphatase inhibitors were removed by two TBS washes. Immune complexes bound to protein G-Sepharose beads were incubated with 0.5 U of protein phosphatase 2A (PP2A; Upstate Biotechnology Inc.) in 20 μl of phosphatase buffer (50 mM Tris-HCl [pH 7.5], 1.0 mM DTT, 1.0 mM $MnCl_2$) for 20 min at 37°C. In control reaction mixtures, the PP2A inhibitor okadaic acid, at a final concentration of 3 nM, was first added. Reactions were terminated by addition of sodium dodecyl sulfate (SDS), and the products were resolved by SDS-PAGE. After electrophoresis, gels were fixed, stained, and analyzed by autoradiography. The radioactivity of the NSP5-related material was quantified with a GS-250 molecular imager (Bio-Rad).

Analysis of viral NSP5 by 2-D PAGE. Radioactively labeled proteins were immunoprecipitated with NSP5 antiserum as described above, dissolved in 9.5 M urea–2.0% Nonidet P-40–5.0% β-mercaptoethanol–2.0% ampholines (pH 3.5 to 10) (Pharmacia), and subjected to two-dimensional (2-D) PAGE, as originally described by O'Farrell et al. (26). In the first dimension, the proteins were separated by isoelectric focusing in 150- by 1.5-mm rod gels containing an ampholyte gradient of pH 3.5 to 10. Proteins were applied at the anode, and separation was allowed to proceed for 10 h at 400 V. In the second dimension, proteins were resolved in SDS–12% polyacrylamide gels by the method of Laemmli (19). After electrophoresis, the gels were dried and subjected to autoradiography.

Phosphoamino acid analysis. Phosphoamino acids were analyzed by the method of Boyle et al. (5). Proteins metabolically labeled from 3 to 6 h postinfection with $^{32}P_i$ were isolated by immunoprecipitation, as described above. They were resolved by SDS-PAGE and then transferred to an Immobilon polyvinylidene difluoride membrane (Millipore). The locations of radioactive proteins were determined, and corresponding membrane strips were excised. Polypeptides bound to the strips were hydrolyzed with 6 N HCl at 110°C for 1 h. After evaporation to dryness, the samples were resuspended in 5 μl of pH 1.9 buffer (formic acid/glacial acetic acid/deionized water ratio, 50:156:1,794 [by volume]) and applied to cellulose thin-layer plates together with phosphoamino acid standards (Sigma). Samples were subjected to 2-D electrophoresis. The first dimension was run in pH 1.9 buffer at 2,000 V for 30 min, and the second dimension was run in pH 3.5 buffer (glacial acetic acid/pyridine/deionized water ratio, 100:10:1,890 [by volume]) at 1,800 V for 20 min. Reference phosphoamino acids were identified by ninhydrine staining, and the ^{32}P-labeled phosphoamino acids were visualized by autoradiography.

Tryptic peptide mapping of phosphoamino acids. Trypsin digestion and 2-D phosphopeptide analysis were performed essentially as previously described (5). Briefly, rotavirus-infected MA104 cells were metabolically labeled with 0.2 mCi of $^{32}P_i$ per 60-mm-diameter petri dish at 3 to 6 h postinfection. Material from clarified TX buffer lysates precipitated with NSP5 antiserum was resolved by SDS-PAGE, and proteins were electrotransferred to a Hybond C-extra nitrocellulose membrane (Amersham). Membrane strips containing the radioactive protein were excised and soaked in 0.5% (wt/vol) polyvinylpyrrolidone–0.6% (vol/vol) acetic acid at 37°C for 30 min. After being washed with water, the membrane piece was incubated in 50 mM NH_4HCO_3 with 1.0 μg of sequencing-grade modified trypsin (Promega). Digestion was carried out for 18 h at 37°C. The peptides were lyophilized, performic acid was added, and the samples were left on ice for 1 h. Then the peptides were evaporated to dryness and redissolved in 50 mM NH_4HCO_3, and trypsin digestion was repeated at 37°C overnight. Finally, peptides were dissolved in pH 1.9 buffer and applied to a thin-layer chromatography plate. Electrophoresis was carried out in pH 1.9 buffer for 16 min at 2,000 V. Ascending chromatography in the second dimension was then performed overnight in isobutyric acid buffer (isobutyric acid/*n*-butanol/pyridine/glacial acetic acid/deionized water ratio, 1,250:38:96:58:558 [by volume]). Tryptic ^{32}P-labeled phosphopeptides were visualized by autoradiography.

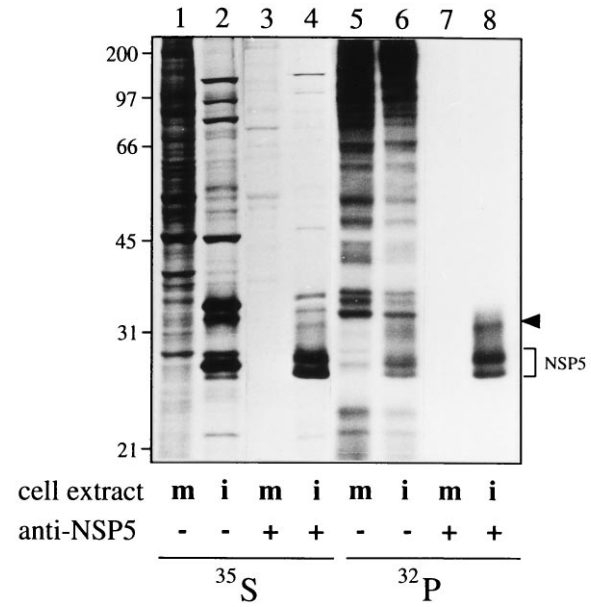

FIG. 1. Synthesis and modification of NSP5 protein. Monkey MA104 cells were infected with rotavirus SA11. Starting at 4 h postinfection, cells were labeled for 2 h with [^{35}S]methionine or $^{32}P_i$. At the end of the labeling period, cytosolic extracts were prepared from mock- (m) and rotavirus-infected (i) cells. After extraction with TX lysis buffer, polypeptides were either resolved directly by SDS-PAGE (−) or first immunoprecipitated with NSP5 antiserum (+). Radioactivity was detected by autoradiography. The positions of size markers (in kilodaltons) are indicated on the left, and those of 26- to 28-kDa NSP5 and a 35-kDa polypeptide (arrowhead) are indicated on the right.

Analysis of protein kinase activity in vitro. NSP5 formed during rotavirus infection for 6 h was isolated by immunoprecipitation as described above, and the NSP5-antibody complexes bound to protein G-Sepharose were used in analysis. GST-NSP5 fusion protein formed in *Escherichia coli* cells was purified by affinity chromatography on glutathione-Sepharose by the procedure recommended by the manufacturer (Pharmacia). Protein kinase activity was assayed in 25 μl of kinase buffer (20 mM HEPES [pH 7.5], 10 mM $MnCl_2$ or $MgCl_2$, 1.0 mM DTT, 1.0 μM ATP) with 10 μCi of [γ-^{32}P]ATP for 10 min at 25°C. The reaction was stopped by adding 25 μl of SDS-containing electrophoresis sample buffer. Then the samples were boiled for 5 min and subjected to SDS-PAGE.

Adsorption of proteins in MA104 cell extracts to GST and GST-NSP5. Proteins from mock- and rotavirus-infected MA104 cells were labeled with either [^{35}S]methionine or $^{32}P_i$ for 2 h, starting at 4 h after infection. Cells were extracted with TX lysis buffer as described above for immunoprecipitation. GST and GST-NSP5 proteins formed in bacteria were purified by affinity chromatography on glutathione-Sepharose. The eluted polypeptides were incubated for 2 h at 4°C with a cleared MA104 cell lysate equivalent to 2.5×10^5 cells. Incubation was continued for 20 min at 20°C after addition of 20 μl of glutathione-Sepharose slurry (50% [vol/vol]). Unadsorbed material was removed by washing the Sepharose beads four times with TBS buffer. Then the beads were pelleted, resuspended with 25 μl of SDS sample buffer, and boiled for 5 min, and released proteins were resolved by SDS-PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue, dried, and analyzed by autoradiography.

RESULTS

Detection of NSP5 in rotavirus-infected cells. Polypeptides synthesized during rotavirus infection of monkey MA104 cells were labeled with [^{35}S]methionine or $^{32}P_i$. Total cytoplasmic extracts and fractions precipitated with NSP5 antiserum were then subjected to SDS-PAGE (Fig. 1). The incorporation of [^{35}S]methionine showed that cellular protein synthesis was inhibited and that viral protein synthesis predominated after infection. The earlier identified 26- and 28-kDa forms of NSP5 (13) were specifically immunoprecipitated. At the time point of the experiment (6 h postinfection), the syntheses of these two NSP5 forms appeared to be approximately equal. Between the 28-kDa form of NSP5 and a polypeptide with an approximate

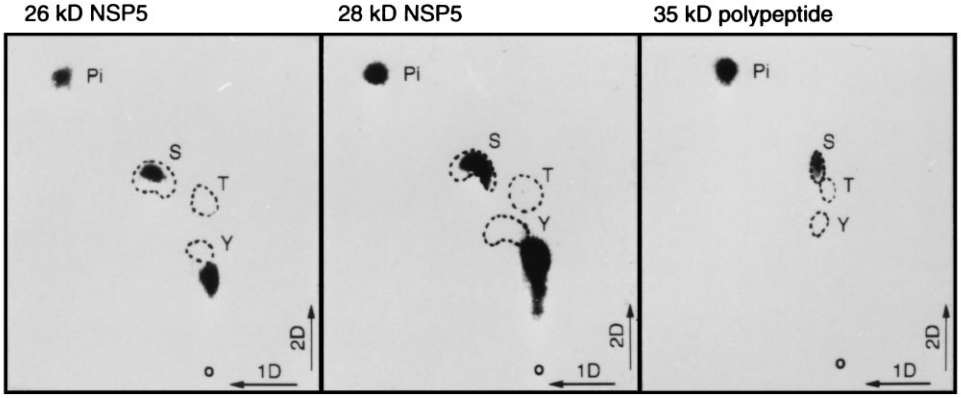

FIG. 2. Phosphoamino acid analysis of NSP5. MA104 cells infected with rotavirus were labeled with $^{32}P_i$ at 3 to 6 h postinfection. The NSP5 complex was immunoprecipitated from cell lysates, and the 26-, 28-, and 35-kDa (kD) proteins were isolated after SDS-PAGE. They were subjected to acid hydrolysis, and amino acids were resolved by 2-D electrophoresis on cellulose thin-layer plates. The positions of the origin (o) and ninhydrin-stained unlabeled phosphoserine (S), phosphothreonine (T), phosphotyrosine (Y), and free phosphate (P_i) are shown. Radioactivity was detected by autoradiography. The directions of migration in the first (1D) and second (2D) dimensions are indicated by arrows.

size of 35 kDa, there was a smear of radioactivity (Fig. 1, lane 4).

The immunoprecipitated NSP5 labeled with ^{35}S also contained a number of polypeptides that were not present in extracts of mock-infected cells. They were probably virus-encoded proteins which coimmunoprecipitated with NSP5. Their sizes corresponded to the rotavirus VP2, VP3, VP6, NSP2, and NSP3 polypeptides that all colocalize with NSP5 to viroplasms (11).

Labeling with ^{32}P showed that rotavirus infection affected phosphorylation of cellular polypeptides to a lesser extent than it did their synthesis. In keeping with earlier observations (34), NSP5 antiserum brought down phosphorylated polypeptides with mobilities corresponding to 26 and 28 kDa during SDS-PAGE. The 28-kDa material contained somewhat more label than did the 26-kDa form. In addition to these two NSP5 forms, there was a 35-kDa phosphorylated species and a heterogeneous set of polypeptides between the 28- and 35-kDa positions (Fig. 1, lane 8). None of these were present in extracts of mock-infected cells.

Phosphoamino acid analysis. NSP5 contains serine, threonine, and tyrosine residues, although the serines predominate. To determine which types of residues were modified, NSP5 was immunoprecipitated from ^{32}P-labeled cells and further purified by SDS-PAGE. The 26- and 28-kDa forms of NSP5 and the 35-kDa polypeptide were isolated and then hydrolyzed. Phosphoamino acids were separated by 2-D electrophoresis on cellulose thin-layer plates (Fig. 2). Autoradiography showed that in all three types of polypeptides, the only ^{32}P-labeled amino acid was serine. No phosphothreonine or phosphotyrosine residues were detected even after prolonged exposure of autoradiograms. The labeled substance migrating close to phosphotyrosine probably consisted of acid-insoluble material that contaminated the soluble fraction.

Dephosphorylation of NSP5 protein in vitro. To investigate whether the 28- to 35-kDa polypeptides reacting with NSP5 antiserum had reduced electrophoretic mobilities as a result of phosphorylation, we incubated the immunoprecipitated material with PP2A. This serine/threonine phosphatase has a broad specificity (25). SA11-infected cells were labeled with [^{35}S]methionine or $^{32}P_i$ at 4 to 6 h postinfection. Protein extracts were mixed with NSP5 antiserum, and immune complexes were adsorbed to immobilized protein A. Material labeled with ^{35}S or ^{32}P was incubated with PP2A in the absence or presence of 3 nM okadaic acid. The protein phosphatase removed all label from the ^{32}P-labeled polypeptides, except for the radioactivity of the 26-kDa form of NSP5 (Fig. 3, lane 8). In the presence of the PP2A inhibitor okadaic acid, no dephosphorylation occurred (Fig. 3; compare lanes 7 and 9). PP2A treatment of ^{35}S-labeled polypeptides led to a corresponding result. In an okadaic acid-sensitive reaction, PP2A eliminated materials with mobilities corresponding to 28 to 35 kDa (Fig. 3, lane 4). There was a concomitant accumulation of radioactivity at the 26-kDa position. Quantitation of radioactivity showed that at least 70% of the ^{35}S radioactivity from the heavier material was shifted to the 26-kDa band. The weak remaining band at 28 kDa (Fig. 3, lane 4) may be unphosphorylated, since there was no ^{32}P radioactivity at the corresponding position (lane 8). Together, the results from this experiment show that the 28- to 35-kDa material consisted of highly phosphorylated forms of NSP5.

Analysis of NSP5 protein by 2-D electrophoresis and separation of tryptic peptides. The ^{35}S- and ^{32}P-labeled polypeptides precipitated with NSP5 antiserum were also analyzed by 2-D PAGE (Fig. 4). The component with an electrophoretic mobility corresponding to 26 kDa resolved into several spots with isoelectric points in the range of 4.6 to 6.0. Polypeptides with an apparent size of 28 kDa were on average more acidic, showing at least three forms at pI 4.6 to 5.6. In polypeptides of both sizes, the acidic forms were most abundant. These forms also contained most of the phosphate label (Fig. 4, bottom), suggesting that sequential addition of phosphate groups was the basis for formation of the acidic NSP5 species. The 35-kDa polypeptide seen as a distinct band after 1-D PAGE (Fig. 1 and 3) probably corresponded to the radioactive material streaking toward the upper right corner of the autoradiogram (Fig. 4).

To delineate the number of phosphorylation sites and the relative extent of phosphorylation, the phosphorylated 28-kDa form of NSP5 was trypsin digested. The resulting peptides were separated on a cellulose thin-layer plate developed by electrophoresis in the first dimension and chromatography in the second dimension (Fig. 5). Four to six major phosphopeptides and several minor phosphopeptides were present. This result agrees with the 2-D PAGE analysis (Fig. 4), where several NSP5 isoforms were identified.

Protein kinase activity of NSP5 isolated from infected cells. The possibility that the extensive phosphorylation of NSP5 was catalyzed by this protein itself prompted an analysis of protein

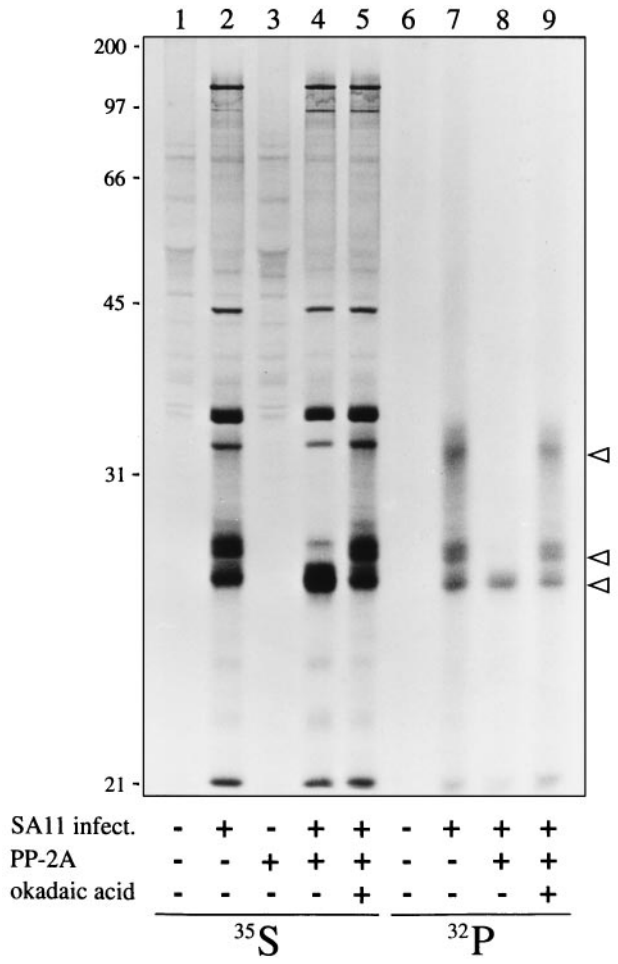

FIG. 3. Dephosphorylation of NSP5 in vitro. Cells were infected (infect.; +) and labeled with [^{35}S]methionine and $^{32}P_i$, as described in the legend to Fig. 1. Immunoprecipitated material was either analyzed directly by SDS-PAGE (−) or first treated with 0.5 U of PP2A for 20 min at 37°C (+). Control reactions were performed in the presence (+) of 3 nM okadaic acid. After electrophoresis, the gel was analyzed by autoradiography. The positions of size markers (in kilodaltons) are indicated on the left. The positions of 26-, 28-, and 35-kDa polypeptides are indicated by arrowheads on the right.

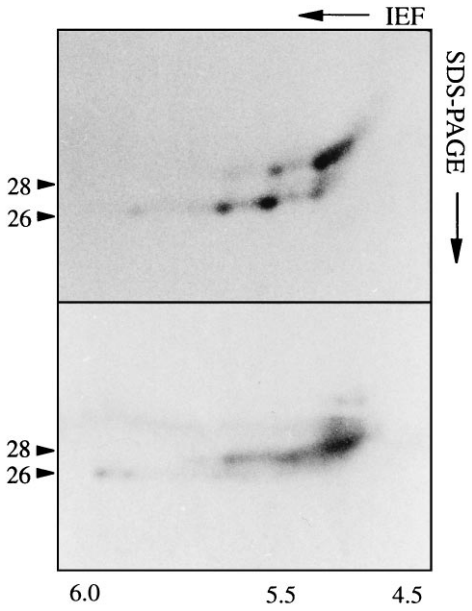

FIG. 4. Analysis by 2-D PAGE of NSP5 protein formed in rotavirus-infected cells. Cells were infected and labeled with [^{35}S]methionine (top) or $^{32}P_i$ (bottom), as described in the legend to Fig. 1. Protein was immunoprecipitated with NSP5 antiserum and then separated by 2-D PAGE. The directions of separation in the first dimension by isoelectric focusing (IEF) and in the second dimension by SDS-PAGE are indicated by arrows. Isoelectric points determined with standard proteins are shown below the gel. The sizes (in kilodaltons) of polypeptide markers are shown on the left.

kinase activity in vitro. NSP5 was immunoprecipitated from rotavirus-infected MA104 cells. The immune complexes immobilized on protein G-Sepharose were incubated in kinase buffer containing [γ-^{32}P]ATP for 10 min at 25°C. SDS-PAGE analysis of the polypeptides showed (Fig. 6) a weakly labeled band migrating to 26 kDa and an intensely labeled broad band at 28 to 35 kDa, both corresponding to the NSP5 protein. Evidently, a protein kinase activity was present in the NSP5 immunoprecipitates. No corresponding polypeptides were phosphorylated in extracts of mock-infected cells.

The protein kinase activity was tested in the presence of Mg^{2+} and Mn^{2+} ions. The result of this experiment showed (Fig. 6) that the protein kinase was active with either divalent ion, although Mn^{2+} appeared to be more effective.

Protein kinase activity of NSP5 protein expressed in *E. coli*. The GST-NSP5 fusion protein purified by affinity chromatography provided a tool to determine whether the protein kinase activity was characteristic of NSP5. The fusion protein expressed in *E. coli* should be free from contaminating cellular protein kinases. GST and GST-NSP5 proteins purified by glutathione-Sepharose chromatography were incubated with kinase buffer. In the reaction mixtures, we used [γ-^{32}P]ATP at 1.0 and 5.0 μM concentrations. SDS-PAGE analysis of the reaction products showed (Fig. 7) that radioactivity was incorporated into GST-NSP5, but not into GST. ATP at a concentration of 1.0 μM was clearly suboptimal for the reaction. Even at the higher ATP concentration, the autophosphorylating activity of NSP5 was quite low, considering the large amount of protein used in the reaction mixture.

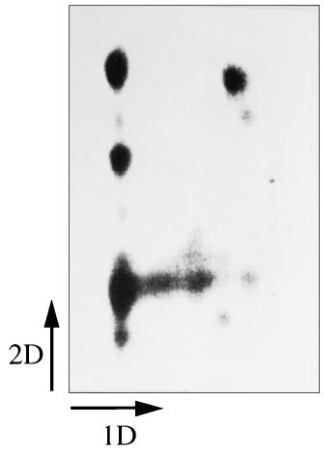

FIG. 5. Analysis of phosphorylation sites in NSP5 by separation of tryptic peptides. NSP5 was ^{32}P labeled, and the 28-kDa form was isolated, as described in the legend to Fig. 2. The purified material was digested with trypsin, and the resulting peptides were resolved on a thin-layer plate by electrophoresis at pH 1.9 in one dimension (1D) followed by ascending chromatography in the second dimension (2D). Radioactivity was detected by autoradiography.

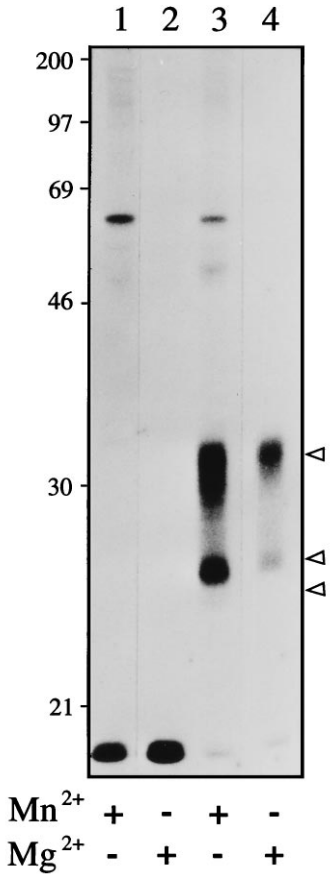

FIG. 6. Protein kinase activity of NSP5 complex isolated from rotavirus-infected cells. Cells were infected and NSP5 was immunoprecipitated as described in the legend to Fig. 1. Immune complexes from uninfected (lanes 1 and 2) and rotavirus-infected (lanes 3 and 4) cells were incubated for 10 min with [γ-^{32}P]ATP in a kinase buffer containing (+) either Mn^{2+} or Mg^{2+}. The reaction products were then subjected to SDS-PAGE. The positions of the 35-, 28-, and 26-kDa bands corresponding to the NSP5 polypeptides are indicated on the right by arrowheads; the mobilities of molecular size markers (in kilodaltons) are indicated on the left. −, absent.

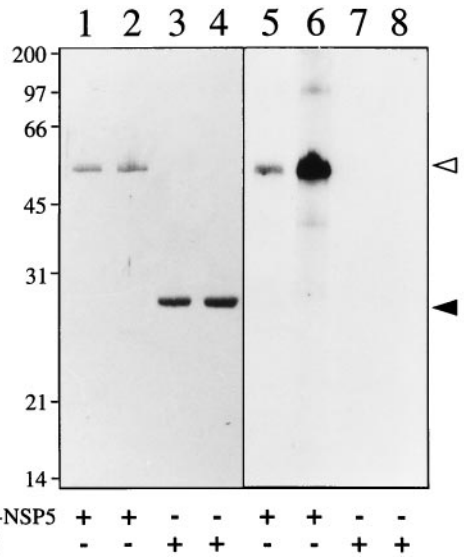

FIG. 7. Protein kinase activity of purified GST-NSP5 fusion protein. Affinity-purified GST-NSP5 (lanes 1, 2, 5, and 6) and GST (lanes 3, 4, 7, and 8) proteins were incubated in 25 μl of protein kinase buffer containing 1 (lanes 1 and 3) or 5 (lanes 2 and 4) μM [γ-^{32}P]ATP. The reaction products were analyzed by SDS-PAGE in a 12% polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue (lanes 1 through 4), and radioactivity was visualized by autoradiography (lanes 5 through 8). The positions of GST-NSP5 (open arrowhead) and GST (filled arrowhead) are indicated on the right. The mobilities of molecular size markers (in kilodaltons) are indicated on the left. +, present; −, absent.

Binding activity of bacterial NSP5 polypeptide. We investigated the ability of the purified GST-NSP5 fusion protein to associate with cellular and viral polypeptides induced during rotavirus infection. The GST and GST-NSP5 polypeptides were incubated for 2 h with soluble proteins extracted from mock-infected MA104 cells and for 6 h after rotavirus infection. Two sets of cell extracts, one prepared after labeling with [^{35}S]methionine and the other prepared after incubation with $^{32}P_i$, were used. After incubation, the GST moiety was attached to glutathione-Sepharose again and the unbound material was removed by several washing steps. Finally, the material bound to glutathione-Sepharose was analyzed by SDS-PAGE.

The results of this analysis are presented in Fig. 8, with the top showing the results of staining with Coomassie brilliant blue. Besides GST and GST-NSP5, four abundant cellular polypeptides bound to glutathione-Sepharose, either directly or after association with GST. Using [^{35}S]methionine-labeled proteins from uninfected and rotavirus-infected MA104 cells, a relatively large number of polypeptides were adsorbed (Fig. 8, bottom). Since cellular protein synthesis is strongly inhibited after rotavirus infection, it is not surprising that the patterns of labeled proteins shifted after the infection of cells. Although several proteins of potential viral origin bound to glutathione-Sepharose, GST, or GST-NSP5, there were only two polypeptides of 26 to 28 kDa that associated specifically with GST-NSP5. With extracts of ^{32}P-labeled, uninfected cells, only little radioactive material was recovered. In extracts of infected cells, there were labeled materials with electrophoretic mobilities in the range of 26 to 35 kDa that attached specifically to GST-NSP5. This material probably consisted of NSP5. Apparently, GST-NSP5 could form relatively stable oligomers with NSP5 from infected cells but did not bind with similar affinity to other viral or cellular polypeptides.

DISCUSSION

NSP5 is synthesized in relatively large amounts during rotavirus infection of monkey kidney cells. Immunofluorescence analysis showed that most of NSP5 is located in viroplasms (23, 34), sites of viral RNA synthesis and provirion assembly. NSP5 is present in replicative intermediates of rotavirus (11), but its function is not known.

Orbiviruses, which also belong to the *Reoviridae* family, produce a nonstructural phosphoprotein, NS2. For bluetongue virus, it is the only virus-encoded phosphoprotein (17). NS2 of bluetongue, epizootic hemorrhagic disease, and African horsesickness viruses is phosphorylated at serine residues (9, 31, 32). NS2 expressed by a recombinant baculovirus is a single-stranded RNA-binding protein. This activity was independent of the phosphorylation state (32). However, NS2 expressed in *E. coli* cells and then purified bound to single-stranded RNA preferentially in its unphosphorylated form (31). Rotavirus NSP5 might also be an RNA-binding protein, since it was reported to be retained on poly(U)-Sepharose (23). Although a comparison of the deduced amino acid sequences of rotavirus NSP5 and orbivirus NS2 showed an overall similarity of only 15 to 20%, they may have related functions. Bluetongue

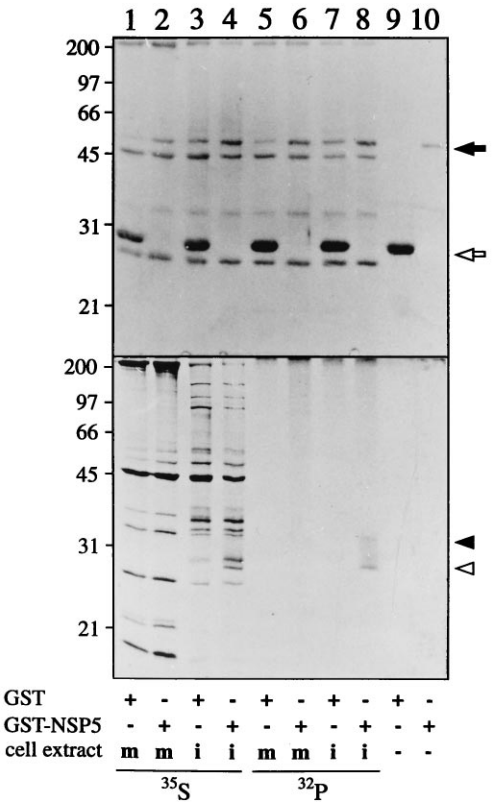

FIG. 8. Protein complex formation with GST-NSP5 fusion polypeptide. MA104 cells were infected, radioactively labeled, and extracted, as described in the legend to Fig. 1. Proteins from mock- (m) and rotavirus-infected (i) cells metabolically labeled with [^{35}S]methionine or $^{32}P_i$ were incubated for 2 h at 4°C with (+) or without (−) affinity-purified GST or GST-NSP5. After adsorption to glutathione-Sepharose, bound material was washed extensively, released by boiling in SDS sample buffer, and then analyzed by SDS-PAGE. The gel was stained with Coomassie brilliant blue (top) and analyzed by autoradiography (bottom). The positions of GST-NSP5 (filled arrow), GST (open arrow), and the 35- (filled arrowhead) and 28 (open arrowhead)-kDa NSP5 polypeptides are indicated on the right. The migrations of molecular size markers (in kilodaltons) are shown on the left.

virus NS2 is localized within the viral inclusion bodies in infected mammalian cells (32). Like in rotavirus, these inclusions are sites for virion assembly, including replication of viral RNA.

Rotavirus NSP5 is unusually serine rich and undergoes posttranslational modifications, including phosphorylation. Our experiments showed that NSP5 was phosphorylated only at serine residues. No phosphorylation of threonine or tyrosine residues was detected (Fig. 2). Trypsin cleavage of NSP5 resulted in four to six major ^{32}P-labeled peptides and a few weak ^{32}P-labeled peptides (Fig. 5), indicating that NSP5 can be phosphorylated at several serine residues. Complementary information from 2-D PAGE analysis (Fig. 4) showed that individual NSP5 polypeptides were phosphorylated at several serine residues. More than five isoforms with different isoelectric points were observed. None of these represented unphosphorylated NSP5, which has a theoretical pI of 6.7. Even the most basic of the polypeptides detectable in the 2-D gels was phosphorylated, as judged by ^{32}P labeling and a pI of approximately 6.0. The results from trypsin cleavage and 2-D PAGE together suggest that NSP5 contains at least five serines that are major phosphorylation sites. Besides these sites, several serine residues were phosphorylated to a lesser extent.

NSP5 antiserum reacted with 26- and 28-kDa polypeptides and with polypeptides having electrophoretic mobilities in the range of 28 to 35 kDa (Fig. 1 and 3). This immunoprecipitated material had incorporated both [^{35}S]methionine and $^{32}P_i$.

In earlier studies of immunoprecipitated NSP5 (13, 23, 34), the 28- to 35-kDa polypeptides were observed by Welch et al. (34) after labeling with [^{35}S]methionine. The reason for the heterogeneous sizes of NSP5 has been assumed to be posttranslational modification. Here we report that treatment of isolated NSP5 with PP2A converts the larger forms to 26-kDa NSP5 (Fig. 3). In this process, ^{32}P labeling disappeared from the 28- to 35-kDa polypeptides. This result suggests that conversion of the 26-kDa form of NSP5 to polypeptides with slower electrophoretic mobilities was caused by addition of multiple phosphate groups. It is interesting that only the 26-kDa form of NSP5 contains PP2A-resistant phosphate groups. They might be phosphorylated serine groups not recognized by PP2A or protected from its activity, e.g., by bound antibodies.

Besides being phosphorylated, NSP5 is modified by *N*-acetylglucosamine at serine or threonine groups. The addition of carbohydrate residues was reported to account for the shift in size from 26 to 28 kDa (13). The O glycosylation of NSP5 appears to consist of monomeric *N*-acetylglucosamine (13) and probably occurs in the cytoplasm (15). However, our data suggest that the 26- to 28-kDa shift was caused by phosphorylation (see above). The function of O glycosylation in NSP5 is unknown. It might be important for interaction of the protein with carbohydrate binding sites or have a structural role. An interesting potential regulatory function of O glycosylation is that it excludes phosphorylation of the same site (6).

Protein kinase activity was observed in the material immunoprecipitated with the NSP5 antiserum from rotavirus-infected cells. The substrates of this reaction were the NSP5 polypeptides, particularly the 28- to 35-kDa forms. The relative levels of phosphorylation of NSP5 polypeptides during metabolic labeling of cells (Fig. 3) and in the cell-free experiment (Fig. 6) were similar. This observation suggests that the isolated proteins with in vitro phosphorylation activities are the same ones that participate in the in vivo process. In vitro phosphorylation experiments and immunoprecipitation of proteins after ^{32}P labeling in vivo did not provide information on the origin of the protein kinase.

In this communication, we have established that a GST-NSP5 fusion polypeptide has protein kinase activity. Assay of this activity using polypeptide produced in *E. coli* resulted in autophosphorylation. This result supports the idea that NSP5 is the component of the complex isolated from infected cells that contains the kinase activity. Apparently, posttranslational modification of NSP5 is not essential for protein kinase activity. It might still be important for full enzyme activity or for interaction with protein substrates or enzyme regulators. NSP5 polypeptide chains without posttranslational modification can interact with each other, since GST-NSP5 made in bacteria oligomerized with NSP5 extracted from infected cells (Fig. 8). However, O glycosylation or phosphorylation might be required for interaction with other target proteins.

Protein kinases contain a common catalytic domain which typically is found in a 250- to 300-amino-acid-long polypeptide segment, including the binding sites for ATP and the protein substrate (14). NSP5 is smaller. Therefore, potential regulation of its protein kinase activity is likely to be achieved by complex formation with other polypeptide(s) that provide the regulatory function. Analysis of the NSP5 amino acid sequence, using the computer algorithm described by Bairoch et al. (1), showed no significant homology with other protein kinases. Hence,

NSP5 appears to be exceptional in the large group of protein kinases that have the typical amino acid motives (14).

We have not yet mapped the autophosphorylation sites of purified NSP5 to see whether they correspond to the pattern in NSP5 isolated from infected cells. It is possible that cellular protein kinases are involved in the in vivo phosphorylation. In the deduced amino acid sequence of NSP5 from rotavirus SA11 and other strains, there are potential protein kinase C phosphorylation sites (S-X-R/K-R/K-X-X-S and K/R-X-S) at serine residues 22, 30, 75, 100, and 136. There are also serine residues at positions 56, 154, and 165 that are potential substrates of casein kinase II (S-X-X-D/E) (1). Protein kinase C and casein kinase II have been shown to be active with the P phosphoprotein of vesicular stomatitis virus (2) and various paramyxoviruses (7, 8, 24, 33). For both vesicular stomatitis virus and respiratory syncytial virus, phosphorylation of the P protein is essential for activation of the viral polymerase complex (2, 12, 24, 30). It is possible that rotavirus NSP5 also has a function in replication of viral RNA, but there is no experimental evidence for this hypothesis.

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