Phosphorylation of Sendai Virus Phosphoprotein by Cellular Protein Kinase C \( \zeta \)*

Clayton C. Huntley, Bishnu P. De, and Amiya K. Banerjee‡

From the Department of Molecular Biology, Research Institute, The Cleveland Clinic Foundation, NC20, Cleveland, Ohio 44195

The phosphoproteins (P) of nonsegmented negative strand RNA viruses are viral RNA polymerase subunits involved in both transcription and replication during the virus life cycle. Phosphorylation of P proteins in several negative strand RNA viruses by specific cellular kinases was found to be required for P protein function. In the present study, using bacterially expressed unphosphorylated P protein of Sendai virus, a mouse parainfluenza virus, we have shown that the major cellular kinase that phosphorylates P protein in *in vitro* is biochemically and immunologically indistinguishable from protein kinase C (PKC) \( \zeta \) isoform. PKC \( \zeta \) was packaged into the Sendai virion and remained associated with purified viral ribonucleoprotein, where it phosphorylated both the P and the nucleocapsid protein in *in vitro*. When PKC \( \zeta \)-specific inhibitory pseudosubstrate peptide was introduced into LLC-MK\(_2\) cells prior to Sendai virus infection, production of progeny virus was dramatically attenuated, and kinetic analysis revealed that primary transcription was repressed. These data indicate that phosphorylation of the Sendai virus P protein by PKC \( \zeta \) plays a critical role in the virus life cycle.

Sendai virus, a member of the paramyxoviridae, contains a non-segmented negative-strand RNA genome of about 15 kilobases that is tightly enwrapped by the viral nucleocapsid protein, NP (60 kDa). Two other virally encoded proteins, a large protein L (250 kDa) and a phosphoprotein P (79 kDa) associate with the ribonucleoprotein (RNP)\(^1\) and function as the RNA-dependent RNA-polymerase complex both in transcription and replication of the encapsidated genomic RNA (1, 2). During transcription, P protein is directly required for RNA synthesis acting as a subunit of the viral polymerase, the P-L complex (3). On the other hand, recent biological and biochemical data suggest that replication of the genome RNA within the nucleocapsid requires two distinct protein complexes, P-NP and P-L, with the latter serving as the RNA polymerase while P-NP complex is needed for encapsidation of newly synthesized genome RNA (4). Deletion analysis of the P protein (568 amino acids) has revealed that P-NP interaction requires cooperation between two noncontiguous domains (amino acids 345–411 and 479–568) in the C-terminal half of P (5, 6). Interestingly, the L-binding region of P (412–478) lies between the two NP-binding domains (7). The N-terminal half of the P protein contains most of the sites phosphorylated by a cellular kinase (8, 9) and acidic domains therein seem to be required for RNA synthesis (10).

Since the P protein appears to have several functions in the life cycle of negative strand RNA viruses, numerous studies have investigated the role of phosphorylation by cellular kinase in the regulation of P protein activities and in the formation of active transcription and replication complexes. P protein phosphorylation by casein kinase II has been demonstrated in vesicular stomatitis virus (VSV) (11, 12), respiratory syncytial virus (13–17), and measles virus (17). Furthermore, mutational analysis and biochemical transcription reconstitution in *in vitro* have led to the conclusion that transcriptional activity is dependent on casein kinase II-mediated phosphorylation of P proteins for both VSV (11, 12, 18) and respiratory syncytial virus (19). In addition, it seems that phosphorylation in VSV is essential for oligomerization of the P protein leading to its binding to the L protein and the NP-RNA template (20–23). Recent data suggest that phosphorylation occurs within the N-terminal acidic domain of the P protein of VSV (New Jersey serotype) which causes a major structural change of the protein leading to its activation (20). Interestingly, human parainfluenza virus type 3 (HPIV3) P protein is phosphorylated by a different cellular kinase, protein kinase C (PKC) \( \zeta \) isoform (24), and PKC \( \zeta \) activity is required for viral replication *in vivo*.

Here we have investigated the cellular kinase activity responsible for phosphorylation of the P protein of Sendai virus, a prototypic mouse parainfluenzavirus belonging to the paramyxovirus family. Using *Escherichia coli*-expressed Sendai virus P protein in an unphosphorylated (P\(_0\)) form as substrate, we fractionated cellular protein kinase activities competent for phosphorylating P\(_0\) *in vitro*. The major activity was identified as PKC \( \zeta \) by biochemical and immunological assay. We further observed that PKC \( \zeta \) was packaged into Sendai virion and remains tightly associated with the viral RNP. By inhibiting PKC \( \zeta \) activity *in vivo* by a specific inhibitory peptide, the production of progeny Sendai virions was dramatically reduced indicating a critical role for PKC \( \zeta \) in the life cycle of the virus.

**EXPERIMENTAL PROCEDURES**

*Materials—* Phosphatidylserine (PS) and diacylglycerol (DAG) were supplied by Sigma. (\( \gamma^{32}\)P)ATP (3000 Ci/mmol) was from DuPont NEN. Recombinant PKC \( \alpha \), \( \eta \), and \( \zeta \) were expressed in baculovirus and obtained from Walter Kolch. Peptides A (RKGLRQKN) and Z (RRGARWRRK) were from Quality Controlled Biochemicals. Peroxidase-conjugated goat anti-rabbit Ig, proteinase K, and Rnasin were purchased from Boehringer Mannheim. Anti-PKC \( \alpha \), \( \beta \), \( \delta \), \( \epsilon \), \( \gamma \), and \( \zeta \) antibodies were from Life Technologies, Inc. Reticuloocyte lysate was from Promega.

\( \dagger \) To whom reprint requests should be addressed: Dept. of Molecular Biology, The Cleveland Clinic Foundation, 9500 Euclid Ave., NC20, Cleveland, Ohio 44195

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Cloning of Sendai P Gene and Expression in E. coli—Sendai virus RNP was isolated from virion by treatment with an equal volume of 2 × buffer (20 mM Tris, pH 8.0, 10% glycerol, 0.8 M NaCl, 3.7% Triton X-100, 1.6 mM DTT) at room temperature for 10 min, then centrifuged through 30% glycerol on a 10% glycerol cushion for 2 hr at 40,000 rpm at 4 °C in a Beckman SW50.1 rotor. RNP from the 30%/100% interface was treated with extraction buffer (0.5% SDS, 10 mM MgCl₂, 0.25% bovine serum albumin, 50 mM ATP, 100 mM NaCl, and 2 units RNasin) for 20 min at 37 °C, and genomic RNA isolated by phenol/chloroform extraction and ethanol precipitation. A cDNA clone of the P gene was obtained by reverse transcription-PCR using the primer 5′-TGGATCAGTGGCCCTACATCTCCTA for first strand synthesis and the primer 5′-GAATATGATGAGTGTCTGTTGATCGTGTCTGTC for second strand synthesis. The latter primer adds a heptahistidine tag at the C terminus of the Sendai P protein. The PCR product was cut by restriction endonucleases NdeI and BamHI, then ligated into the pET-17b vector (Novagen). The P gene sequence was confirmed by dideoxy sequencing. Bacterially expressed P protein, which was present in the unpurified form (P₀), was purified using a Ni²⁺-affinity column according to the manufacturer’s protocol (Novagen).

Purification of Cellular Kinase—CV-1 cells (2 × 10⁶) were lysed by freezing and thawing in buffer containing 10 mM Tris-HCl, pH 8.0, and 10 mM NaCl. The lysate was centrifuged at 10,000 × g for 5 min and the supernatant further clarified by centrifugation at 100,000 × g for 1 hr on a 30%/100% glycerol cushion in 20 mM Hepes-KOH, pH 7.5, 1 mM DTT. The supernatant (S100) was then dialyzed overnight against buffer A (25 mM Tris-HCl, pH 8.0, 5% glycerol, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl), then loaded onto a DEAE-cellulose column (5 ml) equilibrated with buffer A, followed by washing with 5 bed volumes of buffer A. The column was developed with a linear gradient of 0–1 M NaCl in buffer A and fractions collected. Active fractions which phosphorylated the Sendai P₀ were pooled and loaded onto a phosphocellulose column (3 ml) which was then washed with 0.2 M NaCl buffer A. This column was developed with a linear gradient of 0.2–1.0 M NaCl buffer A and fractions collected. In an effort to further purify the active kinase fraction, P₀ phosphorylating fractions eluted from the phosphocellulose column were rechromatographed on a DEAE-cellulose column and active fractions were then dialyzed overnight against buffer B (10 mM KPO₄, 1 mM DTT, 5% glycerol), then loaded onto a hydroxylapatite column (2 ml). This column was washed with buffer B, developed with a linear gradient of 0.01–0.4 M KPO₄, pH 8.0, 3 mM MgCl₂, 6 M NH₄Cl, 14 mM KCl, and 2 mM DTT, for 2 h at 45,000 rpm and 4 °C in a Beckman SW 50.1 rotor. Twenty fractions of equal volume were collected from top to bottom of the gradient. Aliquots of every second fraction were resuspended in Laemmli sample buffer and subject to Western blot using PKC-ζ-specific antibody as described above.

In Vitro RNA Synthesis and Product Analysis—An in vitro transcription was done to detect RNA synthetic activity in sucrose gradient fractions of disrupted Sendai virion. A 50-µl reaction containing 0.1 M Hepes-KOH, pH 8.0, 0.1 M KCl, 5 mM MgCl₂, 1 mM DTT, 1 mM ATP, CTP, and GTP, 0.01 mM UTP, 15 ml of [α-³²P]UTP, 0.5 µl of RNasin, 2 µl of reticulocyte lysate, and 5-µl gradient fraction was incubated at 30 °C for 3 h. At the end of the reaction, products were precipitated in ETOH, denatured with formamide, then fractionated on a 5% acrylamide-urea sequencing gel. Relative transcriptional activity was quantitated by PhosphorImager analysis.

Interference with Sendai Replication by PKC-ζ-Specific Peptide Inhibitor—The cPKC and PKC-ζ-specific pseudosubstrate peptides, A and Z, respectively, were delivered into subconfluent (90%) monolayers of LLC-MK₁ cells using the Transport reagent kit (Life Technologies, Inc.) according to the manufacturer’s protocol. Cells in a 24-well plate were treated with the peptides plus Transport reagent for 15 min in a volume of 200 µl, followed by the addition of 20 µl of stop solution. Cells were then allowed to recover by incubation with 400 µl of Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, for 30 min at 37 °C. Cells were then infected with Sendai virus, pH 6.5, 1 µl plaque-forming unit, in 200 µl of Dulbecco’s modified Eagle’s medium without serum for 1 h. Unadsorbed virus was removed by washing once with Dulbecco’s modified Eagle’s medium, then 300 µl of Dulbecco’s modified Eagle’s medium containing 10% serum was added for incubation. Released progeny viruses were harvested at 30 h post-inoculation.

Detection of Intracellular Viral RNA Synthesis—To monitor the accumulation of positive or negative sense Sendai viral RNAs, a cDNA clone of the Sendai NP gene coding region was constructed by reverse transcription of purified genomic RNA followed by PCR amplification as described above for the cloning of the viral P gene. The primer 5′-GGCTTAGGCCCCATGCGCGGGTGTGTAAGG was used for first strand synthesis and the primer 5′-GCTGATGCTATCGATATTCTTCC for second strand synthesis. The PCR product was cut by restriction endonucleases APA 1 and NSI 1, then ligated into the pcDNAII vector (Invitrogen) to create pSNP. The NP gene sequence was confirmed by dideoxy sequencing. To generate 32P-labeled riboprobes for the detection of genomic (negative sense) viral RNAs, pSNP was linearized by restriction with NSI 1 followed by T7 transcription. Conversely, linearization by APA 1 and Sp6 transcription were used to produce a positive sense riboprobe (positive sense) viral transcripts.

Subconfluent LLC-MK₁ cells in 6-well plates (35 mm) were transfected with peptides A or Z (4 µM) for 1 h then infected with Sendai virion as described above. At various time points (1, 2, 4, 6, and 9 h) total intracellular RNA/mRNA was extracted by treatment of the cells with RNA Stat-60 (Tel-Test “B” Inc., Friendswood, TX) according to the manufacturer’s protocol. Equal aliquots of the RNA samples were subsequently denatured with formamide/formaldehyde, applied to a dot blot apparatus (Life Technologies, Inc.), and probed separately for the presence of positive and negative sense transcripts. Relative accumulation of Sendai viral transcripts at each time point was quantitated by PhosphorImager analysis and the signal from uninfection/peptide-treated control cells used to subtract background counts.

Heme-adsorption Plaque Assay—Sendai virus harvested from peptide-treated and control cells were treated with trypsin (2.5 µg/ml) for 30 min at 37 °C before serial dilution. Confluent monolayers of LLC-MK₁ cells (6-well plates) grown in medium 199 (5% fetal calf serum) were infected with diluted virus in a volume of 0.4 ml for 90 min at 37 °C with occasional gentle agitation. Virus inoculum was removed and 2 ml of 0.5% agar (Difco Bacto Agar, in 1 × media 199, no serum, and 2 ml of guinea pig red blood cells) was immediately added, then incubated for 30 min at room temp. Red blood cells were removed and washed twice with phosphate-buffered saline. Plaques were scored as well defined areas of red blood cells.

RESULTS

In Vitro Phosphorylation of Sendai P Protein Expressed in E. coli—To determine the cellular kinase phosphorylating the Sendai virus P protein, we prepared a cDNA clone of full-length P gene by reverse transcription of genomic Sendai viral RNA followed by PCR amplification. A C-terminal heptahistidine tag
was added as described under “Experimental Procedures.” Recombinant P protein was expressed in E. coli in its unphosphorylated (P0) form and purified by affinity chromatography on a Ni2+ affinity column. A nearly homogeneous preparation of Sendai P protein was obtained as judged by Coomassie Blue-stained acrylamide gel (data not shown). The P0 protein was then used as a substrate for characterization of cellular kinases purified from CV-1 cell extract. The soluble (S-100) fraction of CV-1 cells was collected and further fractionated by passage through DEAE-cellulose where phosphorylation of Sendai P0 protein by those fractions was assayed. As shown in Fig. 1A, cellular kinase phosphorylating P0 protein eluted in a broad peak at ~0.25 M NaCl. The active fractions were pooled and further fractionated over a phosphocellulose column, where the major P0 phosphorylating activity eluted in the unbound (flow-through) fraction. Two minor activities at ~0.3 M NaCl (fraction number 15) and ~0.6 M NaCl (fraction number 30) were also observed (Fig. 1B). To characterize the major cellular kinase(s) present in the flow-through fraction, we determined the effect of different inhibitors on the P0 phosphorylating activity (Fig. 2A). It can be seen that staurosporine (400 nM) completely inhibited P protein phosphorylation while heparin (5 μg/ml) had no apparent effect. These data demonstrated that a PKC-like activity is present in the unbound fraction which phosphorylates the P0 protein. The active fractions from the phosphocellulose column were subjected to an additional round of DEAE-cellulose and hydroxylapatite column purification. Fold purification was monitored throughout the chromatographic steps and was approximately 1000-fold after the final hydroxylapatite column as shown in Table I. Due to the low yield of kinase activity following hydroxylapatite column fractionation, the phosphocellulose flow-through fraction was used for most purposes described herein. However, the enzymatic properties of both purified fractions were compared and found to be indistinguishable.

To directly confirm the presence of PKC in the phosphocellulose column flow-through fraction, it was subjected to immunoblot analysis using antibodies against specific PKC isoforms (α, β, δ, ε, γ, and ζ). These PKC isoforms were chosen because of their wide distribution in a variety of tissue types (26). As shown in Fig. 2B and Fig. 4, only the antibody specific for PKC ζ isoform detected a band of approximately 68 kDa in the flow-through fraction. An antibody raised against the α subunit of casein kinase II did not cross-react with proteins present in the unbound fraction. All antibodies were tested for their reactivity with corresponding authentic kinases (data not shown). Similar Western blot analysis using antibodies against PKC isoform η were carried out and found to be negative (data not shown). The phosphorylation of P0 by PKC ζ was further confirmed using purified recombinant PKC ζ along with other isoforms including α and η in the in vitro kinase reactions (Fig. 2C). While all three isoforms were found to be capable of phosphorylating P0, PKC ζ had the greatest activity for P0 (see “Experimental Procedures”) indicating involvement of PKC ζ in P0 phosphorylation. Next, we examined the cofactor requirements of the cellular kinase to confirm that it is indeed a member of the novel PKC family (26, 27, 28). As shown in Fig. 3, phosphorylation of P0 by the cellular kinase was stimulated by about 3-fold in the presence of PS, whereas the addition of Ca2+ or DAG had no stimulatory effect. In fact, addition of Ca2+ and DAG was slightly inhibitory (lane 6). Furthermore, the addition of EGTA (500 mM) had no effect on P0 phosphorylation (data not shown). These data are consistent with the cofactor requirements of PKC ζ.

**Presence of PKC ζ in Purified RNP—**To investigate whether PKC ζ was specifically packaged into Sendai virion, we disrupted twice pelleted, purified virus in the presence 0.4 M NaCl and 1.85% Triton X-100, then purified the RNP complex by gradient centrifugation as described under “Experimental Procedures.” Even numbered gradient fractions were subjected to SDS-PAGE analysis then stained by Coomassie Blue (Fig. 4A). Viral nucleocapsid (NP) and phosphoproteins (P) appear almost exclusively in the bottom, pellet fraction. In contrast, the viral matrix protein (M) and envelope proteins, hemeagglutinin-neuraminidase, and fusion can be seen in fractions near the top of the gradient indicating that the viral envelope was disrupted by the detergent and salt treatment. Immunoblot analysis of gradient fractions with a PKC ζ-specific antibody

![Figure 1](http://www.jbc.org/)

**Fig. 1. Purification of cellular kinase.** Fractions eluted from DEAE-cellulose (A), phosphocellulose (B), and hydroxylapatite (C) columns were tested for protein kinase activity using bacterially expressed Sendai P0 protein as substrate. Phosphorylated proteins were analyzed by 10% SDS-PAGE and detected by autoradiography of the stained and dried gel. The migration position of Sendai P is shown. Column flow-through (FT) and eluted fraction numbers are as indicated above each lane.

![Figure 2](http://www.jbc.org/)

**Fig. 2. Characterization of cellular kinase(s) in phosphocellulose column flow-through fraction.** A, effect of different inhibitors on the phosphorylation of Sendai P0. Heparin (5 μg/ml) and staurosporine (400 nM) were included in the kinase reaction. The migration of P is shown. B, Western blot analysis of the phosphocellulose column flow-through fraction using antibodies specific for PKC α, β, δ, ε, γ, and ζ isoforms, and by antibody against casein kinase II. The position of PKC ζ is indicated. C, phosphorylation of Sendai P0 by recombinant PKC α, η, and ζ isoforms. Equal amounts (activity) based upon phosphorylation of peptide α (data not shown) were used for the labeling of Sendai P0. Sendai P migration is indicated to the left.
Phosphorylation of Sendai Virus P Protein

Table I

Purification of cellular kinase

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Fig. 3. Identification of cellular kinase as PKC ζ by cofactor requirements. Bacterially expressed Sendai P0 was 32P-labeled in vitro by a cellular kinase in the phosphocellulose column unbound fraction in the presence or absence of PS (100 μg/ml), DAG (100 μg/ml), and CaCl2 (200 μM) as indicated. Where CaCl2 was not added, EGTA (500 mM) was included in the reaction mixture. Radiolabeled proteins were denatured in Laemmli sample buffer then fractionated by 10% SDS-PAGE. Phosphorylation was visualized by autoradiography, and kinase activity quantified by PhosphorImager. Baseline activity (labeling of P0 without addition of cofactors, lane 1) was denoted as 100%.

Fig. 4. PKC ζ is tightly associated with RNP in Sendai virion. Detergent disrupted Sendai virion were centrifuged through a 15–30% continuous sucrose gradient. Twenty fractions of equal volume were collected with 1 representing the top and 20 representing the bottom of the gradient. A, even numbered gradient fractions were fractionated by 10% SDS-PAGE and Coomassie stained to reveal the protein profile of gradient fractions. Hemeagglutinin-neuraminidase (HN), fusion protein (F), matrix protein (M), phosphoprotein (P), and nucleocapsid (NP). B, Western blot analysis of even numbered gradient fractions developed with specific anti-PKC ζ antibody. The position of PKC ζ is indicated to the left. C, bacterially expressed Sendai P0 phosphorylating activity (hatched columns) and NP mRNA transcriptional activity (solid columns) were assayed in vitro using even numbered gradient fractions as the source of kinase and Sendai RNP. The relative levels of P protein phosphorylation and NP mRNA transcription in each fraction were determined by PhosphorImager analysis, with values in fraction 20 denoted as 100%.

Inhibitory Peptide—To investigate whether the observed phosphorylation of Sendai P protein is important in the life cycle of the virus, we used specific pseudosubstrate peptides to inhibit PKC activity during virus replication as described under “Experimental Procedures.” Pseudosubstrate peptides are derived from sequence motifs found near the N terminus of all PKC isoforms, and are similar to the phosphorylation site consensus sequence, except that serine or threonine residues are changed to alanine. This motif cannot be phosphorylated and represents an autoregulatory feature by blocking the catalytic (substrate binding) site (26). Pseudosubstrate peptides have been shown to be quite efficient inhibitors of PKC activity both in vitro and in situ (29, 30). We delivered PKC ζ-specific inhibitory peptide (peptide Z) into LLC-MK2 cells prior to Sendai virus infection. In control cells, the pseudosubstrate peptide A, which inhibits conventional PKC activity (e.g. PKCs α, β, and γ) was used (31)
and replication of Sendai was measured by heme-adsorption plaque assay. As shown in Fig. 6, peptide Z dramatically reduced production of progeny Sendai virus in a concentration-dependent manner, while the presence of peptide A had no apparent effect even at the highest concentration tested demonstrating that PKC\(_\text{z}\) indeed plays a critical function in the Sendai virus life cycle.

A kinetic study was undertaken to analyze whether positive or negative sense transcriptional activity (i.e., transcription or replication) was debilitated by the inhibition of cellular PKC\(_\text{z}\) activity. LLC-MK2 cells were transfected with peptides A and Z (4 mM) then infected with Sendai virion, and total intracellular RNA collected at early time points from 1 to 9 h post-inoculation. Samples were then subjected to Northern dot blot analysis using strand specific positive and negative strand-specific riboprobes derived from the Sendai viral NP coding sequence. Accumulation of progeny positive (A) and negative (B) strand RNAs was monitored by PhosphorImager analysis. C, ratio of positive to negative strand signal.

The discovery of divergence in the types of cellular kinase parasitized by several negative-strand RNA viruses presents an opportunity to study the molecular basis of this critical host-virus interaction. Previous studies have shown that the P proteins of VSV (11, 12) and respiratory syncytial virus (14) are phosphorylated by cellular casein kinase II. On the other hand, the cellular kinase phosphorylating the HPIV3 P protein was identified as the \(\text{z}\) isoform of PKC (24, 32). In all of these viruses an essential role for these kinases was established.

Recently, the P protein of measles virus has been shown to be phosphorylated by casein kinase II (17) as well as by PKC\(_\text{z}\) (2). Similarly, cellular casein kinase II and PKC\(_\text{z}\) have been shown to be involved in phosphorylating the P protein of canine dis-
temper virus, a canine measles virus. Thus, phosphorylation by specific cellular kinase(s) appears to be an important factor in the life cycle of negative strand RNA viruses infecting a variety of organisms with a wide range of organ and tissue tropisms. We were interested to extend these observations to the murine Sendai virus, a type 1 parainfluenzavirus distantly related to HPIV3, which is extensively used in the laboratory as a prototype of paramyxoviridae. Sequence analysis of P protein from the two viruses reveals only 23% sequence similarity (33). However, these two proteins appear to be structurally similar as shown by their conserved distribution of hydrophilic regions in hydropathicity plots. In the present study we investigated whether similar cellular kinase(s) are used to phosphorylate the P protein. We observed three distinct cellular fractions having substrate affinity for Sendai virus P protein, one major and two minor. Inhibition of protein kinase activity in the major fraction by staurosporine indicated that the kinase was a member of the PKC family. This large family of serine/threonine kinases is subdivided into the conventional PKCs that require Ca<sup>2+</sup>, phospholipid, and DAG or phorbol esters for activation, and the novel PKCs that do not require Ca<sup>2+</sup> or DAG for activation (26–28). These different requirements for activation are reflected in the presence or absence of cofactor-binding domains in the variable N-terminal half of PKC isozymes (26).

By biochemical and immunological means the major Sendai virus P protein kinase was identified as a member of the nPKC family, and was precisely characterized as nPKC ζ. We also observed that PKC ζ was specifically packaged into virion and remained tightly associated with RNP despite detergent treatment and gradient centrifugation. The role of PKC ζ in the Sendai virus life cycle was directly demonstrated when we delivered PKC ζ-specific inhibitory peptide into LLC-MK<sub>2</sub> cells in tissue culture and observed a dose-dependent decrease in the accumulation of progeny Sendai virion. To further investigate the role that PKC ζ plays in the viral life cycle, we examined the kinetics of viral transcription and replication product accumulation in the presence of PKC ζ inhibitory and control pseudosubstrate peptides (Fig. 7). We observed that both positive and negative strand accumulation was greatly inhibited by the PKC ζ inhibitory peptide. This result is not unexpected because reduced synthesis of either messenger or genomic RNA will lead inevitably to a reduction in the other. However, because synthesis of positive strand was reduced both to a greater extent and at an earlier time, the data suggest that PKC ζ activity plays a role in the primary transcription of Sendai viral mRNAs and may explain why PKC ζ is specifically packaged into mature virion. The identities of the two minor P<sub>0</sub> phosphorylating activities which constitute approximately 10% of the total kinase activity (Fig. 1B, fractions 15 and 30) have not yet been determined. However, heparin inhibition and Western blot analysis of the kinase activity in fraction 30 suggest that P<sub>0</sub> phosphorylating activity may be casein kinase II (data not shown). Further work is in progress to characterize these minor protein kinases and study their roles, if any, in Sendai virus gene expression.

By deletion mapping, Curran et al. (10) have identified two acidic domains within the N-terminal acidic domains of Sendai P protein, amino acids 1–77 or 78–145, either of which could support mRNA transcription in vitro. They observed both increased and decreased phosphorylation of the P protein that could not be correlated with the reconstituted transcription activity. However, because these studies used whole cell extracts containing the P protein mutants, it is possible that the function of the acidic domains was provided in trans by acidic cellular proteins (34). In fact there is precedence for this concept. Replacement by an acidic protein, tubulin, of the N-terminal domain in VSV P protein, both in cis and in trans, supported transcription of the VSV genome (35). Thus, the deletion analysis may not be well suited to investigate the role of phosphorylation in the Sendai virus P protein. Further study, using defined site-directed mutagenesis of potential phosphorylation sites in Sendai P protein, in combination with in vitro transcription and/or replication assay may help to solve this problem.

Recently, Byrappa et al. (36) examined the pattern of phosphorylation in the Sendai P protein and reported seven strongly phosphorylated sites at serine residues, six of which were phosphatase sensitive. In addition, numerous less strongly phosphorylated sites were reported, suggesting that P protein may have more than one biologically relevant phosphorylated form. Using recombinant vaccinia virus to express Sendai virus, Byrappa et al. (37) reported identification of one major phosphorylation site as serine 249. Involvement of a proline-directed protein kinase activity, such as the family of cyclin-dependent and mitogen-activated protein kinases was suggested. Mutation of serine 249 or proline 250 eliminated phosphorylation at serine 249, with concomitant increase in the level of phosphorylation at several other sites. Whether phosphorylation of serine 249 has a relevant biological function has not yet been addressed. The apparent discrepancy between their results and our findings presented in this article may have resulted from the different approaches followed. We have used bacterially expressed P<sub>0</sub> and fractionated cell lysates to identify protein kinase activities. This technique has been applied successfully to identify cellular kinases phosphorylating P proteins of VSV, respiratory syncytial virus, and HPIV3, where in each case the biological relevance of the specific cellular kinase activity has been demonstrated (11, 12, 19, 24).

Using the similar techniques, our data provide strong evidence that PKC ζ also plays a critical role in the life cycle of Sendai virus. The function of the proline-directed kinase in the Sendai virus life cycle remains to be elucidated.

In addition to the phosphorylation of P protein, we have observed that the nucleocapsid protein is also phosphorylated by a protein kinase tightly associated with viral RNP (Fig. 5). The pattern of cofactor requirements and Western blot analysis suggests that the kinase involved is a member of the nPKC family. To date very little is known about the function of nucleocapsid protein phosphorylation or of the kinase(s) involved (2, 38). Moreover, Gambert et al. (39) recently reported that soluble measles virus N protein contained only phosphoserine residues, whereas RNP associated N protein contained both phosphoserine and phosphothreonine modifications. This additional phosphorylation may be related to a conformational change that measles virus N protein undergoes prior to rapid incorporation into viral nucleocapsids (40). Intriguingly, in persistent infection of neuronal cells by measles virus resulting in subacute sclerosing panencephalitis, a fatal human disease, N protein gains additional phosphorylation on tyrosine residue(s) (41). Thus, it seems that more than one kinase may be involved in nucleocapsid protein phosphorylation.

Utilization of PKC ζ specifically by Sendai virus for P protein phosphorylation is interesting because the PKC family contains 10 or more different isozymes whose distribution and relative levels vary greatly among tissue and cell types (26). However, PKC ζ appears to be ubiquitously distributed having been detected in brain, lung, skin, spleen, thymus, and other tissues (26), making it an attractive candidate for molecular parasitism. While the role of PKC ζ in cellular function and its
normal protein substrate(s) are not yet well characterized, recent work has provided evidence that PKC ζ physically interacts with and is a downstream target of Ras (31, 42, 43) and that PKC ζ participates in NF-κB activation in human immuno deficiency virus-infected monocytes (44) and in the in vivo stimulation of mitogen-activated protein kinase and mitogen-activated protein kinase kinase (45). The latter work suggests that PKC ζ is an important intermediary both in mitogenic signaling and in inflammatory pathways. Therefore, an intriguing question is whether intracellular PKC ζ levels are altered in response to infection by Sendai and HPIV3. It is interesting to note that infection of macrophages by bovine parainfluenza type 3 results in the selective depletion of an as yet unidentified calcium-independent phospholipid-dependent novel PKC (46). It remains to be seen if depletion is related to the selective packaging of a PKC isozyme. Because PKC ζ activity appears to be required during the Sendai virus life cycle, this study may provide a window of opportunity to manipulate this specific virus-host interaction in a large family of viruses that have a significant health and economic impact to society.

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REFERENCES