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Borna Disease Virus P-protein Is Phosphorylated by Protein Kinase Cϵ and Casein Kinase II*

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Borna disease virus (BDV) is a newly classified nonsegmented negative-strand RNA virus (order of Mononegavirales) that persistently infects specific brain regions and circuits of warm-blooded animals to cause behavioral disturbances. Viruses within the order of Mononegavirales have phosphoproteins that typically serve as transcription factors and are modulated in functional activity through phosphorylation. To identify the kinases involved in BDV phosphoprotein (BDV-P) phosphorylation, *in vitro* phosphorylation assays were performed using recombinant phosphoprotein produced in *Escherichia coli* as substrate and cytoplasmic extracts from a rat glioma cell line (C6) or rat brain extracts as sources of kinase activity. These experiments revealed that BDV-P was phosphorylated predominantly by protein kinase C (PKC) and to a lesser extent by casein kinase II. Partial purification of the PKC from rat brain extract suggested that the BDV-P phosphorylating kinase is PKCϵ. A role for PKC phosphorylation *in vivo* was confirmed by using the PKC-specific inhibitor GF109203X. Furthermore, peptide mapping studies indicated that BDV-P is phosphorylated at the same sites *in vitro* as it is *in vivo*. Mutational analysis identified Ser^{26} and Ser^{28} as sites for PKC phosphorylation and Ser^{70} and Ser^{86} as sites for casein kinase II phosphorylation. The anatomic distribution of PKCϵ in the central nervous system may have implications for BDV neurotropism and pathogenesis.

Borna disease virus (BDV)[1] is the prototype of a new family, Bornaviridae, within the nonsegmented negative-strand RNA viruses (Mononegavirales) (1, 2), which is characterized by low productivity, neurotropism (3, 4), a nuclear localization for transcription and replication (5, 6), and posttranscriptional modification of subgenomic RNAs by splicing (7, 8). The potential host range for BDV is likely to include all warm-blooded animals. Accumulating evidence suggests that it may be a human pathogen (9–15).

Phosphoproteins of nonsegmented negative-strand RNA viruses are typically integral components of the viral polymerase complex (16). The activity of these proteins, and in some cases their three-dimensional structure, is dependent upon phosphorylation (17–20). Casein kinase II (CKII)-mediated phosphorylation of VSV-P leads to its multimerization *in vitro* and promotes transcription (17, 20), possibly by facilitating binding of VSV-P to the VSV polymerase (21). In human respiratory syncytial virus (RSV), CKII-mediated phosphorylation of phosphoprotein is also a prerequisite for transcriptional activity (18, 22). Although the human parainfluenza virus type 3 (HPIV3) phosphoprotein is phosphorylated by protein kinase C (PKC)ζ rather than by CKII (19), the effect of HPIV3 phosphoprotein phosphorylation is similar in significance to phosphorylation of VSV-P and RSV phosphoprotein; inhibition of PKCζ by pseudopeptides results in abrogation of viral replication (19).

Because phosphorylation of the BDV phosphoprotein (BDV-P) is likely to be an important step in the life cycle of BDV we have identified the kinases involved in this process and mapped the BDV-P phosphorylation sites. Our findings indicate that BDV-P is phosphorylated predominantly by PKCϵ and to a lesser extent by CKII.

EXPERIMENTAL PROCEDURES

In Vivo Phosphorylation Experiments—BDV-infected C6 cells (23) (5×10^5) were washed twice with phosphate-buffered saline (PBS), incubated in modified RPMI medium 1640 (Irvine Scientific) without P_i (2 ml) in the presence or absence of GF109203X (1–5 μM/ml) (Biomol) for 6 h, and then labeled with inorganic ^{32}P (100 μCi/ml) (NEN Life Science Products) for 3 h. Following two washes with PBS, cells were scraped into PBS, collected by centrifugation, and lysed in 100 μl of 10 mM Tris, pH 8.0, 2 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μM okadaic acid (gift of A. Lepple, University of California, Irvine). BDV-P was immunoprecipitated overnight at 4 °C with rabbit antiserum to BDV-P diluted 1:400 in 10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride. After incubation with Protein G-Sepharose (Sigma) for 1 h at 4 °C, bound protein was collected by centrifugation and then released by boiling in Laemmli buffer (24). Proteins were size-fractionated by 15% SDS-PAGE for analysis by autoradiography.

Plasmid Constructions—BDV-P mutants were generated by PCR using plasmid p23 (25) containing the complete phosphoprotein open reading frame in pBluescript (Stratagene). The BDV-P mutant P11 (lacking 11 amino acids at the amino terminus) was generated using a 5′-primer containing an *Nde*I site (p23-*Nde*I) and a 3′-primer containing a T7 RNA polymerase binding site (T7 primer). All other BDV-P mutants were generated by amplifying overlapping PCR fragments using the T7 primer or p23-*Nde*I primer and combinations of the primers described below. In a final PCR, the complete BDV-P ORF mutant sequence was amplified with the p23-*Nde*I primer and T7 primer and cloned into the *Nde*I/*Hin*dIII site of pET15b (Invitrogen). Introduction of the correct sequence for each mutant was confirmed by dideoxy

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[1] The abbreviations used are: BDV, Borna disease virus; VSV-P, vesicular stomatitis virus phosphoprotein; CKII, casein kinase II; RSV, respiratory syncytial virus; HPIV3, human parainfluenza virus type 3; BDV-P, BDV phosphoprotein; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PS, phosphatidylserine; DAG, diacylglycerol; AcMBP, N-terminally acetylated myelin basic protein; WIB, Western immunoblot; BDV-PK, BDV-P protein kinase; PKC, protein kinase C; PBS, phosphate-buffered saline.

sequencing. Primers used to generate the PKC mutants were P-26/28 (5′-AACGAGCGGGGGCACCAAGACC-3′), P-55 (5′-ATCGCAGACCCAGAC-3′), P-130 (5′-CTCCGATGCCATCAGAATCC-3′), and P-144 (5′-GGATCGCGCCATGAAGAC-3′). Primers used to generate the CKII mutants were P-11 (5′-AGAATCATATGCTGGAGGACGAAGAA-3′), P-58 (5′-ATCGCAGACCCAGAC-3′), P-70 (5′-CTAGCGAATGATGAG-3′), and P-86 (5′-AATGCCATGATCGAGG-3′).

Purification of Recombinant Proteins from Escherichia coli—Histidine-tagged BDV-P (25) and BDV-P mutants were purified from the soluble supernatant of transformed *E. coli* by nickel-agarose chromatography as described previously except that Nonidet P-40 was not used (26). The amino-terminal histidine tract was removed using avidin-coupled thrombin (Invitrogen). BDV-P was incubated for 1 h with 0.5 unit of thrombin in 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20% glycerol, 2.5 mM $CaCl_2$, 250 mM imidazole. The thrombin digestion reaction was stopped with 2 mM phenylmethylsulfonyl fluoride, and the avidin-coupled thrombin was removed with biotin-agarose according to the manufacturer's protocols (Invitrogen). Cleaved BDV-P was bound to Q-Sepharose (Pharmacia Biotech Inc.) in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ and then eluted with 550 mM NaCl. Pilot studies indicated that phosphorylation activity was equivalent with uncleaved and thrombin-cleaved BDV-P; thus, thrombin-cleaved protein was used only for peptide mapping experiments. Methods used for purification of phosphoproteins of VSV and HPIV3 have been described (19).

Purification of PKC and CKII from Extracts of C6 Cells and Rat Brain—PKC was purified from cytoplasmic extracts of C6 (rat glial) cells as described (19). Briefly, 10^8 cells were washed in PBS, collected in 4 ml of Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and lysed by three freeze-thaw cycles. Cell extracts were centrifuged at either 10,000 × *g* for 10 min (S10 extract) or 100,000 × *g* for 60 min (S100 extract) at 4 °C. The S100 supernatant was dialyzed against 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% (v/v) glycerol, 0.1 mM EDTA, 1 mM dithiothreitol (Buffer A) overnight at 4 °C and then subjected to DEAE-cellulose chromatography. The highest PKC activity was present in fractions eluted between 200 and 250 mM NaCl. These fractions were pooled and loaded onto a phosphocellulose column equilibrated with Buffer A containing 0.2 M NaCl. Whereas PKC activity was found in the flow-through fraction of this column, heparin-sensitive kinase (CKII) activity was eluted with increasing concentrations of NaCl starting at 300 mM. The flow-through fraction of the phosphocellulose column was again subjected to DEAE-cellulose chromatography, and PKC was eluted as already described. The pooled fractions were dialyzed against 10 mM K_2PO_4, pH 7.5, 5% glycerol, 1 mM dithiothreitol and applied to a hydroxylapatite column equilibrated with the same buffer. PKC activity was eluted between 250 and 350 mM potassium phosphate.

Methods used to purify PKC from rat brain were similar to those employed to purify PKC from C6 cells except that whole rat brain was lysed by Dounce homogenization in buffer A rather than by alternate freeze-thaw cycles.

SDS-PAGE Immunoblot Analyses—SDS-PAGE immunoblot analyses were performed using 10–20 μg of cell lysates (S10 extract) or 500 ng of purified protein fraction from the final hydroxylapatite column (rat brain material). Polyclonal antibodies against PKCε, PKCδ, PKCζ (Life Technologies, Inc.), and PKCη (Panvera) were used at concentrations suggested by the manufacturer. Recombinant PKCs (PKCε, PKCδ, and PKCζ) expressed in a baculovirus vector system were a gift of W. Koelch, München.

Phosphorylation Assays—For kinase experiments, 100–200 ng of BDV-P and variable amounts of cellular kinases or recombinant PKCs were incubated in 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.25% bovine serum albumin, 50 μM ATP, 5–10 mCi of [γ-^{32}P]ATP (3000 Ci/mmol), and when indicated, 200 mM $CaCl_2$, phosphatidylserine (PS, 10 μg/ml), or diacylglycerol (DAG, 100 μg/ml) for 60 min at 30 °C in a total volume of 30 μl. After addition of 1.5 μl of 10 mM ATP and 10 μl of 4 × Laemmli buffer, reactions were analyzed by SDS-PAGE and autoradiography.

Peptide phosphorylation assays were performed as described (39). Briefly, the kinase activities were assayed under standard reaction conditions (30-μl volume) in the presence of PS (10 μg/ml), DAG (100 μg/ml), EGTA (0.2 mM), and substrate peptides at 200 μM or substrate protein at 200 μg/ml. After incubation for 30 min at 30 °C, a 3-μl aliquot was spotted onto P81 paper (Whatman). Radioactivity bound to paper was counted after washing for 5 min with 75 mM H_3PO_4 five times. Peptides AcMBP-(4–14) (Boehringer Mannheim), Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide, Sigma), α-peptide [Ser-25]PKC-(19–31) (Life Technologies, Inc.) and ε-peptide [Ser-159]PKC-(149–164) (Peninsula Laboratories), and proteins histone H1 (Boehringer Mannheim), and histone IIA (Boehringer Mannheim) were used as substrates.

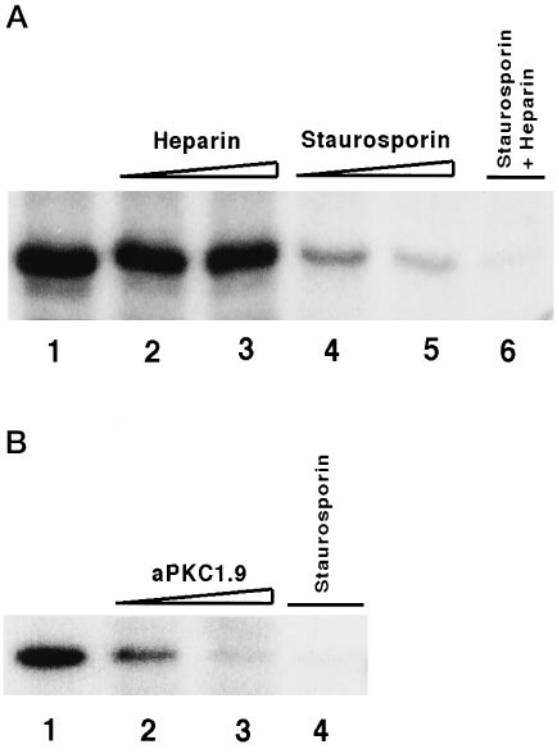

FIG. 1. **Borna disease virus P-protein is phosphorylated *in vitro* predominantly by a PKC.** *A*, 100 ng of BDV-P were phosphorylated with an S10 extract of C6 cells for 1 h at 30 °C in the presence or absence of heparin or staurosporin. *Lane 1*, no heparin, no staurosporin; *lane 2*, 50 μg/ml heparin; *lane 3*, 100 μg/ml heparin; *lane 4*, 200 nM staurosporin; *lane 5*, 400 nM staurosporin; *lane 6*, 50 μg/ml heparin, 400 nM staurosporin. *B*, 100 ng of BDV-P were phosphorylated with an S10 extract of C6 cells for 1 h at 30 °C in the presence or absence of an antibody to the catalytic subunit of protein kinase C (αPKC1.9). *Lane 1*, no antibody; *lane 2*, 0.4 μg of αPKC1.9; *lane 3*, 2 μg of αPKC1.9; *lane 4*, 400 nM staurosporin.

Peptide Mapping—^{32}P-Radiolabeled BDV-P was fractionated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membrane-bound BDV-P was digested *in situ* with trypsin, and peptides were recovered as described by Boyle *et al.* (27). Trypsin-digested peptides were fractionated on cellulose plates (J. T. Baker Inc.) by electrophoresis at pH 1.9 at 900 V for 25 min in a precooled peptide map apparatus and then subjected to chromatography in 1-butanol (37.5%, v/v), pyridine (25%, v/v), glacial acetic acid (7.5%, v/v), and deonized water (30%, v/v) for 6–8 h. Plates were dried and analyzed by autoradiography.

RESULTS

BDV-P Is Phosphorylated in Vitro Predominantly by a PKC—Recombinant BDV-P was purified from *E. coli* to use as substrate to study phosphorylation of BDV-P *in vitro*. Incubation of BDV-P with an S10 extract of C6 cells resulted in its phosphorylation (Fig. 1*A*, *lane 1*). Initially, two kinase inhibitors were used to gain insight into the nature of the kinase that phosphorylates BDV-P. Whereas staurosporin, a potent inhibitor of PKC and other kinases (28), had a strong inhibitory effect on BDV-P phosphorylation (Fig. 1*A*, *lanes 4* and *5*), heparin which efficiently inhibits CKII had only a minimal effect (Fig. 1*A*, *lanes 2, 3,* and *6*). The combination of staurosporin and heparin resulted in nearly complete inhibition of BDV-P phosphorylation (Fig. 1*A*, *lane 6*). Incubation with an antibody to the conserved catalytic subunit of PKC (αPKC1.9) resulted in a concentration-dependent inhibition of BDV-P phosphorylation (Fig. 1*B*, *lanes 2* and *3*). The extent of inhibition with saturating concentrations of αPKC1.9 (2 μg) was similar to that observed with 400 nM staurosporin (Fig. 1*B*, *lane 4*). Inhibition was also observed following addition of the specific PKC inhib-

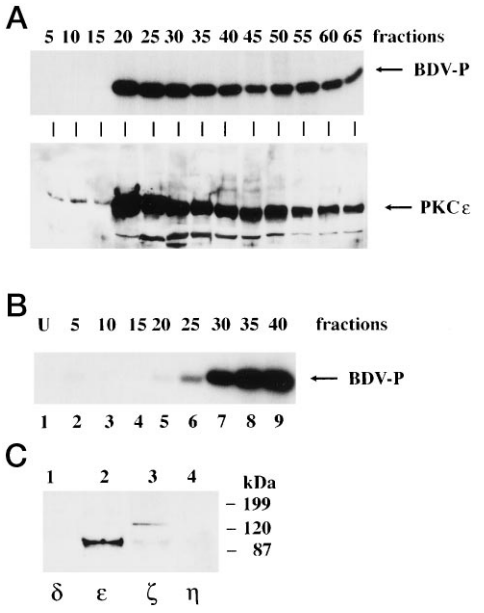

FIG. 2. **Partial purification of the BDV-P-phosphorylating kinase from rat brain.** Rat brain extracts (S100) were subjected to sequential chromatography through DEAE, phosphocellulose, DEAE (*A*), and hydroxylapatite (*B*). *A*, *upper panel*: *lanes 1–13*, after application of a sodium chloride gradient (0–1000 mM), 50-μl column fractions were collected and monitored for kinase activity. *Lower panel*, WIB analysis of the corresponding elution fractions in the *upper panel* using PKCε-specific antibodies. *B*, 50-μl column fractions were collected and monitored for kinase activity. *Lane 1*, unbound fraction; *lanes 2–9*, elution fractions after application of a potassium phosphate gradient (0–500 mM). *C*, WIB analysis with fraction 40 (500 ng) from *B* using subtype-specific antibodies against PKCδ, PKCε, PKCζ, and PKCη (*lanes 1–4*).

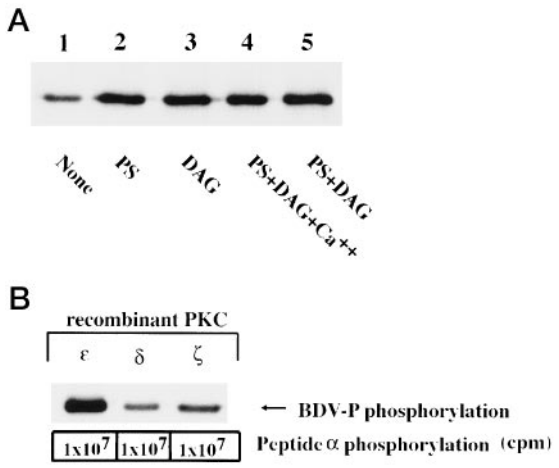

FIG. 3. *A*, PKC-dependent phosphorylation of phosphoprotein is stimulated by phosphatidylserine and diacylglycerol. BDV-P was phosphorylated with the partially purified BDV-P kinase from the final hydroxylapatite chromatography step (fraction 40) in the absence (*lane 1*) or presence of PKC activators (*lanes 2–5*). Activators were 10 μg/ml PS (*lane 2*), 100 μg/ml DAG (*lane 3*), 10 μg/ml PS, 100 μg/ml DAG, and 200 μM calcium (*lane 4*), and 50 μg/ml PS and 100 μg/ml DAG (*lane 5*). *B*, phosphorylation of BDV-P by recombinant PKCε, PKCδ, and PKCζ. The kinase concentration employed for BDV-P phosphorylation by PKCε (*lane 1*), PKCδ (*lane 2*), and PKCζ (*lane 3*) was normalized to α-peptide phosphorylation (10^7 cpm).

itor GF109203X to 6.0 μM final concentration. CKII-mediated phosphorylation of VSV-P was not affected by αPKC1.9 antibodies (data not shown). These results indicate that a cellular kinase of the PKC family is the major kinase involved in the incorporation of phosphate into the BDV-P.

PKC-dependent Phosphorylation of BDV-P Is Calcium-independent—The PKC enzymes include two subgroups, which differ in their response to calcium (29). To determine whether phosphorylation of BDV-P was mediated by a calcium-dependent PKC (PKCα, -β, and -γ) or calcium-independent PKC (PKCζ, -δ, -ε, and -η) (29), phosphorylation experiments with C6 or rat brain S100 extracts were performed in the presence of EGTA or supplemental calcium. Neither 0.5 mM EGTA nor calcium to 200 μM concentration had any effect on kinase activity (data not shown).

Purification of the BDV-P-phosphorylating PKC—The BDV-P-phosphorylating PKC was partially purified from C6 cells (data not shown) and rat brain by sequential chromatography through columns composed of DEAE, phosphocellulose, DEAE, and hydroxylapatite. Although elution profiles were similar with C6 and rat brain extracts, levels of phosphorylation activity were higher in fractions representing rat brain extracts. Fig. 2 displays results with rat brain extracts. Phosphorylation of BDV-P by the kinase in the elution fractions of the second DEAE column (Fig. 2*A*, *upper panel*) correlated with the presence of PKCε in each fraction as judged by Western immunoblot (WIB) using a PKCε subtype-specific antibody (Fig. 2*A*, *lower panel*). In contrast, WIB analysis of the same fractions using PKCζ subtype-specific antibodies revealed a different elution pattern, which did not correlate with the BDV-P phosphorylation activity (data not shown). In the final chromatography step, the kinase was eluted with 250 mM potassium phosphate (Fig. 2*B*, fraction 30 and beyond). The highest concentration of protein was eluted at 180 mM potassium phosphate (data not shown). BDV-P phosphorylation activity by the kinase eluted in fraction 40 was inhibited by staurosporin and αPKC1.9 antibodies but not by heparin (data not shown). To identify the PKC present in fraction 40, PKC subtype-specific antibodies (PKCε, PKCδ, PKCζ, PKCη) were used for WIB analysis. Only PKCε was detected in fraction 40 (Fig. 2*C*, *lane 2*).

To assess whether the purified BDV-P-phosphorylating PKC might be sensitive to the known PKC activators PS or DAG, the kinase activity was measured in experiments where substrate was not a limiting factor (Fig. 3*A*). Addition of 10 μg/ml PS (*lane 1*), 100 μg/ml DAG (*lane 2*), and 10 μg/ml PS and 100 μg/ml DAG in the presence (*lane 3*) or absence (*lane 4*) of 200 μM calcium stimulated phosphorylation of BDV-P 3–4-fold.

Comparison of Enzymatic Activity of the Recombinant PKCε, PKCδ, and PKCζ with the Partially Purified BDV-P Protein Kinase (BDV-PK)—The characteristics of the BDV-P protein kinase present in extracts from C6 cells and rat brain suggested its identity as PKCε. To further investigate the activity of individual PKC isotypes with respect to BDV-P, recombinant PKCε, PKCδ, and PKCζ were examined for the capacity to phosphorylate BDV-P (Fig. 3) and a panel of phosphate acceptors (Table I). When employed at concentrations normalized for phosphorylation of PKC α-peptide, the efficiency of PKCε in BDV-P phosphorylation was 5–10-fold higher than PKCδ and PKCζ (Fig. 3*B*). Whereas the efficiencies of BDV-PK and PKCε in PKC ε-peptide phosphorylation were similar (BDV-PK, 106%; PKCε, 118%), phosphorylation of ε-peptide by PKCδ and PKCζ was less efficient (PKCδ, 60%; PKCζ, 80%). BDV-PK and PKCε were also similar in inefficiency of AcMBP phosphorylation (BDV-P, 10%; PKCε, 13%). In contrast, PKCδ and PCKζ were more efficient in AcMBP phosphorylation (PKCδ, 58%; PKCζ, 45%). All kinases tested (BDV-PK, PKCε, PKCδ, and PKCζ) were inefficient in phosphorylation of histone H1 and histone IIA (4–8%), phosphate acceptors that are phosphorylated by cAMP- or cGMP-dependent protein kinase rather than PKC.

In Vivo Phosphorylation of BDV-P—Phosphorylation of proteins may differ *in vitro* or *in vivo*. Therefore, to test whether

TABLE I
Relative enzymatic activity of BDV-PK with various phosphate acceptors

Protein kinase activities were assayed in the presence of PS (10 μg/ml) and DAG (100 μg/ml). Results were normalized to the activity with α-peptide as substrate. Substrates were added at 200 μg/ml (proteins) or 200 μM (peptides).

Substrate	Protein kinase activity			
	BDV-PK	ϵ	δ	ζ
		%		
α	100	100	100	100
ϵ	106	118	60	80
AcMBP	10	13	58	45
Kemptide	15	11	3	10
Histone H1	8	8	6	8
Histone IIA	7	6	4	8

BDV-P is also phosphorylated by PKC *in vivo*, infected C6 cells were incubated with the specific PKC inhibitor GF109203X and inorganic [^{32}P]phosphate. BDV-P was immunoprecipitated from these cells using a monospecific antibody to BDV-P and subjected to SDS-PAGE and autoradiography. Increasing concentrations of GF109203X resulted in decreased BDV-P phosphorylation (Fig. 4, *lanes 3–6*) indicating a role for PKC in phosphorylation of BDV-P.

Peptide mapping studies were pursued to determine whether BDV-P is phosphorylated by PKC at the same sites *in vivo* and *in vitro*. For this purpose, BDV-P from infected C6 cells phosphorylated *in vivo* and recombinant BDV-P phosphorylated *in vitro* using a PKC-enriched fraction (C6 cells, second DEAE column) were separated by SDS-PAGE, transferred to nitrocellulose membranes, digested *in situ* with trypsin, and separated on thin layer chromatography plates for analysis by autoradiography. Peptide maps were identical (Fig. 5*A*) with three dominant spots. Similar maps were observed when recombinant BDV-P was phosphorylated with the crude S10 extract of C6 cells (data not shown).

Previous work had shown that BDV-P is phosphorylated *in vivo* at serine residues (30). Thus, the five potential sites of BDV-P phosphorylation were investigated using BDV mutants in which serine residues were changed to alanine by PCR mutagenesis. Experiments with a C6 S100 extract depleted of CKII revealed that the double mutant BDV-P (26/28) was not efficiently phosphorylated (Fig. 5*B*). Peptide mapping of BDV-P (26/28) revealed that this site corresponds to the major peptide spot *1* (Fig. 5*A*). Due to the lack of a trypsin cleavage site between Ser^{26} and Ser^{28} it was not possible to differentiate between the two potential phosphorylation sites with peptide maps.

The Heparin-sensitive Kinase Is CKII—The observations that staurosporin did not completely block phosphorylation of BDV-P and that the residual kinase activity was heparin-sensitive (Fig. 1) suggested the possibility of minor phosphorylation by CKII. To investigate this further, CKII was purified from the C6 S100 extract by phosphocellulose chromatography. The majority of PKC-mediated kinase activity was found in the void volume (Fig. 6*A*, *lane 1*). This activity was inhibited by staurosporin (Fig. 6*A*, *lane 3*) but not by heparin (Fig. 6*A*, *lane 2*). In contrast, kinase activity in fraction 50 was inhibited by heparin (Fig. 6*B*, *lane 2*) and not by staurosporin (Fig. 6*B*, *lane 3*). The kinase activity in fraction 50 was also inhibited by antibodies to CKII (Fig. 6*B*, *lane 4*). To identify the sites of CKII phosphorylation, BDV-P mutants were generated by PCR that either lacked the first 11 amino acids (deleting two potential CKII sites) or substituted alanine for serine residues at positions 58, 70, and 86. Incubation of these BDV-P mutants and wild-type recombinant BDV-P with a commercial CKII revealed that the serine residues at positions 70 and 86 are the major sites of phosphorylation by CKII (Fig. 6*C*). Neither mutation had an impact on the PKC-dependent phosphorylation (data not shown).

Similarly, mutations at sites found to impair PKC-dependent phosphorylation of BDV-P (residues 26 and 28) had no effect on CKII-mediated phosphorylation (data not shown). Phosphorylation of wild-type BDV-P by a commercial CKII was similar in the presence or absence of S10 C6 cell extracts (data not shown).

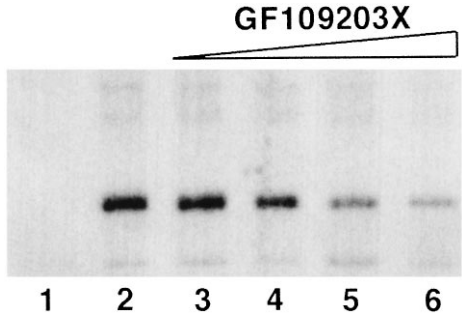

FIG. 4. **Phosphorylation of BDV-P *in vivo* is partially inhibited by GF109203X.** BDV-infected C6 cells (C6-BDV) were incubated 4 h with [^{32}P]phosphate and then lysed for immunoprecipitation of BDV-P with rabbit antibodies to BDV-P (*lanes 2–6*) or normal rabbit sera (*lane 1*). *Lanes 3–6*, C6-BDV cells were incubated with increasing concentrations of GF109203X (1, 2, 3, and 4 μM) 6 h before addition of [^{32}P]phosphate.

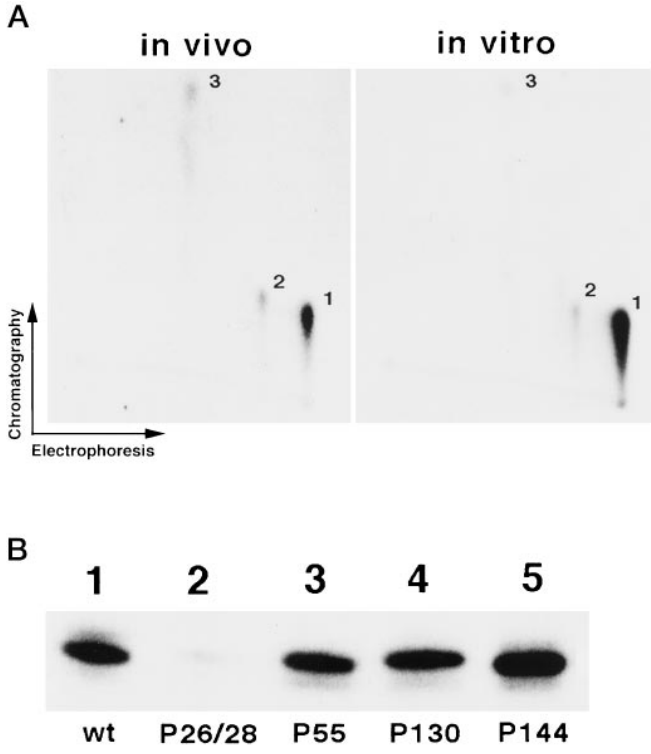

FIG. 5. *A*, BDV-P is phosphorylated at similar sites *in vitro* and *in vivo*. Peptide maps of BDV-P immunoprecipitated from infected cells (*in vivo*) and recombinant phosphoprotein phosphorylated using a PKC-enriched protein fraction (*in vitro*) are shown. Trypsin-digested peptides were separated on thin layer chromatography and analyzed by autoradiography. *Numbers* indicate sites of major peptide signals. *B*, serine residues 26 and 28 are major sites for PKC-dependent phosphorylation of BDV-P. Wild-type and mutant forms (serine to alanine substitution) of BDV-P were phosphorylated *in vitro* using an S100 extract of C6 cells depleted of CKII. *Lane 1*, wild-type (*wt*) BDV-P; *lane 2*, double mutation at residues 26 and 28; *lane 3*, mutation at residue 55; *lane 4*, mutation at residue 130; *lane 5*, mutation at residue 144.

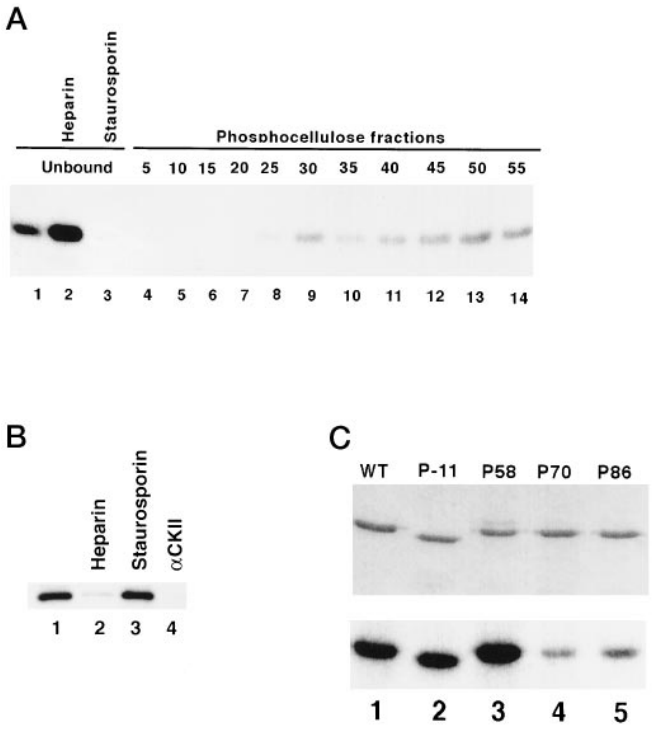

FIG. 6. **The heparin-sensitive phosphorylation of BDV-P is due to CKII.** *A*, kinase activity is present in both unbound (*lane 1*) and bound elution column (*lanes 4–14*) fractions after phosphocellulose fractionation of a DE52-concentrated S100 extract of C6 cells using a 100–1000 mM NaCl gradient. The kinase activity in the unbound fraction is sensitive to staurosporin (200 nM, *lane 3*) but not heparin (20 μg/ml, *lane 2*). *B*, phosphorylation of BDV-P by elution fraction 50 in the absence (*lane 1*) or presence of heparin (20 μg/ml, *lane 2*), staurosporin (200 nM, *lane 3*), or antibodies to CKII (*αCKII, lane 4*). *C,* serine residues 70 and 86 are major sites for CKII-dependent phosphorylation of BDV-P. Wild-type (*WT*) and mutant forms (serine to alanine substitution) of BDV-P were phosphorylated *in vitro* using CKII (0.01 unit/μl). *Lane 1*, BDV-P wild type; *lane 2*, mutant lacking the first 11 amino acids (P-11); *lane 3*, mutation at residue 58 (P58); *lane 4*, mutation at residue 70 (P70); *lane 5*, mutation at residue 86 (P86). *Lower panel*, phosphorylation activity as measured by autoradiography; *upper panel*, protein concentration as measured by Coomassie Blue staining.

DISCUSSION

The objective of this study was to characterize the cellular kinases responsible for phosphorylation of BDV-P. *In vitro* experiments with recombinant BDV-P and crude extracts from C6 cells indicated that BDV-P was phosphorylated by both PKC and CKII; however, phosphorylation appeared to be mediated primarily by PKC. BDV-P phosphorylation was largely inhibited *in vitro* by antibodies directed against the catalytic subunit of PKC. Exposure of infected cells to increasing concentrations of GF109203X, a specific PKC inhibitor (31), resulted in decreased phosphorylation of BDV-P. Analysis of extracts from C6 cells and rat brain enriched for BDV-P phosphorylation activity revealed that the BDV-PK is PKCε. The enzymatic activity of BDV-PK was correlated with the presence of PKCε rather than that of PKCδ, PKCζ, or PKCη. In addition, the *in vitro* enzymatic activity profiles of purified BDV-PK and recombinant PKCε were similar with respect to BDV-P and a panel of phosphate acceptors. Peptide maps of BDV-P phosphorylated *in vitro* and *in vivo* were identical and revealed one major and two minor phosphopeptides. Experiments with BDV-P mutants lacking potential PKC sites indicated that the major phosphopeptide represents a PKC site.

The major phosphopeptide found in peptide maps from *in vivo* phosphorylated BDV-P contained the potential PKC sites, Ser^{26} and Ser^{28}. We have only examined phosphorylation of BDV-P from BDV strain He/80. Because this viral strain lacks a suitable cleavage site between Ser^{26} and Ser^{28}, it was not possible to directly determine which site was phosphorylated through peptide mapping. However, the observation that Ser^{26} is not present in BDV strain V (32) suggests that BDV-P is likely to be phosphorylated at Ser^{28} *in vivo*.

Site-directed mutagenesis of recombinant BDV-P established Ser^{70} and Ser^{86} as the principal sites for phosphorylation by CKII *in vitro*. A potential basis for the observation that CKII-dependent phosphorylation is less pronounced than phosphorylation mediated by PKC is that CKII sites are more sensitive to phosphatases. However, this explanation appears to be unlikely given that levels of BDV-P phosphorylation with recombinant CKII did not differ in the presence or absence of crude C6 cell extract. Alternatively, the difference in the efficiency of PKC and CKII phosphorylation *in vitro* may reflect accessibility of individual sites in the folded protein. Whether BDV-P is phosphorylated by CKII *in vivo* remains to be determined; however, the observation that kinase activity cannot be eliminated *in vivo* using PKC inhibitors is consistent with a role for CKII in BDV-P phosphorylation.

Phosphoproteins of other nonsegmented, negative-strand RNA viruses are typically phosphorylated *in vitro* only by one kinase, CKII (*e.g.* measles (33) and RSV (34)) or PKC (*e.g.* HPIV3 (19)). An exception is VSV-P where initial phosphorylation by CKII induces a conformational change of VSV-P (New Jersey) that opens sites for secondary phosphorylation by an L-associated kinase (35). BDV-P is phosphorylated *in vitro* by both PKCε and CKII. Although there are no data concerning the possibility that phosphorylation by one kinase effects a conformational change in BDV-P, the activities of these kinases appear to be independent because mutants lacking PKC sites are phosphorylated by CKII, and conversely, mutants lacking CKII sites are phosphorylated by PKC.

The observation that BDV-P is predominantly phosphorylated by PKCε does not imply that CKII phosphorylation is inconsequential. There is precedent for functionally significant phosphorylation of individual cellular transcription factors by more than one kinase. For example, phosphorylation of cyclic AMP-responsive element binding proteins Jun and Fos by different kinases has been shown to regulate their activities (36). We can only speculate as to the role of phosphorylation of BDV-P at multiple sites by different kinases. BDV is the only nonsegmented negative-strand RNA virus known to have a nuclear localization for transcription and replication. Perhaps phosphorylation of BDV-P by one kinase impacts its translocation to the cell nucleus. Consistent with such a hypothesis is the observation that the PKC phosphorylation sites are located within the putative nuclear localization signal of phosphoprotein. Phosphorylation by a second kinase might then trigger assembly with other cellular or viral proteins (for example, the BDV polymerase) to form an active transcription factor complex. Finally, it is intriguing to speculate that phosphorylation events may play a role in BDV tropism for limbic circuitry. Indeed, the anatomic distributions of PKCε (37) and BDV are similar in rat brain (3, 38), the best described model system for Borna disease. As recombinant BDV systems are established these hypotheses will be tested using BDV-P mutants lacking specific phosphorylation sites.

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