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 cytoplastic 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 Ser26 and Ser28 as sites for PKC phosphorylation and Ser76 and Ser66 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.

EXPERIMENTAL PROCEDURES

In Vivo Phosphorylation Experiments—BDV-infected C6 cells (23) (5 x 10^6) were washed twice with phosphate-buffered saline (PBS), incubated in modiﬁed RPMI medium 1640 (Irvine Scientiﬁc) without P, (2 ml) in the presence of absence of GF109203X (1 μmol/ml) for 6 h, and then labeled with inorganic 32P (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 NdeI site (p23-NdeI) 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-NdeI primer and combinations of the primers described below. In a final PCR, the complete BDV-P ORF mutant sequence was amplified with the p23-NdeI primer and T7 primer and cloned into the NdeI/HindIII site of pET15b (Invitrogen). Introduction of the correct sequence for each mutant was confirmed by dyeoxy
was counted after washing for 5 min with 75 mM H3PO4 five times. It was spotted onto P81 paper (Whatman). Radioactivity bound to paper was recovered as described by Boyle et al. (27). Trypsin-digested peptides were fractionated on cellulose plates (J. T. Baker Inc.) in 20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 10 mM 2-mercaptoethanol, 20% glycerol, 2.5 mM CaCl2, 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 MgCl2 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, 108 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 x g for 10 min (S10 extract) or 100,000 x 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 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 K2PO4, 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.

Methods used for purification of phosphoproteins of VSV and HPIV3 have been described (19).

Peptide Mapping—[32P]-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 deionized 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. 1A, 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. 1A, lanes 4 and 5), heparin which efficiently inhibits CKII had only a minimal effect (Fig. 1A, lanes 2, 3, and 6). The combination of staurosporin and heparin resulted in nearly complete inhibition of BDV-P phosphorylation (Fig. 1A, lane 1). Incubation with an antibody to the conserved catalytic subunit of protein kinase C (ePKC1.9) resulted in a concentration-dependent inhibition of BDV-P phosphorylation (Fig. 1B, lanes 2 and 3). The extent of inhibition with saturating concentrations of ePKC1.9 (2 μg) was similar to that observed with 400 nM staurosporin (Fig. 1B, lane 4). Inhibition was also observed following addition of the specific PKC inhib-
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 DEAE, phosphocellulose, DEAE, and hydroxyapatite. Although elution profiles were similar through columns composed of DEAE, phosphocellulose, DEAE, and hydroxylapatite (data not shown) and rat brain by sequential 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 PKCe, PKCd, and PKCζ. The kinase concentration employed for BDV-P phosphorylation by PKCe (lane 1), PKCd (lane 2), and PKCζ (lane 3) was normalized to α-peptide phosphorylation (10^4 cpm).

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 hydroxyapatite 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 PKCe, PKCd, and PKCζ. The kinase concentration employed for BDV-P phosphorylation by PKCe (lane 1), PKCd (lane 2), and PKCζ (lane 3) was normalized to α-peptide phosphorylation (10^4 cpm).

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. 3A). 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 PKCe, PKCd, 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 PKCe. To further investigate the activity of individual PKC isoforms with respect to BDV-P, recombinant PKCe, PKCd, 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 PKCe in BDV-P phosphorylation was 5–10-fold higher than PKCd and PKCζ (Fig. 3B). Whereas the efficiencies of BDV-PK and PKCe in PKC ε-peptide phosphorylation were similar (BDV-PK, 100%; PKCe, 115%), phosphorylation of ε-peptide by PKCd and PKCζ was less efficient (PKCd, 60%; PKCζ, 50%). BDV-PK and PKCe were also similar in inefficiency of AcMBP phosphorylation (BDV-PK, 10%; PKCe, 13%). In contrast, PKCd and PKCζ were more efficient in AcMBP phosphorylation (PKCd, 58%; PKCζ, 45%). All kinases tested (BDV-PK, PKCe, PKCd, and PKCζ) were inefficient in phosphorylation of histone H1 and histone H1A (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
BDV-P is also phosphorylated by PKC \textit{in vivo}, infected C6 cells were incubated with the specific PKC inhibitor GF109203X and inorganic [\textsuperscript{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 \textit{in vivo} and \textit{in vitro}. For this purpose, BDV-P from infected C6 cells phosphorylated \textit{in vivo} and recombinant BDV-P phosphorylated \textit{in vitro} using a PKC-enriched fraction (C6 cells, second DEAE column) were separated by SDS-PAGE, transferred to nitrocellulose membranes, digested \textit{in situ} with trypsin, and separated on thin layer chromatography plates for analysis by autoradiography. Peptide maps were identical (Fig. 5A) 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 \textit{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. 5B). Peptide mapping of BDV-P (26/28) revealed that this site corresponds to the major peptide spot 1 (Fig. 5A). Due to the lack of a trypsin cleavage site between Ser\textsuperscript{26} and Ser\textsuperscript{28} it was not possible to differentiate between the two potential phosphorylation sites with peptide maps.

The \textit{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. 6A, lane 1). This activity was inhibited by staurosporin (Fig. 6A, lane 3) but not by heparin (Fig. 6A, lane 2). In contrast, kinase activity in fraction 50 was inhibited by heparin (Fig. 6B, lane 2) and not by staurosporin (Fig. 6B, lane 3). The kinase activity in fraction 50 was also inhibited by antibodies to CKII (Fig. 6B, 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. 6C). 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).
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 PKCe.

The enzymatic activity of BDV-PK was correlated with the presence of PKCe rather than that of PKCb, PKCd, or PKCγ. In addition, the in vitro enzymatic activity profiles of purified BDV-PK and recombinant PKCe 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 vitro phosphorylated BDV-P contained the potential PKC sites, Ser26 and Ser86. We have only examined phosphorylation of BDV-P from BDV strain He/80. Because this viral strain lacks a suitable cleavage site between Ser26 and Ser86, it was not possible to directly determine which site was phosphorylated through peptide mapping. However, the observation that Ser26 is not present in BDV strain V (32) suggests that BDV-P is likely to be phosphorylated at Ser28 in vivo.

Site-directed mutagenesis of recombinant BDV-P established Ser70 and Ser86 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 ζ-associated kinase (35). BDV-P is phosphorylated in vitro by both PKCe 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 PKCe 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 PKCe (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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