Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5204–5208, May 1995
Biochemistry

Cellular protein kinase C isoform ζ regulates human parainfluenza virus type 3 replication

(protein phosphorylation/viral replication/RNA virus)

BISHNU P. DE*, SEEMA GUPTA, SANHITA GUPTA, AND AMIYA K. BANERJEE*

Department of Molecular Biology, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195

Communicated by George R. Stark, The Cleveland Clinic Foundation, Cleveland, OH, March 3, 1995

ABSTRACT **Phosphorylation of the P proteins of nonsegmented negative-strand RNA viruses is critical for their function as transactivators of the viral RNA polymerases. Using unphosphorylated P protein of human parainfluenza virus type 3 (HPIV3) expressed in *Escherichia coli*, we have shown that the cellular protein kinase that phosphorylates P *in vitro* is biochemically and immunologically indistinguishable from cellular protein kinase C isoform ζ (PKC-ζ). Further, PKC-ζ is specifically packaged within the progeny HPIV3 virions and remains tightly associated with the ribonucleoprotein complex. The P protein seems also to be phosphorylated intracellularly by PKC-ζ, as shown by the similar protease digestion pattern of the *in vitro* and *in vivo* phosphorylated P proteins. The growth of HPIV3 in CV-1 cells is completely abrogated when a PKC-ζ-specific inhibitor pseudosubstrate peptide was delivered into cells. These data indicate that PKC-ζ plays an important role in HPIV3 gene expression by phosphorylating P protein, thus providing an opportunity to develop antiviral agents against an important human pathogen.**

Human parainfluenza viruses (HPIVs) are members of the paramyxovirus family (1, 2). Understanding the molecular events that control HPIV replication is particularly important because they are significant human pathogens causing diseases such as croup, pneumonia, and bronchiolotis in children (3). No suitable vaccines are currently available for these classes of viruses. HPIV type 3 (HPIV3), most pathogenic among the HPIVs, contains a linear genomic RNA (15,461 nt) of negative polarity and three structural proteins, NP (68-kDa nucleocapsid protein), L (251-kDa large protein), and P (90-kDa phosphoprotein), all of which are enclosed within a lipid-containing envelope (1, 2). The L and P proteins together constitute the viral RNA-dependent RNA polymerase complex that, similar to that of other paramyxoviruses, transcribes the NP-bound genomic RNA (4–6); L is the RNA polymerase, whereas P is a required transcription factor or transactivator of L. Host cytoskeletal proteins can act as positive regulators in viral mRNA synthesis—e.g., actin in HPIV3 (7) or tubulin in Sendai and measles viruses (8, 9).

Phosphorylation of the P proteins of nonsegmented negative-strand RNA viruses is believed to be mediated by cellular protein kinases and is an important step in the formation of the active RNA polymerase complex (10–12). Cellular casein kinase II (CKII) has been implicated in phosphorylation of P proteins of vesicular stomatitis virus (VSV) (10) and respiratory syncytial virus (11, 12). In the well-studied VSV system, use of unphosphorylated P protein (P_0) obtained from a cDNA-derived expression in *Escherichia coli* has demonstrated that a cascade phosphorylation pathway is operative for P-protein activation *in vitro* (13). The first step in the pathway is the phosphorylation mediated by cellular CKII, leading to a structural alteration of the P protein (P1 form). The second step involves further phosphorylation of P1 by the protein kinase activity associated with the L protein, leading to the formation of the fully phosphorylated P2 form. This phosphorylation pathway is obligatory for the activation of P protein to function in the RNA polymerase complex. Here we report that HPIV3, in contrast, selects a specific isoform (ζ) of cellular protein kinase C (PKC-ζ) for phosphorylation of its P protein and also packages the same isoform within the virion. Specific inhibition of PKC-ζ activity in the infected cells abrogates HPIV3 replication. Since the PKC-ζ activity in cells can be downregulated without affecting significantly the normal cellular function (14, 15), this pathway may be selectively targeted in developing antiviral agents for clinical use.

MATERIALS AND METHODS

Expression of HPIV3 Gene in *E. coli*. The HPIV3 P cDNA clone was a gift from Mark Galinski (The Cleveland Clinic Foundation) and was subcloned into the bacterial expression vector pET-3a as described (16), except that the cloned gene was further engineered to encode a stretch of seven histidine residues at the carboxyl terminus of the P protein. The bacterially expressed P_0 was purified on a Ni^{2+} affinity column (Novagen).

Protein Kinase Assay. *In vitro* phosphorylation of HPIV3 P_0 (2 μg) and other substrates was done in a 30-μl reaction mixture containing 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 0.25% bovine serum albumin, 50 μM ATP, 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq), and, as indicated, 200 μM $CaCl_2$, phosphatidylserine (PS, 100 μg/ml), diacylglycerol (DAG, 100 μg/ml), and purified cellular kinase. The mixture was incubated at 30°C for 1 hr, and the radiolabeled proteins were analyzed by SDS/10% PAGE. Synthetic peptide substrates were used at 40 μM. Incorporation of radioactivity into peptides was measured by phosphocellulose paper binding assay (17).

Purification of Cellular Protein Kinase. Simian CV-1 cells (2×10^8) were lysed by freezing and thawing in 10 ml of 10 mM Tris·HCl, pH 7.5/10 mM NaCl. The lysate was centrifuged at 10,000 × *g* for 10 min and the supernatant was further clarified by centrifugation at 100,000 × *g* for 1 hr. The 100,000 × *g* supernatant (S100) was dialyzed overnight against buffer A [50 mM Tris·HCl, pH 7.5/50 mM NaCl/5% (vol/vol) glycerol/0.1 mM EDTA/1 mM dithiothreitol]. The dialyzed supernatant was loaded onto a DEAE-cellulose column (4 ml) equilibrated with buffer A and the column was washed with 5 column volumes of buffer A. The column was developed with a linear gradient of 0–0.4 M NaCl in buffer A (20 ml; 0.1 ml per fraction). The protein kinase that phosphorylated P_0 was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HPIV, human parainfluenza virus; VSV, vesicular stomatitis virus; CKII, casein kinase II; PKC, protein kinase C; P_0, unphosphorylated P protein; DAG, diacylglycerol; PS, phosphatidylserine; RNP, ribonucleoprotein.

*To whom reprint requests should be addressed.

eluted at ≈0.25 M NaCl. The active fractions were pooled and loaded onto a phosphocellulose column (4 ml) which was then washed with 6 ml of buffer A plus 0.2 M NaCl. The column was developed with a linear gradient of 0.2–1.0 M NaCl (20 ml; 0.1 ml per fraction). The HPIV3 P_0 phosphorylating activity, present in the unbound fraction, was then chromatographed on a second DEAE-cellulose column and a heparin-agarose column (18). The activity was present mainly in the unbound fraction of the heparin-agarose column, whereas only ≈20% of the total activity was present in the bound fraction that was eluted in a broad peak at ≈0.6 M NaCl. The heparin-agarose unbound fraction was used in all subsequent experiments, and the protein concentration in this fraction was estimated at ≈20 μg/ml.

RESULTS

***In Vitro* Phosphorylation of HPIV3 Protein Expressed in *E. coli*.** To determine which cellular kinase(s) mediates phosphorylation of the P protein of HPIV3, we expressed recombinant P_0 in *E. coli* and purified it as detailed in *Materials and Methods*. An S100 fraction from CV-1 cells was prepared and was purified by chromatography on DEAE-cellulose, where the kinase activity that phosphorylated P_0 of both VSV and HPIV3 was eluted at ≈0.25 M NaCl (Fig. 1*A*). However, when further fractionated in a phosphocellulose column, the HPIV3 P_0 phosphorylating activity was clearly separated from the VSV P_0 phosphorylating activity (i.e., CKII) and was eluted in the unbound fraction. The HPIV3 P_0-specific kinase was further purified by chromatography on a second DEAE-cellulose column and a heparin-agarose column. This purified protein kinase (referred to as cellular kinase) was used in all subsequent experiments. The cellular kinase was found to contain a major 64-kDa protein, as detected in a silver-stained SDS/polyacrylamide gel (see below).

To characterize the cellular kinase, first we determined the effect of various protein kinase inhibitors on the phosphorylation of HPIV3 P_0 (Fig. 2*A*). The phosphorylation of P_0 was virtually abolished in the presence of staurosporine (200 nM),

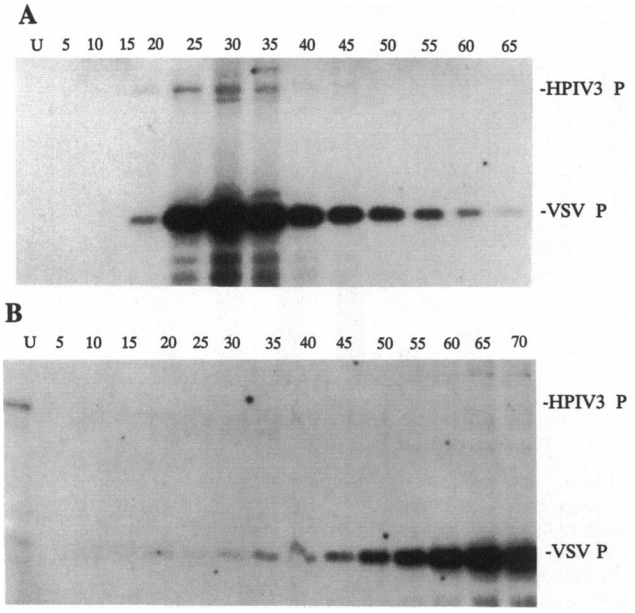

FIG. 1. Purification of cellular kinase. Each fraction (2-μl aliquot) eluted from DEAE-cellulose (*A*) and phosphocellulose (*B*) columns was tested for protein kinase activity using bacterially expressed P_0 proteins of HPIV3 and VSV (10). Phosphorylated proteins were analyzed by SDS/10% PAGE and detected by autoradiography of the stained and dried gel. Migration positions of P proteins are shown. U, unbound fraction; numbers above lanes represent the eluted fractions.

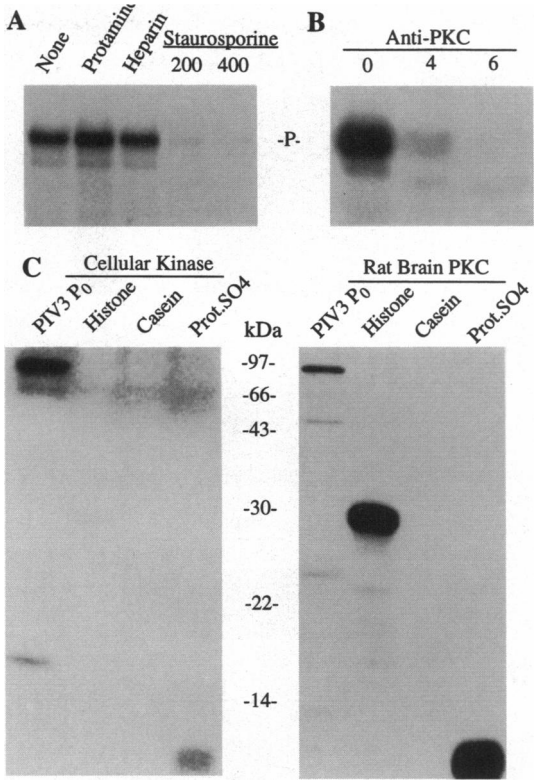

FIG. 2. Properties of the cellular kinases. (*A*) Effect of various inhibitors on the phosphorylation of HPIV3 P_0. Protamine (25 μg/ml), heparin (40 μg/ml), or staurosporine (200 or 400 nM) was included in the kinase reaction mixture. (*B*) Inhibition of cellular kinase activity by anti-PKC antibody (Boehringer Mannheim) that inhibits all isoforms was added (0, 4 or 6 μg) to the reaction mixture. (*C*) Phosphorylation of various substrates (2 μg each) by cellular kinase (2 μl) (*Left*) and rat brain PKC (0.01 μg); (Promega) (*Right*). The ^{32}P-labeled proteins were analyzed as in Fig. 1. Size markers are indicated.

whereas protamine and heparin, specific inhibitors of VSV L-protein-associated kinase (13) and CKII, respectively, had no inhibitory effect. These data suggest that a PKC-like activity present in the cellular kinase is involved in the phosphorylation of HPIV3 P_0. Next, we examined the presence of PKC activity in the cellular kinase more directly by using an active-site-specific anti-PKC antibody. The phosphorylation of HPIV3 P_0 was abolished in the presence of the anti-PKC antibody (Fig. 2*B*), indicating that the phosphorylation is mediated by PKC. The substrate specificity of the cellular kinase was then tested and compared with that of a commercial rat brain PKC containing primarily α, β, and γ isoforms. HPIV3 P_0 was the best phosphate acceptor among the substrates tested for the cellular kinase (Fig. 2*C*). The substrate specificity of the commercial PKC was quite different. These results indicate that the HPIV3 P_0 phosphorylation is probably mediated by a specific isoform of PKC other than conventional PKC, present in the purified cellular kinase.

Phosphorylation of HPIV3 P Is Mediated by Cellular PKC-ζ. To establish the isoform class of the PKC that phosphorylates HPIV3 P_0, we carried out Western blot analyses using isoform-specific antibodies. The four antibodies, raised against the isoforms α, β, δ, and ζ, were selected because these isoforms are known to exist in many tissues and cell types. Only anti-PKC-ζ recognized a 64-kDa polypeptide (Fig. 3*A Left*), indicating that the cellular kinase is immunologically similar to PKC-ζ. Consistent with this finding, the protein pattern of the purified cellular kinase showed a major 64-kDa protein in a silver-stained SDS/polyacrylamide gel (Fig. 3*A Right*). This is also in agreement with the previous finding that the 64-kDa

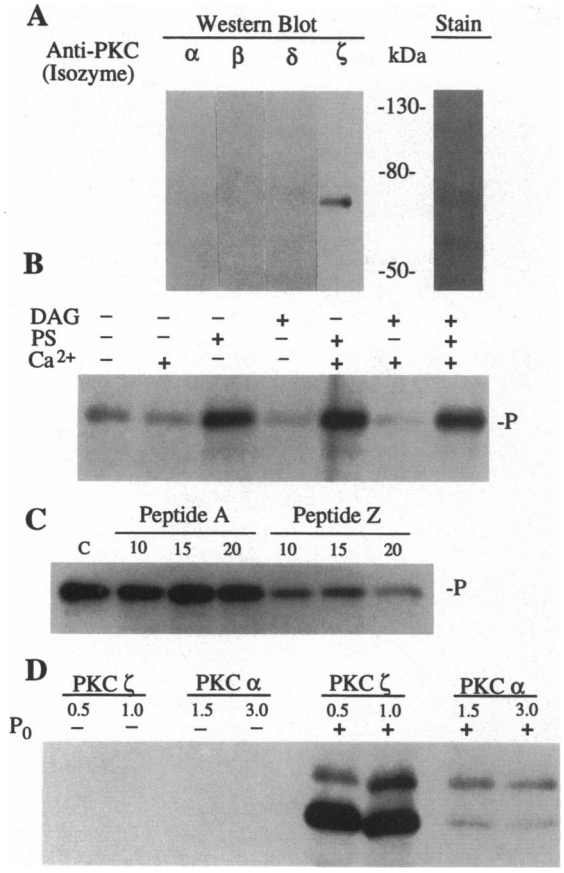

FIG. 3. Characterization of the cellular kinase. (*A*) (*Left*) Western blot analysis with PKC isoform-specific antibodies. The cellular kinase (20 μl) was electrophoresed in an SDS/10% polyacrylamide gel and transferred to a GeneScreen membrane (DuPont) for Western blot analyses with isoform-specific antibodies (GIBCO/BRL) according to the protocol supplied by DuPont. Detection was by chemiluminescence (ECL; Amersham). Prestained size markers are indicated. (Right) Silver-stained gel. (*B*) Requirement of cofactors for the phosphorylation of HPIV3 P_0. Phosphorylation of HPIV3 P_0 (2 μg) was done in the absence or presence of DAG, PS, and Ca^{2+}. Radiolabeled proteins were analyzed as in Fig. 1. (*C*) Specific inhibition of cellular kinase activity by PKC-ζ-specific peptide inhibitor. Peptides A and Z (15), containing the amino acid sequences RKGALRQKN and RRGARRWRK, respectively, were synthesized (Quality controlled Biochemicals, Hopkinton, MA) and added at 10, 15, or 20 μM to the kinase reaction mixture. Lane C, control. (*D*) Phosphorylation of HPIV3 P_0 by baculovirus-expressed recombinant PKC-ζ. Aliquots (μl) of recombinant PKC isoforms, based upon identical phosphorylation of peptide α, were used for the phosphorylation of HPIV3 P_0. Reaction mixtures for PKC-α contained PS, DAG, and Ca^{2+}, while those for PKC-ζ contained PS only. The slower migrating phosphorylated band corresponds to the full-length P.

form predominantly exists in the cytosol (15, 19). The other, minor protein bands probably were contaminants still present in the purified fraction. Next, we determined the requirement of cofactors for the phosphorylation of HPIV3 P_0. Phosphorylation of P_0 by purified cellular kinase was stimulated about 5-fold in the presence of PS, whereas Ca^{2+} and DAG had no such effect (Fig. 3*B*). Consistent with these data, the Ca^{2+} chelator EGTA (0.5 mM) had no inhibitory effect on phosphorylation (data not shown). These results confirm that the PKC-ζ isoform is involved in HPIV3 P_0 phosphorylation. To investigate whether specific inhibition of PKC-ζ activity in purified cellular kinase is sufficient to inhibit the phosphorylation of HPIV3 P_0, we used two pseudosubstrate peptide inhibitors, peptides Z and A, the former being a specific inhibitor of PKC-ζ and the other an inhibitor of PKC isoforms α, β, and γ (15). Phosphorylation of HPIV3 P_0 was inhibited >75% by the PKC-ζ-specific peptide, peptide Z, whereas peptide A did not inhibit the phosphorylation (Fig. 3*C*). Phosphorylation of HPIV3 P_0 by PKC-ζ was then directly demonstrated by using recombinant PKC-ζ in the *in vitro* phosphorylation assay. Recombinant PKC-ζ efficiently phosphorylated HPIV3 P_0, while under identical conditions only a very low level of phosphorylation was observed with recombinant PKC-α (Fig. 3*D*). The faster migrating PKC-ζ phosphorylated band was a proteolytically degraded form of P, as confirmed by immunoprecipitation (data not shown). We conclude that PKC-ζ is specifically involved in the phosphorylation of HPIV3 P_0.

Presence of PKC-ζ in Purified Virions. To investigate whether PKC-ζ is also packaged in the virion, we determined the protein kinase activity in the virion particles. Purified virus particles were disrupted with 0.4 M NaCl in the presence of 1.8% Triton X-100 and the ribonucleoprotein (RNP) complex was purified (5). The packaging of a protein kinase was apparent when RNP was incubated with [γ-^{32}P]ATP, which resulted in the phosphorylation of P protein (Fig. 4*A*). The phosphorylating activity was stimulated about 2-fold in the presence of PS and did not require Ca^{2+} or DAG. Further, the kinase activity was inhibited >90% by staurosporine (400 nM) (Fig. 4*A*), whereas heparin (40 μM) had no inhibitory effect (data not shown). The stimulatory effect of PS was lower than the cellular kinase-mediated phosphorylation of P_0 (Fig. 3*B*).

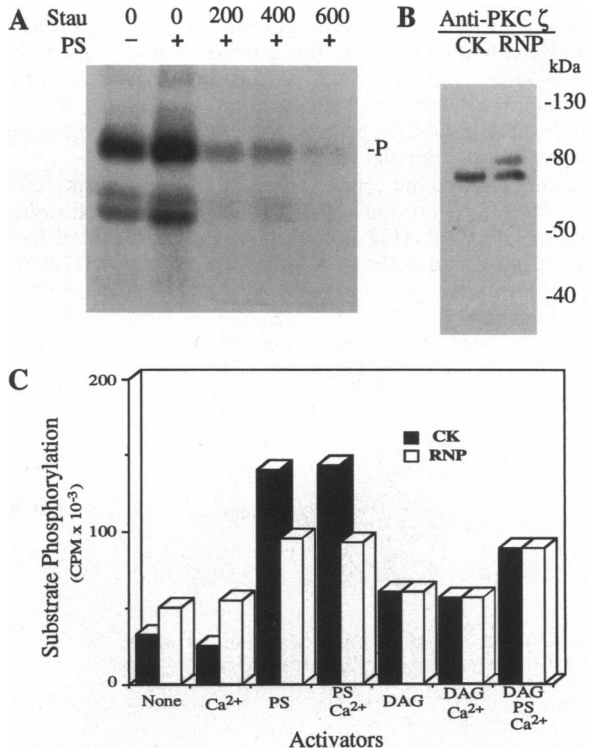

FIG. 4. Packaging of PKC-ζ in HPIV3 virions. (*A*) Phosphorylation of virion-associated P. The RNP (5 μg) of purified virions (5) was used in the kinase reaction with 0–600 nM staurosporin (Stau) and PS as indicated. Radiolabeled proteins were analyzed by SDS/10% PAGE followed by autoradiography. (*B*) Identification of PKC-ζ in the RNP by Western blot with anti-PKC-ζ antibody. Purified RNP (30 μg) and 20 μl of the cellular kinase (CK) were subjected to SDS/PAGE and Western blot analyses on GeneScreen membrane. The immunoblots were developed with anti-PKC-ζ antibody as in Fig. 3. (*C*) Phosphorylation of peptide α by cellular and virion-associated protein kinases. Cellular kinase (CK, 0.04 μg) or RNP (5 μg) was used for the phosphorylation of peptide α (40 μM). Phosphorylation was monitored by phosphocellulose paper binding assay (17). PS (100 μg/ml), DAG (100 μg/ml), and Ca^{2+} (200 μM) were present as indicated.

The reason for this seems to be the presence of endogenous PS derived from viral membrane that remained associated with the RNP (20). These results indicate that the protein kinase which is tightly packaged in the virion is similar in properties to PKC-ζ. The phosphorylation of RNP-associated P by the packaged protein kinase suggests that at least a fraction of the P proteins in the virion is in the unphosphorylated form. We confirmed the isoform type of protein kinase associated with the virion by Western blot analysis using isoform-specific antibodies. Two polypeptides were detected in the RNP by anti-PKC-ζ (Fig. 4*B*), indicating that two different forms of PKC-ζ (64 and 78 kDa) are packaged in the virion. The existence of PKC-ζ in these two molecular forms has been reported prevously (15, 18, 19). In Western blot analyses CKII and PKC isoforms α, β, and δ were not detected, suggesting specific packaging of PKC-ζ in the virion (data not shown). In a separate series of experiments we studied the phosphorylation of two PKC-specific synthetic peptides, an N-acetylated myelin basic protein peptide [Ac-MBP-(4–14)] and peptide α {[Ser^{25}]PKC-(19–31)}, by the cellular and virion-associated protein kinases. The former peptide is not a substrate for PKC-ζ, whereas both the peptides are phosphorylated by most of the other isoforms (21). The cellular and the virion-associated protein kinase phosphorylated peptide α with almost similar cofactor requirements (Fig. 4*C*) but failed to phosphorylate Ac-MBP-(4–14) (data not shown), indicating that PKC-ζ is the sole cellular protein kinase involved in HPIV3 P phosphorylation and is packaged within the virion. This confirms that the protein kinase packaged in the virion is indeed the PKC-ζ isoform.

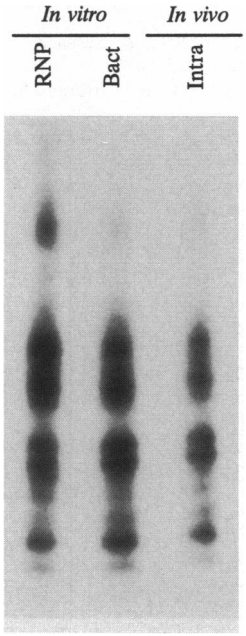

FIG. 5. Peptide mapping of *in vitro* and *in vivo* phosphorylated P proteins. For *in vitro* labeling either bacterially expressed P_0 (Bact, 2 μg) was phosphorylated by cellular kinase or the RNP-associated P (RNP, 5 μg) was phosphorylated by the virion-associated kinase. For *in vivo* labeling (Intra), CV-1 cells (10^5) were infected with HPIV3 (20 plaque-forming units per cell) and then incubated for 18 hr with 500 μCi of [^{32}P]orthophosphate in phosphate-free minimal essential medium containing actinomycin D (5 μg/ml). The cells were washed with phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris·HCl, pH 7.5/0.15 M NaCl/1 mM EDTA/1% Triton X-100/0.1% SDS/1% sodium deoxycholate) (300 μl). The cell debris was removed and the supernatant was treated with mouse anti-P serum for 2 hr. The complex was precipitated with protein A-Sepharose and electrophoresed in an SDS/10% polyacrylamide gel. The labeled P proteins were excised and subjected to digestion with *S. aureus* V8 protease during electrophoresis in an SDS/15% polyacrylamide gel; fragments were detected by autoradiography (19).

Intracellular Phosphorylation of P by PKC-ζ. The above experiments clearly show that cellular PKC-ζ phosphorylates bacterially expressed HPIV3 P_0 and is packaged within the virion. To investigate whether PKC-ζ is involved in phosphorylating HPIV3 P during infection, a structural comparison of the *in vitro* and *in vivo* phosphorylated P proteins was carried out by digestion with *Staphylococcus aureus* V8 protease and one-dimensional peptide mapping. HPIV3 P_0 and RNP-associated P were phosphorylated *in vitro*, and the *in vivo* phosphorylated P was obtained by anti-P immunoprecipitation from HPIV3-infected CV-1 cells metabolically radiolabeled with inorganic [^{32}P]phosphate. The *in vitro* and *in vivo* radiolabeled P proteins were resolved in an SDS/10% polyacrylamide gel and the bands were excised. The gel slices containing the P proteins were then subjected to partial digestion by V8 protease, and analyzed in an SDS/15% polyacrylamide gel (22). In all P-protein digests five major phosphorylated bands with similar electrophoretic mobilities were obtained (Fig. 5). These data suggest that the polypeptides phosphorylated *in vitro* and *in vivo* are similar, and that the phosphorylation may be mediated by PKC-ζ.

Inhibition of HPIV3 Replication by PKC-ζ-Specific Peptide Inhibitor. Finally, because phosphorylation of P is believed to be required for the formation of the active RNA polymerase complex for transcription and replication of the viral genome, we investigated whether inhibition of phosphorylation would abrogate HPIV3 viral transcription and replication. However, a reconstituted *in vitro* transcription system with purified viral and cellular proteins, free from contaminating protein kinase activity, is not available at the present time. Accordingly, we studied the replicative cycle of HPIV3 in cells in the presence of the PKC-ζ-specific pseudosubstrate inhibitor peptide Z. Peptide A was used as a control. These two peptides were delivered into CV-1 cells with a peptide delivery kit (BRL), and replication of HPIV3 following infection of these cells was measured by plaque assay. Replication of HPIV3 was completely inhibited in the presence of peptide Z (4 mM), whereas the peptide A had no effect on viral replication at the highest concentration tested (Fig. 6). At 4 mM, no toxic effect of peptide Z was observed, since the cells remained viable as

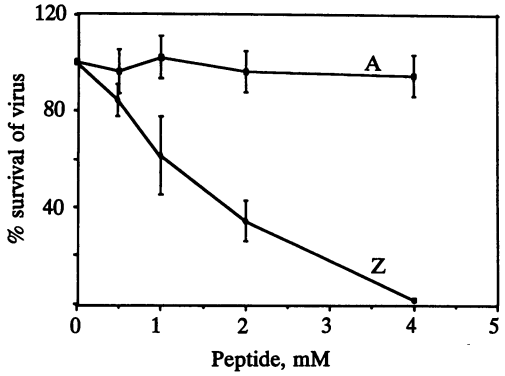

FIG. 6. Inhibition of HPIV3 replication by PKC-ζ pseudosubstrate in cultured cells. Peptides A and Z, as indicated, were delivered into subconfluent monolayers of CV-1 cells with a transport reagent kit (GIBCO/BRL). The cells in a 24-well plate were treated with the peptides plus transport reagent in a 200-μl volume for 15 min, 20 μl of stop solution was added, the medium was replaced with 400 μl of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, and the cells were incubated at 37°C for 30 min. The cells were then infected with HPIV3 (1 plaque-forming unit per cell) in 200 μl of DMEM without serum for 1 hr. Unabsorbed virus was removed by one wash with DMEM, and 300 μl of DMEM containing 10% fetal bovine serum was added. The released virus was harvested at 30 hr, and the infectious particles were measured by plaque assay.

judged by trypan blue exclusion and also supported the replication of VSV almost to the same level as the control cells (data not shown). Thus, all of these studies strongly indicate that the replication of HPIV3 requires PKC-ζ function. The phosphorylation of P protein in infected cells mediated by PKC-ζ appears to be the main target of the inhibitor.

DISCUSSION

We have shown that the cellular protein kinase that phosphorylates HPIV3 P protein in *in vitro* is biochemically and immunologically identical to PKC-ζ. The same protein kinase is involved in the phosphorylation of P protein in cultured cells and is also specifically packaged within the virion. Involvement of PKC-ζ in the regulation of gene expression of HPIV3 underscores the point that P proteins of negative-strand RNA viruses (with nonsegmented RNA genomes) interact with specific cellular protein kinases for their activation (10–12, 23). In this respect, HPIV3 bears both similarities and differences with the well-studied VSV system. Like VSV (23), HPIV3 also packages the protein kinase specifically involved in the phosphorylation of its P protein; however, the VSV P protein is phosphorylated by cellular CKII (13). Thus, it seems that the P proteins of the two viruses have evolved so that phosphorylation-mediated activation of the protein is regulated by two widely different cellular protein kinases.

The cellular CKII is a ubiquitous protein kinase and present in relatively high levels (24), whereas PKC exists in at least eight different isoforms whose relative levels vary among tissue and cell types (25, 26). Four isoforms (α, β1, β2, and γ), designated conventional PKC, require Ca^{2+}, phospholipid, and DAG or phorbol ester for activation. Other forms (δ, ε, ζ, η), designated novel PKC, have structures closely related to but clearly distinct from conventional PKC and do not require Ca^{2+} for activation (25, 26). The protein kinases in the conventional PKC family, which are mostly membrane associated, are the major regulatory enzymes involved in the control of many physiological processes including differentiation, tumor promotion, and membrane receptor function (25, 27). However, the cellular function and the physiological substrate for PKC-ζ have not been precisely characterized. Our studies reveal that HPIV3 P protein is an excellent substrate for PKC-ζ. Since the virus replicates in the cytoplasm of the cell, it appears that the soluble form of PKC-ζ in the cytoplasm is directly involved in P-protein phosphorylation. Why does HPIV3 selectively use PKC-ζ for P-protein phosphorylation among a multitude of protein kinases present in the cytoplasm? In the VSV system, cellular CKII-mediated phosphorylation of the P protein appears to alter the secondary structure of the protein, leading to its activation (T. Das and A.K.B., unpublished observation). In the same context, it is possible that the HPIV3 protein may have evolved to be phosphorylated by PKC-ζ for similar structural alteration.

Cellular protein kinases play a vital role in the gene expression of rhabdoviruses and paramyxoviruses. Phosphorylation of the RNA polymerase subunit P mediated by cellular protein kinase is presumably a necessary event for activation leading to transcription and replication. For HPIV3, a direct role of PKC is demonstrated by the fact that a PKC-ζ-specific pseudosubstrate, peptide Z, completely inhibits HPIV3 replication in tissue culture cells (Fig. 6). Since PKC-ζ is needed for HPIV3 replication, it is possible to search for cell lines where PKC-ζ activity is extremely low or develop cell lines where PKC-ζ activity is specifically depleted by ablation of its mRNAs (15, 28). The study of the regulation of HPIV3 gene expression in those cell lines following transfection with plasmids containing PKC-ζ cDNA would certainly help us understand the role of PKC-ζ in the life cycle of HPIV3. In this respect, it is interesting that respiratory syncytial virus, another highly pathogenic respiratory-disease virus, uses cellular CKII for P-protein phosphorylation (11, 12). Since both viruses infect respiratory epithelial cells, this raises the question whether respiratory syncytial virus and HPIV3 infect specific target cell types of the infected organ which provide the required source of the essential protein kinase. Detailed studies in this direction are needed to understand the mechanism of host–virus interaction. That PKC-ζ is involved in the life cycle of HPIV3 provides an opportunity to interfere with viral growth, for instance with synthetic peptides (15) or other agents designed to inhibit PKC-ζ activity.

Recombinant PKC isoforms were kindly provided by Drs. Alan Wolfman and Walter Kolch. This work was supported by U. S. Public Health Service Grant AI32027 (A.K.B.).

1. Galinski, M. S. & Wechsler, S. L. (1991) in *The Paramyoxviruses*, ed. Kingsbury, D. W. (Plenum, New York), pp. 41–82.
2. Banerjee, A. K., Barik, S., & De, B. P. (1991) *Pharmacol. Ther.* **51**, 47–70.
3. Chanock, R. M. & McIntosh, K. (1985) in *Virology*, ed. Field, B. N. (Raven, New York), pp. 1241–1253.
4. Hamaguchi, M., Yoshida, T., Nishikawa, K., Naruse, H. & Nagai, Y. (1983) *Virology* **128**, 104–117.
5. De, B. P., Lesoon, A. & Banerjee, A. K. (1991) *J. Virol.* **65**, 3268–3275.
6. Curran, J., Marg, J. & Kolakofsky, D. (1992) *Virology* **189**, 647–656.
7. De, B. P., Burdsall, A. L. & Banerjee, A. K. (1993) *J. Biol. Chem.* **268**, 5703–5710.
8. Moyer, S. A., Baker, S. C. & Lessard, J. L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5405–5409.
9. Moyer, S. A., Baker, S. C. & Horikami, S. M. (1990 *J. Gen. Virol.* **71**, 775–783.
10. Barik, S. & Banerjee, A. K. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6570–6574.
11. Villanueva, N., Navarro, J., Mendez, E. & Albert, I. G. (1994) *J. Gen. Virol.* **75**, 555–565.
12. Mazumder, B. & Barik, S. (1994) *Virology* **205**, 104–111.
13. Barik, S. & Banerjee, A. K. (1992) *J. Virol.* **66**, 1109–1118.
14. Diaz-Meco, M. T., Berra, E., Municio, M. M., Sanz, L., Lozano, J., Dominguez, I., Diaz-Golpe, V., deLera, M. T. L., Alcami, J., Paya, C. V., Seisdedos, F. A., Virelizier, J. L. & Moscat, J. (1993) *Mol. Cell. Biol.* **13**, 4770–4775.
15. Dominguez, I., Diaz-Meco, M. T., Municio, M. M., Berra, E., de Herreros, A. G., Cornet, M. E., Sanz, L. & Moscat, J. (1992) *Mol. Cell. Biol.* **12**, 3776–3783.
16. Barik, S. & Banerjee, A. K. (1991) *J. Virol.* **65**, 1719–1726.
17. Casnellie, J. E. (1991) *Methods Enzymol.* **200**, 115–120.
18. Nakanishi, H. & Exton, J. H. (1992) *J. Biol. Chem.* **267**, 16347–16354.
19. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3099–3103.
20. Wiley, D. C. (1985) in *Virology*, ed. Fields, B. N. (Raven, New York), pp. 45–67.
21. Kochs, G., Hummel, R., Meyer, D., Hug, H., Marme, D. & Sarre, T. F. (1993) *Eur. J. Biochem.* **216**, 597–606.
22. Fischer, S. G. (1983) *Methods Enzymol.* **100**, 424–430.
23. Gupta, A., Das, T. & Banerjee, A. K. (1995) *J. Gen. Virol.* **76**, 365–372.
24. Tauzon, P. T. & Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res.* **23**, 123–164.
25. Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665.
26. Bell, R. M. & Burns, D. J. (1991) *J. Biol. Chem.* **266**, 4661–4664.
27. Reich, N. C. & Pfeffer, L. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8761–8765.
28. Mischak, H., Goodnight, J. A, Kolch, W., Baron, G. M., Schaechtle, C., Kazanietz, M. G., Blumberg, P. M., Pierce, J. H. & Mushinski, J. F. (1993) *J. Biol. Chem.* **268**, 6090–6096.