Phosphorylation of Canine Distemper Virus P Protein by Protein Kinase C-ζ and Casein Kinase II

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Transcription by nonsegmented negative-strand RNA viruses is mediated by the viral RNA-dependent RNA polymerase and transcriptional cofactor P. The P protein is activated by phosphorylation, an event initiated by cellular kinases. The kinase used differs among this group of RNA viruses; vesicular stomatitis virus and respiratory syncytial virus utilize casein kinase II (CKII), whereas human parainfluenza virus type 3 utilizes PKC isoform zeta (PKC-ζ) for activation of its P protein. To identify the cellular kinases involved in the phosphorylation of the canine distemper virus (CDV) P protein, we used recombinant CDV P in phosphorylation assays with native kinase activities present in CV1 cell extracts or purified CKII and PKC isoforms. Here, we demonstrate that the CDV P protein is phosphorylated by two cellular kinases, where PKC-ζ has the major and CKII the minor activities. In contrast, the P protein of another member of the morbillivirus genus, measles virus, is phosphorylated predominantly by CKII, whereas PKC-ζ has only minor activity. Selective inhibition of PKC-ζ activity within CV1 cells eliminated permissiveness to CDV replication, indicating an in vivo role for PKC-ζ in the virus replication cycle. The broad tissue expression of PKC-ζ parallels the pantropic nature of CDV infections, suggesting that PKC-ζ activity is a determinant of cellular permissiveness to CDV replication.

INTRODUCTION

The transcription and genomic replication of nonsegmented negative-strand RNA viruses is mediated by a virus-encoded RNA-dependent RNA polymerase complex consisting of viral phosphoprotein (P) and RNA polymerase (L). The P protein is a transcription cofactor or a transactivator for L (Curran et al., 1992; De et al., 1991; Hamaguchi et al., 1983; Takacs et al., 1995). In vesicular stomatitis virus (VSV), a prototype for negative-strand RNA viruses, P phosphorylation is initiated by cellular kinases (Barik and Banerjee, 1991, 1992; Gao and Lenard, 1995; Gupta et al., 1995). The initial phosphorylation of P (P0) is mediated by casein kinase II (CKII), resulting in a structurally distinct form of P (P1). The P1 is then phosphorylated by an L-associated kinase (LAK) to become the fully phosphorylated P2 form (Barik and Banerjee, 1992; Gao and Lenard, 1995). The essential role of CKII in VSV transcription was demonstrated in reconstituted systems, where purified ribonucleoprotein templates (i.e., N protein-encapsidated genomic RNA) were combined with purified native L protein and a nonphosphorylated recombinant P protein. The addition of CKII was necessary and sufficient to support VSV transcription (Gao and Lenard, 1995). Phosphorylation of P by CKII results in P oligomerization which is, in turn, necessary for the association of P with template and L protein and the formation of an active polymerase complex (Das et al., 1995a; Gao and Lenard, 1995). The role of LAK in VSV transcription/replication remains to be determined.

The particular cellular kinase responsible for the P0 phosphorylation varies among negative-strand RNA viruses, illustrated by members of the paramyxovirus family. Human parainfluenza virus type 3 (HPIV3) utilizes exclusively protein kinase C-ζ for P0 phosphorylation (De et al., 1995; Huntley et al., 1995). In situ inhibition of PKC-ζ activity using an isoform-specific peptide inhibitor eliminates cellular permissiveness to HPIV3 replication. Furthermore, PKC-ζ is incorporated into the HPIV3 virion (De et al., 1995). For respiratory syncytial virus (RSV), CKII phosphorolates P0 and is directly responsible for regulating viral transcriptional activity in reconstituted systems (Barik et al., 1995; Mazumder and Barik, 1994; Vilanueva et al., 1994). Casein kinase II was also shown to be the predominant source of measles virus (MV) P0 phosphorylation using crude cell extracts as a source of enzyme (Das et al., 1995b). However, a reconstitution system to show the requirement of CKII-mediated P phosphorylation for transcription is not currently available for MV. Protein kinase C recognition sites are present within the deduced amino acid sequence of the MV P gene although their recognition by cellular kinases has not been established.

Analysis of the deduced amino acid sequence of the canine distemper virus P gene reveals multiple CKII and PKC (non-isoform-specific) recognition sites. The close
structural relatedness of canine distemper virus (CDV) and MV P proteins (Rima, 1983; Yamanaka et al., 1992) suggests similar kinase utilization for P phosphorylation. However, the heterogeneity of kinase utilization by the paramyxoviruses requires that this prediction be substantiated and the potential relevance of PKC phosphorylation events be addressed. The objective of the present work was to identify cellular kinase(s) phosphorylating the CDV P protein, to compare these phosphorylation events to those of MV P protein, and to establish the relevance of these kinase activities to CDV replication.

**MATERIAL AND METHODS**

**Virus – cell systems**

The Onderstepoort strain of canine distemper virus (Ond-CDV) was used to infect continuous cell lines derived from African green monkey kidney: Vero (ATCC CCL81) and CV1 (ATCC CCL70). Both cell lines support equivalent degrees of cytopathic and productive infection by Ond-CDV. CDV-infected cells were used as a source of viral nucleocapsid (NC)/genomic RNA for the production of a full-length P gene cDNA, while uninfected cells were used as a source of cellular kinases in the phosphorylation of recombinant CDV and MV P protein. Cells were grown at 37° in complete Eagle’s essential medium supplemented with 10% FCS.

Production of recombinant CDV P in Escherichia coli

The production and characterization of recombinant P protein from the Edmonston strain of MV has been described (Das et al., 1995b). For the production of a nonphosphorylatable recombinant CDV P protein, the Ond-CDV NC was purified by CsCl isopycnic centrifugation as previously described (Oglesbee et al., 1989a,b). Genomic RNA was isolated by resuspending NC in TE buffer (10 mM Tris – HCl, pH 8.0, 1 mM EDTA) containing 0.6% SDS and 0.2 µg/µl proteinase K, incubating the mixture at 37° for 20 min, and then using two rounds of phenol/chloroform extraction. The full-length genomic RNA was used as template for the reverse transcription (RT)-PCR amplification of the P gene. The initial RT reaction contained 50 mM Tris – HCl (pH 8.5), 8 mM MgCl₂, 30 mM KCl, 1 mM DTT, 1 mM dNTPs, 2 units/µl AMV reverse transcriptase (Boehringer Mannheim, Germany), 1.25 U/µl RNAse inhibitor, and 25 ng/µl primer CDV-P5 (5’-GGCG CCAA TAT GCC AGA GGA ACA GGA CTA C). The reaction was incubated at 37° for 30 min followed by heat inactivation at 85° for 10 min. The reaction product was then used as template for the PCR amplification of P gene cDNA. The reactions contained 10 mM Tris – HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 1 mM dNTPs, 0.01 U/µl Taq DNA polymerase (Boehringer Mannheim), and 5 ng/µl of both the 5’ (CDV-P5) and 3’ (CDVP3Hs; 5’-CCC AAG CTT TTA ATG ATG ATG ATG ATG ATG AGC ATG AGT AAT ACT TTT GAC) primers. Primers were synthesized by Operon Technologies, Inc. (Alameda, CA). The PCR incubation (Perkin – Elmer Thermal Cycler) consisted of 24 thermal cycles, each cycle including denaturation at 94° for 2 min, annealing at 50° for 2 min, and elongation at 72° for 2 min. A 5-min initial denaturation and an additional elongation period of 5 min following the last cycle were also incorporated.

The CDV P gene cDNA was gel purified, restricted with HindIII and NdeI, then ligated into HindIII/NdeI linearized pET-17b (Novagen) expression vector. This vector contains an ampicillin-resistance marker (bla) and a bacterial origin of replication, allowing its use in the amplification of the construct. The ligation reaction product was transformed into E. coli DH5-α for screening and amplification. Desired constructs were identified by restriction analysis using BamHI; restriction maps of the P gene and vector predict the release of a 4- and a 0.91-kb fragment following BamHI digestion. Desired constructs were introduced into E. coli BL21(DE3) cells for the production of P protein. The DE3 cells contain a T7 RNA polymerase gene under control of a lacUV5 promoter; the pET-17b vector contains a strong T7 RNA polymerase promoter directed at the polylinker. The pET-17b-CDVP-containing DE3 cells were grown at 37° in LB media containing 100 µg/ml ampicillin and 0.3% glucose. Production of P protein was then induced with 1 mM IPTG for 2 hr and the recombinant P protein recovered by Ni-affinity chromatography as previously described (De et al., 1995). Column fractions were resolved by SDS-PAGE followed by both Coomassie staining and Western blot analysis using CDV P monospecific hyperimmune serum (Oglesbee et al., 1989b).

**Purification of cellular kinase activities**

The cellular kinase activities phosphorylating the recombinant CDV and MV P proteins were purified from cytoplasmic extracts of CV1 cells using DEAE-cellulose and phosphocellulose ion-exchange chromatography as previously described (De et al., 1995). Measurement of phosphorylation was based upon the incorporation of [γ-32P]ATP into nonphosphorylated recombinant P₀ (see below). Twenty million CV1 cells were disrupted into 10 ml of 10 mM Tris – HCl, pH 7.5, 10 mM NaCl using two freeze-thaw cycles. The lysate was clarified at 10,000 g for 10 min and then at 100,000 g for 1 hr (Beckman Ultracentrifuge, SW50 rotor). The clarified lysate was then dialyzed against buffer A [50 mM Tris – HCl (pH 7.5), 50 mM NaCl, 5% v/v glycerol, 0.1 mM EDTA, 1 mM DTT] for 12 hr and then adsorbed to the DEAE-cellulose column (DE52). This column was developed using a linear gradient of 0 - 0.4 M NaCl in buffer A. The fractions supporting P phosphorylation were pooled and adsorbed onto the phosphocellulose column and the column developed by a linear 0.2 - 1.0 M NaCl gradient.
In vitro phosphorylation of CDV and MV P using CV1-origin kinase

In vitro phosphorylation of 1 μg CDV or MV P protein was performed under reaction conditions optimal for either CKII or PKC. The CKII reactions contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 2 mM DTT, 33.3 μM ATP, and 10 μCi [γ-32P]ATP. PKC reactions differed in that the salt concentration was low (20 mM Tris–HCl and no NaCl), BSA was added (0.25% w/v), and PKC cofactors were added as indicated, including 0.1 μg/μl phosphatidylserine (PS), 0.5 mM CaCl₂, 0.1 μg/μl diacylglycerol (DAG). The PS and DAG were dissolved in 10 mM CHAPS prior to addition to the reactions; detergent does not influence kinase activity in these assays. For the analysis of kinase activities within crude cell extracts, a CKII buffer system supplemented with PKC cofactors was used since these conditions will readily support both CKII and PKC activity. The volume of cell extract used in these reactions (1 μl) was established empirically. Reactions were incubated at 30°C for 1 hr and resolved by 10% SDS–PAGE followed by autoradiography.

In vitro phosphorylation of CDV and MV P using exogenous kinase

Recombinant kinases and kinases purified from cells other than CV1 were used in the phosphorylation of recombinant CDV and MV P protein and recombinant P proteins from other negative-strand RNA viruses to support results of experiments using CV1 cell extracts. The enzymes included recombinant CKII (New England Biolabs, Beverly, MA), recombinant PKC-α, recombinant PKC-γ, recombinant PKC-ζ (Goodnight et al., 1995), and mixed isoforms of PKC isolated from rat brain (Boehringer Mannheim). A filter-binding assay was used to establish the relative activity of the specific PKC isoforms (i.e., PKC-α, PKC-γ, and PKC-ζ) so that reactions could be established containing equivalent activities of each enzyme. One and two microliters of each recombinant PKC isoform was used under the PKC reaction conditions described above, using as substrate the synthetic peptide [Ser²⁵]PKC-(19-31), designated peptide α (Kochs et al., 1993). Peptide α is recognized equally by these PKC isoforms. Reaction products were spotted onto phosphocellulose paper, unincorporated radioactivity removed by washing in 0.35% phosphoric acid (5 min × 2), and the degree of peptide phosphorylation measured by liquid scintillation counting.

In vivo inhibition of protein kinase C activity

Peptide inhibitors of PKC isoforms were introduced into CV1 cells prior to Ond-CDV infection. Peptide A (RKGALRQKN) inhibits conventional PKC isoforms (e.g., PKC-α, PKC-β, and PKC-γ). Peptide Z (RGARRWRK) selectively inhibits PKC-ζ activity. These two peptides (Quality Controlled Biochemicals, Inc.) were delivered into cells using a lipid-based peptide delivery system (TransPort transient cell permeabilization kit; Gibco BRL). Subconfluent monolayers of cells in 24-well plates were incubated at 37°C, 15 min, with varying concentrations of peptide A or Z (0, 1, 2, 3, 4, or 5 mM) in the peptide delivery buffer system. The peptide-containing buffer was then replaced by fresh MEM medium with 10% FCS. Cells were allowed to recover at 37°C for 45 min, then infected with Ond-CDV at a multiplicity of infection (m.o.i.) of 0.01. Viral progeny were harvested at 24 hr postinfection using two freeze–thaw cycles and titrated in a plaque forming assay.

RESULTS

Cloning of the CDV-P gene and purification of recombinant P protein from E. coli

The phosphoprotein P of negative-strand RNA viruses is phosphorylated when purified from infected cells (Banerjee et al., 1991; Rima, 1983). However, when the P protein of VSV, HPIV3, and MV is expressed in bacteria (E. coli), the recombinant P remains nonphosphorylated (Barik and Banerjee, 1991; Das et al., 1995b; De et al., 1995) and is suitable for the in vitro analysis of kinase activities. Therefore, prokaryotic expression of recombinant P was used to analyze CDV P phosphorylation.

A full-length cDNA clone of the CDV-P gene was generated by RT-PCR of genomic RNA purified from nucleocapsids. The primers were designed to add an Ndel site overlapping the initiator methionine codon AUG, and a HindIII site 3’ to the P gene. This design allowed cloning of the RT-PCR product into unique sites in the expression vector. The RT-PCR primers were also designed to add a seven-histidine tag to the carboxyl terminus of the recombinant P protein, facilitating purification of the recombinant protein by Ni-affinity chromatography. The RT-PCR product migrated as a single 1.6-kb band on a 1% agarose gel (data not shown), matching the size of the P gene (1631 bp). The structure of the recombinant plasmid (pET-17b-CDVP) was confirmed by restriction analysis using BamHI, in which a 4.0- and 0.91-kb band were visualized by ethidium bromide staining (data not shown), matching the size of restriction fragments predicted from sequence analysis of the CDV P gene and pET-17b vector.

Expression of recombinant CDV P protein was induced by IPTG after the pET-17b-CDVP-transformed DE3 cells were grown to optimal density. When bacterial cell extracts were fractionated on a Ni-affinity column, a prominent doublet with an average molecular mass of 75 kDa was observed in fractions 2–7 by 10% SDS–PAGE (data not shown). To further characterize the purified protein, CDV P-specific hyperimmune serum was used in Western blot analysis of both recombinant P and native P contained in purified nucleocapsids. The same protein doublets of approximately 75 kDa were recognized in
mediated predominantly by a CKII-like activity, consistent with previously published results (Das et al., 1995b).

Cell extracts were then fractionated, purifying the kinase activities in order to identify the specific kinases responsible for the observed activity. Crude extracts of CV-1 cells were fractionated through a DE52 column. The recombinant CDV and MV P₀ was phosphorylated by fractions eluted with $\approx 0.25 \text{ M } \text{NaCl}$ (data not shown). The active fractions were pooled and passed through a phosphocellulose column. The bound proteins were eluted from the column by a 0.2–1.0 M NaCl linear gradient. Interestingly, both CDV and MV P phosphorylating activity was detected in both the unbound (flowthrough) and bound fractions with peak activities in fraction numbers 25–35, as illustrated in Fig. 2. The unbound and pooled bound fractions were used in all subsequent experiments. The phosphorylation of CDV and MV P by the unbound fraction was inhibited by 400 nM staurosporine, while 20 $\mu$g/ml heparin had no effect on this activity (Fig. 3). Conversely, the kinase activity in peak fraction 30 was inhibited by 20 $\mu$g/ml heparin while staurosporine had no effect (Fig. 3). These data suggested that both PKC (unbound fraction) and CKII (bound fraction) are involved in the phosphorylation of CDV and MV P proteins. Phosphorylation of MV P by PKC was found to be highly sensitive to phosphatase activity present in crude cell extracts (data not shown), suggesting a basis for the detection of only minor PKC activities when using crude extract in MV P phosphorylation reactions.

To determine whether phosphorylation by one kinase

![CDV and MV phosphorylation](image1.png)

**FIG. 1.** Kinase activity of crude cytoplasmic extracts of CV1 cells using CDV and MV recombinant P protein as substrate. Reactions were performed using CKII buffer conditions containing PKC cofactors and $[\gamma^{32}\text{P}]\text{ATP}$. The P proteins were resolved by 10% SDS-PAGE and the phosphorylated reaction products visualized by autoradiography. The 75-kDa CDV P protein and the 70-kDa MV P protein were phosphorylated by the kinase(s) present in the extract. Staurosporine (S) (400 nM) and heparin (H) (20 $\mu$g/ml) were added to reactions as indicated.

CDV and MV P was phosphorylated by PKC and CKII-like activities in CV1 extracts.

Crude cytoplasmic extracts and cytoplasmic extracts fractionated by DEAE-cellulose and phosphocellulose chromatography were used to identify the cellular kinase(s) that phosphorylates CDV P and, for comparison, MV P. For these kinase assays, recombinant CDV or MV P₀ protein was mixed with cytoplasmic extract under buffer conditions optimal for both CKII and PKC activity (i.e., CKII buffer supplemented with PKC cofactors), with or without inhibitors of CKII (i.e., heparin) and PKC (i.e., staurosporine). In crude cell extracts, both CDV and MV P were phosphorylated (Fig. 1). The CDV P phosphorylation was inhibited by 60% in the presence of staurosporine (400 nM) and by 30% in the presence of heparin (20 $\mu$g/ml). Similar results were obtained when using 200 nM staurosporine and 8 $\mu$g/ml heparin (data not shown). In contrast, MV P phosphorylation was inhibited by 20% in the presence of staurosporine and by 90% in the presence of heparin. For both CDV and MV, phosphorylation was totally inhibited when these two inhibitors were used in combination. The magnitude of P phosphorylation was based upon $^{32}\text{P}$ phosphorimager signal intensities of SDS-PAGE banded reaction products. These results suggest that CDV P₀ phosphorylation occurs via a predominant PKC-like activity and a minor CKII-like activity. MV P₀ phosphorylation, on the other hand, appears to be

![Isolation of kinase activities](image2.png)

**FIG. 2.** Isolation of kinase activities in cytoplasmic extracts of CV1 cells. The phosphocellulose column fractions were tested for their kinase activity using recombinant CDV or MV P as substrate. Reactions were performed using CKII buffer conditions containing PKC cofactors and $[\gamma^{32}\text{P}]\text{ATP}$. The CDV and MV P proteins were then resolved by 10% SDS-PAGE and the phosphorylated proteins visualized by autoradiography. Prominent kinase activities were detected in the unbound fraction (U) and by material retained on the column, then eluted by a 0.2–1.0 M NaCl gradient. Numbers above lanes represent the eluant fraction number.
(i.e., PKC or CKII) influences phosphorylation by the other kinase, equal amounts of CDV and MV P proteins were used in reactions containing unbound and bound cell fractions, separately or in combination. No synergistic effect was observed when the PKC- and CKII-containing cell fractions were used in combination; phosphorylation by PKC and CKII-like activities occurred independently. Under optimal conditions for PKC and CKII activity, 1.0 mol of phosphate was incorporated by the PKC-like activity and 0.3 mol by the CKII activity for each mole of CDV P substrate. The stoichiometry of phosphorylation was determined. In the case of MV, 2.0 mol of phosphate were phosphorylated by CKII and PKC, respectively, were included as positive controls. The CDV, MV, and HPIV3 P proteins were phosphorylated. Novel PKCs (μ, ζ, and λ) are dependent upon phosphatidyserine and are not affected by calcium, diacylglycerol, or phorbol esters (Nishizuka, 1992). Reactions were supplemented with calcium or the calcium was depleted using 200 μM calcium chloride or 500 μM EGTA, respectively. Phosphatidyserine was added at 100 μg/ml and the diacylglycerol added to a final concentration 100 μg/ml. The kinase activity in the unbound fraction did not require calcium or diacylglycerol but was stimulated by phosphorylated PKC (Fig.4). These data indicated that the PKC isoform involved in phosphorylation of CDV and MV P belongs to the atypical subgroup.

Western blot analysis of the unbound and peak cytoplasmic fractions was performed to confirm the existence of an atypical PKC in the unbound fraction and CKII in the peak fraction. Five PKC isoform-specific polyclonal antibody preparations were used (Gibco BRL), recognizing α, β1/β2, γ, ε, and ζ. In the unbound fraction, specific signal was demonstrated with only the anti-PKC-ζ antibody (i.e., a double band in which the lower component has an approximate mass of 68 kDa) (Fig.5). Polyclonal antibody against CKII (Yu et al., 1991) detected a 42-kDa band in the peak bound fraction (data not shown), consistent with the mass of CKII and the CKII-like kinase activity detected in this cell fraction.

CDV and MV P protein was phosphorylated by exogenous PKC and CKII

Protein kinase C purified from rat brain (mixed isoforms) and recombinant CKII were used to confirm the role of PKC and CKII in mediating phosphorylation of recombinant CDV and MV P. Nonphosphorylated P substrate from VSV and HPIV3 which are specifically phosphorylated by CKII and PKC, respectively, were included as positive controls. The CDV, MV, and HPIV3 P proteins were phosphorylated.
were phosphorylated by purified PKC (Fig. 6), and staurosporine (400 nM) inhibited the PKC activity. Recombinant CKII phosphorylated the P protein of CDV, MV, and VSV consistent with the kinase activity observed in the phosphocellulose bound fraction. The phosphorylation was inhibited by heparin at 20 μg/ml. The lesser degree of heparin-mediated inhibition of VSV and MV P phosphorylation may reflect a higher affinity of CKII for these recombinant viral proteins.

Recombinant PKC isoforms were used to support the specific role of PKC-ζ in CDV and MV P phosphorylation. Using recombinant α, η, and ζ isoforms, we first determined the relative activity of these enzymes in a filter binding assay using their common pseudosubstrate, peptide α. The degree of peptide α phosphorylation, measured by liquid scintillation counting, is used to determine the relative activity of PKC isoform preparations. The relative activity, by volume, of α, η, and ζ isoforms was 3.5:1.0:11.4. This determination allowed the design of subsequent reactions containing equivalent enzymatic activities and recombinant CDV or MV P protein as substrate. CDV and MV P were phosphorylated by PKC-ζ, with lesser degrees of phosphorylation mediated by PKC-η and PKC-α (Fig. 7). These results further support the involvement of PKC-ζ in CDV and MV P phosphorylation.

In situ inhibition of PKC-ζ eliminates CDV replication

For VSV, RSV, and HPIV3, phosphorylation of P is proposed as an essential step in the activation of the viral polymerase (L) (Barik and Banerjee, 1991, 1992; De et al., 1995; Mazumder and Barik, 1994). Accordingly, in situ inhibition of PKC-ζ activity eliminates HPIV3 but not VSV replication, the latter being dependent upon CKII for P phosphorylation (De et al., 1995). In the present study, pseudosubstrate peptide inhibitors of PKC-ζ (peptide Z) and conventional PKCs (peptide A) were used to deter-

**FIG. 5.** Western blot analysis of PKC isoforms in the unbound fraction. Protein contained within unbound cell fractions phosphorylating CDV and MV P were resolved by 10% SDS – PAGE and transferred to nitrocellulose membranes, and the blots were developed using polyclonal antibodies against PKC-α (α), PKC-β (β), PKC-γ (γ), PKC-ε (ε), and PKC-ζ (ζ). Specific signal was detected by the anti-PKC-ζ antibody.

**FIG. 6.** Phosphorylation of recombinant CDV and MV P by recombinant CKII and purified PKC (mixed isoforms). Reactions were performed in the presence of heparin (H) or staurosporine (S), or in the absence of either inhibitor (–), to confirm the specificity of phosphorylation mediated by the purified kinases. Recombinant P protein from HPIV3 was used as a positive control for PKC reactions, and recombinant P protein from VSV was used as positive controls for CKII reactions.

**FIG. 7.** Phosphorylation of recombinant CDV and MV P using purified recombinant PKC isoforms. Equivalent active units of recombinant PKC isoforms α, η, and ζ were combined with recombinant CDV or MV P in reactions containing [γ-32P]ATP and using PKC buffer conditions. The phosphorylated proteins were resolved by 10% SDS – PAGE and visualized by autoradiography. For each reaction, the 75-kDa CDV P protein is labeled to a degree proportionate to bands of lower molecular mass, suggesting that the latter represent degradation products of the parental protein.
mine whether the PKC-ζ activity is relevant to CDV replication. These inhibitory peptides mimic PKC autoinhibitory regions known as pseudosubstrate domains. Peptide A has a sequence conserved in the pseudosubstrate domain of PKC isoforms α, β, and γ and is an inhibitor of these isoforms when introduced into cells (Dominguez et al., 1992). Peptide Z has a sequence identical to the pseudosubstrate domain of PKC-ζ, being sufficiently different from the pseudosubstrate domains of PKC α, β, and γ to mediate selective inhibition of PKC-ζ within cells (Dominguez et al., 1992). First, these two peptides (i.e., peptide A and Z) were used at varying concentrations (5–20 μM) in the in vitro phosphorylation of CDV P by phosphocellulose unbound cell fraction. Peptide Z inhibited phosphorylation of CDV P by more than 70% at 20 μM, whereas peptide A had no effect. Next these peptides were delivered at varying concentrations into subconfluent monolayers of CV1 cells using a lipid-based peptide delivery system. Cells were infected at an m.o.i. = 0.01 following a 45-min cell recovery interval and viral progeny release was quantitated at 24 hr postinfection. Cells treated with 1–5 mM peptide A demonstrated virus-induced syncytia formation (plaque formation) comparable to the untreated infected controls, whereas cells treated with 1–5 mM peptide Z demonstrated a concentration-dependent reduction in plaque number and size (data not shown). The effect of peptide treatment on infectious viral progeny release was consistent with these results (Fig. 8). Virus harvested from cells treated with 1 mM peptide Z yielded 1200 PFU/1.5 × 10^5 cells, comparable to viral progeny release from infected cells treated with 1–5 mM peptide A or untreated controls. At 2 mM peptide Z, viral progeny release was decreased by 53% and at 3 mM peptide Z viral progeny release was completely abolished. Peptide Z treatment did not affect viability of uninfected control cells.

DISCUSSION

We have demonstrated involvement of two cellular kinases, CKII and PKC-ζ, in the phosphorylation of CDV P protein. Of the two kinases, PKC was detected as the major and CKII as the minor activities in CDV P phosphorylation by crude cell extracts (Fig. 1). In contrast, MV P phosphorylation was carried out predominantly by CKII, as reported previously (Das et al., 1995b). In the latter work, CKII phosphorylation of MV P protein was shown using both native enzyme contained in crude cell extracts and recombinant CKII (Das et al., 1995b). In this regard, phosphorylation of CDV and MV P differs despite the biochemical similarity between these two proteins. The amino acid sequences of the CDV and MV P proteins are 44% identical (Hall et al., 1980; Yamanaka et al., 1992), and both share multiple CKII consensus recognition sites (S/T-X-X-D/E; n = 10 for Ond-CDV and Edmonston MV). In addition, the deduced amino acid sequences of both CDV and MV P contain multiple PKC consensus recognition sites (S/T-X-K/R; n = 8 for Ond-CDV and n = 7 for Edmonston MV).

Using fractionated cell extracts (Figs. 2–5), we demonstrate involvement of an atypical PKC in the phosphorylation of both CDV and MV P proteins. Protein kinase C-ζ was present in cell fractions containing the atypical PKC activity based upon biochemical and antigenic analyses. Furthermore, both native PKC (mixed isoforms; Fig. 6) and recombinant PKC-ζ (Fig. 7) phosphorylate CDV and MV P to a high degree of activity. Previous analysis of MV P phosphorylation by native kinase, however, failed to detect PKC activity when using crude cytoplasmic extracts (Das et al., 1995b). In the current study, the atypical PKC activity against MV P was readily detected only after these crude preparations were further refined by ion-exchange chromatography. The disparity between results of using crude versus fractioned cell extracts appears to reflect the presence of phosphatase activity in the crude extract.

The biological relevance of PKC-ζ phosphorylation of CDV P was supported by results of experiments using isoform-specific inhibition of PKC-ζ activity (Fig. 8). Peptide Z, an inhibitor of PKC-ζ, inhibited in vitro CDV P phosphorylation by fractionated cell extracts and caused a dosage-dependent reduction in MV P protein phosphorylation. These inhibitory peptides mimic PKC autoinhibitory regions known as pseudosubstrate domains. Peptide A has a sequence conserved in the pseudosubstrate domain of PKC isoforms α, β, and γ and is an inhibitor of these isoforms when introduced into cells (Dominguez et al., 1992). Peptide Z has a sequence identical to the pseudosubstrate domain of PKC-ζ, being sufficiently different from the pseudosubstrate domains of PKC α, β, and γ to mediate selective inhibition of PKC-ζ within cells (Dominguez et al., 1992). First, these two peptides (i.e., peptide A and Z) were used at varying concentrations (5–20 μM) in the in vitro phosphorylation of CDV P by phosphocellulose unbound cell fraction. Peptide Z inhibited phosphorylation of CDV P by more than 70% at 20 μM, whereas peptide A had no effect. Next these peptides were delivered at varying concentrations into subconfluent monolayers of CV1 cells using a lipid-based peptide delivery system. Cells were infected at an m.o.i. = 0.01 following a 45-min cell recovery interval and viral progeny release was quantitated at 24 hr postinfection. Cells treated with 1–5 mM peptide A demonstrated virus-induced syncytia formation (plaque formation) comparable to the untreated infected controls, whereas cells treated with 1–5 mM peptide Z demonstrated a concentration-dependent reduction in plaque number and size (data not shown). The effect of peptide treatment on infectious viral progeny release was consistent with these results (Fig. 8). Virus harvested from cells treated with 1 mM peptide Z yielded 1200 PFU/1.5 × 10^5 cells, comparable to viral progeny release from infected cells treated with 1–5 mM peptide A or untreated controls. At 2 mM peptide Z, viral progeny release was decreased by 53% and at 3 mM peptide Z viral progeny release was completely abolished. Peptide Z treatment did not affect viability of uninfected control cells.

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Using fractionated cell extracts (Figs. 2–5), we demonstrate involvement of an atypical PKC in the phosphorylation of both CDV and MV P proteins. Protein kinase C-ζ was present in cell fractions containing the atypical PKC activity based upon biochemical and antigenic analyses. Furthermore, both native PKC (mixed isoforms; Fig. 6) and recombinant PKC-ζ (Fig. 7) phosphorylate CDV and MV P to a high degree of activity. Previous analysis of MV P phosphorylation by native kinase, however, failed to detect PKC activity when using crude cytoplasmic extracts (Das et al., 1995b). In the current study, the atypical PKC activity against MV P was readily detected only after these crude preparations were further refined by ion-exchange chromatography. The disparity between results of using crude versus fractioned cell extracts appears to reflect the presence of phosphatase activity in the crude extract.

The biological relevance of PKC-ζ phosphorylation of CDV P was supported by results of experiments using isoform-specific inhibition of PKC-ζ activity (Fig. 8). Peptide Z, an inhibitor of PKC-ζ, inhibited in vitro CDV P phosphorylation by fractionated cell extracts and caused a dosage-dependent reduction in MV P protein phosphorylation. Peptide A, an inhibitor of conventional PKC isoforms, had no effect in either of these assays.

FIG. 8. The effect of PKC-ζ inhibition on permissiveness of CV1 cells to CDV replication. CV1 cells in the logarithmic phase of growth were treated with 0–5 mM peptide Z, a PKC-ζ inhibitor, or peptide A, an inhibitor of conventional PKC isoforms. The peptides were introduced into the cells using a lipid-based delivery system. Cells were treated for 15 min, allowed to recover for 45 min, and infected at an m.o.i. = 0.01. Infectious viral progeny were quantitated at 24 hr postinfection. Peptide A treatment (●) did not affect viral progeny release. Peptide Z treatment (●) caused a dosage-dependent reduction in progeny release. No viral progeny were produced at 3 mM peptide Z.
Other reports document the ability of peptide Z to reduce the in vitro activity of recombinant PKC-ζ, where peptide A has no effect (De et al., 1995). Furthermore, peptide Z inhibits HPIV3 replication when administered to CV1 cells (De et al., 1995). The specificity of peptide Z for PKC and HPIV3/CDV replication is documented by the lack of effect of peptide Z upon replication of VSV, a virus using CKII for the phosphorylation activation of P (De et al., 1995). Down-regulation of PKC-ζ does not affect metabolic pathways involved in cell proliferation (Berra et al., 1993; Diaz et al., 1993; Dominguez et al., 1992; Larrodere et al., 1990), suggesting that the antiviral effect of PKC-ζ inhibition reflects a specific effect upon CDV replication and not a nonspecific effect upon cellular metabolism. The use of peptide Z in the in vivo inhibition of MV replication has not been examined.

The CK II phosphorylation sites within MV P were previously mapped to Ser residues at positions 86, 151, and 180 (Das et al., 1995b). It would therefore be interesting to determine if CDV P is phosphorylated by CK II at homologous sites. The potential relevance of CKII to the posttranslational modification of MV P was supported by the demonstration of similar patterns of phosphorylation between 32P-labeled proteolytic fragments of recombinant P labeled in vitro and native P labeled in vivo. However, because an in vitro reconstituted transcription system has not yet been established for MV or CDV, the significance of CKII-mediated P phosphorylation to viral gene expression has not yet been demonstrated as in VSV and RSV (Barik and Banerjee, 1992; Mazumder and Barik, 1994). In situ inhibition of CKII activity would be a direct test of the biological relevance of CKII to viral replication. Although heparin is a potent inhibitor of CKII in vitro, its large mass (∼3–18 kDa) precludes its delivery into intact cells (Hindrich, 1990); treatment of CV1 cells with 18 µg/ml low-molecular-mass heparin (i.e., 3 kDa) has no effect upon CKII activity in resultant crude cell extracts, whereas lower concentrations (i.e., 8 µg/ml) ablate CKII activity within in vitro reactions using those same extracts (unpublished observation).

Collectively our results support the in situ interaction between PKC-ζ and CDV P, although a physical interaction has not been directly demonstrated. Protein kinase C-ζ was not detected by Western blot analysis in CDV NC purified from the cytoplasm of infected cells, using a NC purification scheme that did maintain the reversible association between the major inducible 70-kDa heat shock protein and NC (unpublished observation). In HPIV3, PKC-ζ is detected in RNP purified from virions (De et al., 1995), suggesting a less stringent purification approach or an in situ analysis such as immunofluorescence confocal microscopy to be used for CDV. The ζ isoform of PKC is widely distributed in tissues (Bell and Burns, 1991; Nishizuka, 1992) and is detected at high levels in cytoplasmic extracts of continuous cell lines that are permissive to CDV replication, including CV1, Vero, Madin–Darby canine kidney, Madin–Darby bovine kidney, porcine kidney, and murine neuroblastoma cells (unpublished observation). Thus, the expression of PKC-ζ complements the broad cellular and tissue tropism of CDV, suggesting that cellular PKC-ζ activity is an important determinant of permissiveness to CDV infection.

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