The Human Biliverdin Reductase-based Peptide Fragments and Biliverdin Regulate Protein Kinase Cα Activity

THE PEPTIDES ARE INHIBITORS OR SUBSTRATE FOR THE PROTEIN KINASE Cα

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Background: hBVR reduces biliverdin to antioxidant bilirubin. PKCα promotes tumorigenesis and apoptosis. Results: Complex formation between PKCα and hBVR results in transactivation. hBVR-based peptides are identified as substrates or inhibitors of the PKCα in vitro and in the cell. Biliverdin inhibits PKCα. Conclusion: A regulatory loop links PKCα and hBVR in cell signaling. Significance: hBVR-based peptides can be used to regulate PKCα signaling.

PKCα, a Ser/Thr kinase, promotes cell growth, tumorigenesis, and apoptosis. Human biliverdin reductase (hBVR), a Ser/Thr/Tyr kinase, inhibits apoptosis by reducing biliverdin-IX to antioxidant bilirubin. The enzymes are activated by similar stimuli. Reportedly, hBVR is a kinase-independent activator of PKCα and is transactivated by the PKC (Gibbs, P. E., Miralem, T., Lerner-Marmarosh, N., Tudor, C., and Maines, M. D. (2012) J. Biol. Chem. 287, 1066–1079). Presently, we examined interactions between the two proteins in the context of regulation of their activities and defining targets of hBVR phosphorylation by PKCα. LC-MS/MS analysis of PKCα-activated intact hBVR identified phosphorylated serine positions 21, 33, 230, and 237, corresponding to the hBVR Src homology-2 domain motif (Ser230 and Ser237), flanking the ATP-binding motif (Ser21) and in PHPS sequence (Ser33) as targets of PKCα. Ser21 and Ser230 were also phosphorylated in hBVR-based peptides. The Ser230-containing peptide was a high affinity substrate for PKCα in vitro and in cells; the relative affinity was PKCα > PKCBII > PKCζ. Two overlapping peptides spanning this substrate, KRNRYLSF and SFHKSGSL, were effective inhibitors of PKCα kinase activity and PKCα-supported activation of transcription factors Elk1 and NF-κB. Only SFHKSGSL, in PKCα-transfected phorbol 12-myristate 13-acetate-stimulated cells, caused membrane blebbing and cell loss. Biliverdin noncovalently inhibited PKCα, whereas PKCα potentiated hBVR reductase activity and accelerated the rate of bilirubin formation. This study, together with previous findings, reveals an unexpected regulatory interplay between PKCα and hBVR in modulating cell death/survival in response to various activating stimuli. In addition, this study has identified novel substrates for and inhibitors of PKCα. We suggest that hBVR-based technology may have utility to modulate PKCα-mediated functions in the cell.

PKCα, a member of the novel group of PKCs, is a Ser/Thr kinase, and its activation is linked to signaling pathways that govern cell growth, survival, and death (1, 2). In addition to its role in cell growth and apoptotic processes, PKCα has been implicated in regulation of membrane ion channels, activation of transcriptional factors, antigen presentation, and with various cancers (3). hBVR,2 a Ser/Thr/Tyr kinase and a reductase, is a 296-residue soluble polypeptide with an extensive range of input into signal transduction pathways, as well as being a key component of cellular defense mechanisms (4); the reductase activity of hBVR is directly associated with its phosphorylation state (5, 6). Depending on the type of stimulus and cell type, activation of PKCα and hBVR can exert opposing effects on apoptotic events or bring about a similar outcome on cell survival. Accordingly, it is reasonable to foresee an intimate interplay between the two proteins. Moreover, hBVR and PKCα are co-expressed in the cell, and their activation influences an overlapping cast of downstream effector targets.

Previously, we have reported on the potentiation of PKCα activation by hBVR in IGF-1-stimulated cells (7). The activation involved physical interaction between hBVR and PKCα as indicated by co-immunoprecipitation and FRET-fluorescence lifetime imaging spectroscopy analysis. PKCα and hBVR have in common a number of extracellular activators; the list includes reactive oxygen species (ROS), IGF-1, and insulin (4, 5, 8–12). Earlier studies had identified hBVR as an activator of members of the two other major families of PKC kinases, specifically PKCBII and PKCζ, the conventional and atypical

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2 The abbreviations used are: hBVR, human biliverdin reductase; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SH2 domain, Src homology-2 domain.
kinases, in IGF-1- and TNFα-stimulated cells, respectively (13, 14).

In unstimulated cells, PKCs, including PKCδ, are present in an inactive conformation (1, 2). Stimulation of cells with anionic lipid second messengers/cofactors, such as phorbol 12-myristate 13-acetate (PMA) or diacylglycerol, causes conformational changes in PKCs that result in exposure of the activation loop and release of the auto-inhibitory pseudosubstrate sequence in the N-terminal regulatory domain of the protein from the active site (1, 2). The pseudosubstrate domain, placed between the C2-like and C1 regions, maintains the kinase in an inactive conformation by interacting with the substrate recognition site in the catalytic domain, as is the case for all PKCs (15). The two zinc finger motifs in the N-terminal regulatory C1 domain of PKCs are the recognition motifs for the second messengers (16); hBVR is also a Zn²⁺ metalloprotein (17). PKCδ signaling activity is a function of its phosphorylation (1, 2); however, it differs from other members of the PKC family enzymes by also being activated independent of lipids and translocation to the cell membrane (18). Phosphorylation of serine or tyrosine residues influences the translocation of the PKC to organelle targets, enabling it to exert anti-apoptotic/proliferative or pro-apoptotic effects (10, 19).

Activated PKCδ interacts with, and phosphorylates, a number of pro-apoptotic proteins. Therefore, its kinase activity plays a determining role in the regulation of cell death (20); for instance, in PMA-stimulated cells, activation of caspase-3 results in cleavage of PKCδ between the regulatory and catalytic domains of the PKC, leading to translocation of the catalytic domain into the nucleus and hence the onset of apoptosis (21). Conversely, the proliferative effects of PKCδ likely involve its activation of ERK1/2, which are the upstream kinases for a host of transcriptional factors, including Elk1 and NF-κB, that in turn regulate cell growth, proliferation, and survival (22, 23). We have recently characterized hBVR as the scaffold/bridge/anchor for activation of Elk1 by ERK1/2 in the nucleus and also a molecular scaffold/bridge for activation of ERK1/2 by MEK1/2 and PKCδ (7, 24).

By virtue of its catalysis of the conversion of the tetrapyrrrole biliverdin-IX to bilirubin-IX, a quencher of ROS, hBVR limits ROS and free radical-mediated apoptosis (25). Bilirubin-IX plays a central role in cellular defense mechanisms (26–29), and its formation is solely dependent on hBVR activity; reportedly, bilirubin is as effective as glutathione in hindering the toxicity of free radicals (30). Biliverdin is the product of oxidative cleavage of heme (Fe²⁺-protoporphyrin-IX) at the meso-carbon bridge by the two active forms of heme oxygenase, the stress-inducible HO-1 and the constitutive HO-2 (31). hBVR is essential both for activation of HO-1 expression by free radicals (25) and for stabilization of HO-2 (32); HO-2 stabilization is a result of attenuation of ubiquitination and proteasomal degradation. In addition, hBVR is an activator of AP-1- and AP-2-dependent gene expression; the stress-responsive genes, FOS, JUN, and ATF-2/CREB, are downstream targets of hBVR (13, 33).

Consensus phosphorylation targets of several kinases (34, 35) are present in hBVR (7). Three serine residues in consensus phosphorylation targets of protein kinases (36, 37) are present in hBVR. The 21SVR (SXR) sequence flanks the ATP-binding domain of hBVR (13GVGRAG), and the 294SRK is upstream of the hBVR cysteine-rich Zn-binding domain (280HCX10CC) (17). An RX(S/T) motif, which includes Tyr228, is located in the sequence 224KRNNYLSHFKSGSL, a segment of hBVR that we have presently identified as a vital link between PKCδ and hBVR in regulation of their activities. 228YLSF and 198YMKM, when tyrosine-phosphorylated, form SH2 protein-docking sites (38).

Because PKCδ and hBVR both have a broad range of biological activities, their interaction could influence an array of processes that are associated with normal cellular activities, as well as those that are associated with pathophysiology of the cell (1, 2, 4, 39, 40). Accordingly, it is reasonable to postulate that the activation of either enzyme has a bearing on the other. Should this be the case, in conducting this investigation we reasoned that short peptides designed based on the hBVR primary structure could function as surrogates for the intact hBVR polypeptide, capable of modulating PKCδ activity. Our studies have revealed coupled regulation of the activated enzymes. The investigation has led to identification of hBVR-based small peptides, derived from the 224KRNNYLSHFKSGSL sequence, that are highly effective inhibitors of PKCδ kinase activity, whereas the 15-residue-long peptide itself serves as an exceptionally good substrate for the kinase. The inhibitory peptides identified here add to the small battery of peptides that have therapeutic potential for control of PKCδ activity in the cell.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant activated PKCδ for in vitro studies, TNF-α and PMA, were obtained from Calbiochem. The PKCδ peptide substrate, ARRKKGSFFYGG, was purchased from Biomol (Plymouth Meeting, PA). DTT and ATP were obtained from Sigma. Myelin basic protein, phosphatidylserine, and diacylglycerol mixture were from Millipore (Temecula, CA). hBVR-based peptides KNRRYLSF, SFHFKSGSL, and KYCCSRK were synthesized in both unmodified and N-methylated forms by EziBiolab (Westfield, IN); the peptides KNRRYLSHFHSFGSL, GLKRRNYLHFSFGSL, GLKRRNYLFHFSFGSL, and RAGSVRMRDL were obtained from the same source in the unmodified forms only. [γ-³²P]ATP and [³²P]H₂PO₄ (carrier- and HCl-free) were from PerkinElmer Life Sciences. Polyclonal anti-PKCδ antibodies were from Cell Signaling. Anti-human hBVR polyclonal antibodies were obtained as described before (41).

**Plasmids and Mutants**—The hBVR open reading frame was cloned in the pEGFP-C1 and pDsRed-C1 vectors (Clontech) for expression of fluorescent protein-tagged hBVR in cells and as an HA-tagged species in pcDNA3. The GST-hBVR plasmid has been described elsewhere (42). Selected serine residues were mutated to alanine, using the QuikChange kit (Stratagene, Cedar Creek, TX). The human PKCδ open reading frame was also cloned in pcDNA3 and pEGFP-C1, using PCR amplification products derived from a human brain cDNA library (Invitrogen). The constitutively active PKCδ was generated by deletion of amino acids 151–160 in the pseudosubstrate loop (43) from the pcDNA3-PKCδ clone. All plasmids were verified by
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sequencing to ensure both the integrity of inserts and placement in the correct reading frame.

Cell Culture and Transfection— Cultures of HEK293A cells were grown and transfected with plasmids, using Transfectin lipid reagent (Bio-Rad). Overexpression of proteins was verified by Western blotting. Transfected cells were serum-starved in DMEM containing 0.1% FBS for 24 h, before treatment with 100 nm PMA or 20 ng/ml TNF-α for 15 min. Cell lysates were immunoprecipitated as described previously (14).

PKCδ Activity Measurements— PKCδ assay in vitro was performed using 5 ng of purified recombinant human PKCδ (as a GST fusion protein) in a 50-μl reaction containing 50 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.2 mM DTT, sonicated lipid activators (0.5 μg of phosphatidylserine and 0.05 μg of diacylglycerol) or lipid activators plus PMA (as indicated in appropriate experiments), and 50 μM specific PKCδ substrate or hBVR-based peptides at concentrations indicated in the appropriate figures. The reaction was started by the addition of 50 μM ATP labeled with 5 μCi of [γ-32P]ATP and incubated for 15 min at 30 °C, unless otherwise stated. The reaction was terminated either by the addition of 1 volume of 10% phosphoric acid, followed by transfer of the reaction mixture to P81 membranes for scintillation counting (12). For autophosphorylation of PKCδ, [γ-32P]ATP was used to start a 40-min reaction.

PKCδ activity in cells was also measured by immunoprecipitation from cell lysates with anti-PKCδ antibody followed by protein A/G-agarose. The immunoprecipitates were used in kinase reactions, as above, containing 50 μM PKCδ-specific peptide substrate; incorporation of 32P was measured by the P81 method.

To measure the effect of biliverdin on PKCδ activity, the PKC was preincubated in kinase buffer for 5 min first with 0.2 mM DTT, followed by addition of biliverdin (Frontier Scientific, Logan UT) as indicated in the figures, with [γ-32P]ATP being added last to initiate the autophosphorylation reaction. Alternatively, biliverdin was added prior to DTT, or DTT was omitted entirely. The reaction products were resolved by gel electrophoresis and detected by autoradiography.

In Vitro Assays with Recombinant PKCδII and PKCζ Kinases— Recombinant PKCδII (>800 units/mg, Calbiochem) was assayed in vitro in 20 mM HEPES, pH 7.2, 15 mM MgCl₂, 0.2 mM CaCl₂, 1 mM DTT, 25 mM β-glycerophosphate, 50 μg/ml phosphatidylserine, and 5 μg/ml diacylglycerol using the peptide GLKRNRYLSFHFK or myelin basic protein (12.5 μM) substrates and 5 ng of enzyme per 50 μl of reaction. 100 μM ATP (containing [γ-32P]ATP, as above) was used to start the reaction, and incorporation was determined as above using the P81 filter binding assay (12). Similarly, recombinant PKCζ (Millipore) was incubated in 20 mM MOPS, pH 7.2, 15 mM MgCl₂, 0.2 mM EDTA, with the peptide GLKRNRYLSFHFK or myelin basic protein as substrates, again using 5 ng of enzyme per 50-μl reaction (14). Otherwise, reaction conditions were as for PKCδII.

Measurement of hBVR Kinase— Kinase activity of hBVR was assayed, as described earlier (12). hBVR was incubated at 30 °C in a 50-μl reaction containing 50 mM HEPES, pH 8.4, 30 mM MnCl₂, 0.2 mM DTT, 10 μM ATP labeled with 10 μCi of [γ-32P]ATP and PKCδ for 30 min.
tion spectrum data were used in a Mascot search of a custom database, containing individual entries for the three peptides. Mascot search parameters included precursor and fragment ion mass tolerance of 1.5 and 0.8 daltons, respectively, and allowed for one C13 incorporation, fixed carbamidomethyl-cysteine modification, variable methionine oxidation, and serine/threonine/tyrosine phosphorylation. The ion score threshold value was set for 15, with an expect score less than 0.05. Ion peaks containing major peptide species in the four-charge state were analyzed both manually and using Mascot. LC-MS/MS analysis of kinase-treated peptide samples was compared with untreated peptides, using label-free quantification of extracted ion chromatogram analysis and ProteoIQ software.

**Mass Spectrometry Analysis of Peptides from Tissue Culture**—Cells were transfected with pcDNA-PKCδ and serum-starved as described above. They were then treated with 100 nM PMA for 15 min, and the in situ PKCδ assay (42) was used to introduce the peptides into the cells. Cells were washed and incubated for 10 min at 30 °C in 50 μl of kinase assay buffer (137 mM NaCl, 5.4 mM KCl, 10 mM MgCl2, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, 25 mM β-glycerophosphate, 5.5 mM d-glucose, 5 mM EGTA, 1 mM CaCl2, 20 mM HEPES, pH 7.2, 50 μg/ml digitonin, 120 μg/ml PKCδ peptide substrate, and 50 μM ATP). After 1 h, the reaction mix was collected (leaving the cells adhering to the plate), and debris was removed by centrifugation. The supernatant was applied to a Bio-Gel P4 (Bio-Rad) column (equilibrated with 68.5 mM NaCl, 2.7 mM KCl, 0.5 mM EGTA, 0.1 mM EDTA, and 10 mM HEPES, pH 7.2), to separate large proteins from the peptide fraction. Peptide containing fractions were pooled, concentrated, extracted with ethyl acetate and processed for mass spectrometry analysis, using the procedure described above.

**Mapping of Phosphorylation Sites in Intact hBVR by Mass Spectrometry**—GST-tagged hBVR was overexpressed from the plasmid pGEX-hBVR in *Escherichia coli* and purified by affinity chromatography using GSH-arosage. The protein was eluted either with glutathione to give the intact fusion protein or by treatment with thrombin to release intact hBVR. Both preparations were incubated with PKCδ as described above, and the protein was resolved by SDS-gel electrophoresis. To map the complete protein, the following digests were used: chymotrypsin, complete and partial trypsin, and trypsin after treatment of GST-hBVR with acetic anhydride. Stained protein bands were cut from gels, cut into 1-mm square pieces, washed with 50 mM NH4HCO3, and dehydrated. The proteins were reduced with DTT and alkylated with iodoacetamide, and the rehydrated gels were digested with 20 μg/ml trypsin or chymotrypsin (mass spectrometry grade, Promega) in bicarbonate buffer containing 10% acetonitrile for 1 h at 24 °C and then at 37 °C overnight, followed by a further addition of enzyme, and incubated for 3 h. The digested material was extracted from the gel and analyzed by LC-MS/MS, essentially as described for the peptide samples, above, except that 100 ng of digest peptides were loaded on the nanospray column. The Mascot search parameters were adjusted; the ion score cutoff was set at 25 for the custom database, and the Expect value cutoff was set at 0.1 for the complete human protein database. Peptides with an Expect score less than 0.05 were considered positive identification if more than one peptide was identified for a given protein and if identified as a positive spectral match by ProteoIQ software (NuSep). Phosphopeptide fragmentation spectra were accepted if fragment ions allowed for unambiguous mapping of modification sites to a hydroxyamino acid.

**Confocal Microscopy**—HeLa cells were maintained as described above for HEK293 cells. Transfection of HeLa cells was performed at ~80% confluency using FuGENE HD reagent (Promega) following the manufacturer’s instructions. One day after the transfection, the cells were serum-starved for 24 h (0.1% FBS). The peptides (KRNRYLSF, SFHFKSGSL, or KKKRILHC), at a concentration of 10 μM, were added 2 h prior the addition of 100 nM PMA for 15 min. The fluorescence images were collected using a Cell Observer® spinning disc from Zeiss. During the experiments, the cells were kept at 37 °C and 5% CO2. GFP fluorescence was excited using a 488-nm diode laser, and the emission was collected using a 500–550-nm band pass.

**Test of Covalent Binding of Biliverdin to PKCδ**—Association between PKCδ and biliverdin was examined essentially as described by Lamparter et al. (44). Biliverdin was dissolved in 0.1 M NaOH and diluted to 2 μM in PBS, pH 7.4; a 500-μl sample was used to measure the absorption spectrum between 260 and 760 nm. GST-tagged PKCδ was then added to a final concentration of 1 μM and incubated for 5 min at 25 °C, and the spectrum again was measured. To test for covalent association, the sample was adjusted to 1% SDS, loaded on four Sephadex G-50 spin columns equilibrated in PBS, centrifuged, and the excluded fractions were collected and pooled, and the spectrum was again recorded.

**Statistical Analysis**—Data as presented in bar graphs are the means with standard deviations of three experiments, unless otherwise indicated, each with triplicate samples. Data were analyzed by one-way analysis of variance from which Student’s t test was calculated for all sample pairs. Differences within experiments were considered significant if p ≤ 0.05. In the figures, brackets indicate the paired data, and significant differences are indicated by asterisks. Kinetic data for the peptide substrate were fitted to the Michaelis-Menten equation using Prism 3.0 software (GraphPad, San Diego).

**RESULTS**

**Characterization of an hBVR-based Peptide as a PKCδ Substrate**—We had previously observed augmented PKCδ kinase activity and autophosphorylation in IGF-1-stimulated cells (7). That study also detected increased interaction between hBVR and PKCδ in response to IGF-1 and PMA stimulation and further formation of a complex that also included ERK2 and MEK1. We examined the consequence of the hBVR/PKCδ interaction on hBVR phosphorylation, aiming to identify specific targets of the PKC by evaluating several candidate phosphorylation sites on hBVR that are contained within the consensus phosphorylation motifs of PKCδ. Among these potential phosphorylation sites are the three serine residues Ser230, Ser21, and Ser294. The Ser230 site is found in an (S/T)(RYLS) motif in one of the hBVR SH2 domains. Ser21 flanks the ATP-binding domain of hBVR (15GVGRAG) in the SXR (SVR) motif; Ser294 is in the SXX (SRK) motif and is prox-
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FIGURE 1. PKCδ efficiently phosphorylates hBVR-based peptides. a, in vitro, hBVR consensus phosphorylation motifs are targets of PKCδ. PKCδ was incubated with 10 μM hBVR-based peptides, as indicated, for 5 min prior to the addition of radioactive ATP. After incubation, the incorporated radioactivity was measured by the P81 method detailed in the text. b, hBVR-based peptide compares favorably with a commercial PKCδ peptide substrate. Cells transfected with PKCδ expression plasmid were treated with PMA (100 nM, 15 min). PKCδ immunoprecipitated from cell lysate was assayed using the hBVR-based peptide ARRKRKGSFFYGG and a commercially available peptide, ARRKRKGSFFYGG, as substrates. Experimental details are provided in the text.

c, hBVR-based peptide is a high affinity substrate for PKCδ. PKCδ activity was determined in vitro with increasing concentrations of GLKNRNYLSHFK peptide as the substrate. Incorporation of phosphate was measured as in a, and data were fitted to the Michaelis-Menten equation. Identical assays for PKCζ and PKCβI activity used conditions optimal for each (13, 14). Raw data are expressed as a percentage of the V_max for each enzyme, to allow visual comparison of the K_m value for each PKC. d, serine residue in the peptide GLKNRNYLSHFK is a specific target of PKCδ, and N-terminal positively charged residues are essential for its phosphorylation. The hBVR-based peptides with the amino acid substitutions at sites indicated in boldface were tested as substrates for PKCδ kinase activity, as in a. e, hBVR increases kinase activity of constitutively active PKCδ in cells. Cells were co-transfected with a constitutively active pcDNA-PKCΔ151–160 and the hBVR expression plasmid and treated with 100 nM PMA (15 min.). Kinase activity was measured in immunoprecipitates obtained using anti-PKCD antibodies. Experimental details are provided in the text.

In Fig. 1a, the synthetic peptides 18RAGSVRMDRL and 224KRRNRLSHFKSGSL were efficiently phosphorylated by the kinase. In addition, a peptide, including the 284SRK sequence, was also phosphorylated in vitro; subsequent experiments, however, indicated that Ser294 was not phosphorylated in the intact protein. Because Ser230 is an integral part of the YLSF SH2 domain, peptides spanning Ser230 of the hBVR were selected as substrates for more extensive phosphorylation assays. The hBVR-based peptides have a highly basic amino acid sequence; for example, the peptide that corresponds to hBVR amino acids 222–234 (GLKNRNYLSHFK) has four basic residues and is qualitatively similar to the sequence of an accepted consensus PKC phosphorylation site in the commercial peptide ARRKRKGSFFYGG, identified by Nishikawa as being an ideal substrate for PKCδ (45). A kinase assay, using immunoprecipitated PKCδ from cells overexpressing the protein and stimulated with 100 nM PMA for 15 min, was used to assess the phosphorylation rate of the hBVR-based peptide, in comparison with the commercially available PKC consensus peptide substrate. As shown in Fig. 1b, at equimolar concentrations, the hBVR-based peptide was a superior substrate for the PKC, relative to the commercial standard, and this higher reaction rate was further amplified for PMA-activated PKCδ. This observation was further examined by measuring the concentration dependence of hBVR-based peptide phosphorylation. Data obtained for increasing peptide substrate concentrations were fitted to the Michaelis-Menten equation, yielding a K_m of 1.59 ± 0.58 μM (Fig. 1c). This value compares favorably with the reported K_m value for the commercial substrate (0.98 μM (45)). Kinetic analysis of other PKC family members, using the hBVR-derived peptide substrate, revealed that the hBVR peptide is a more favorable substrate for PKCδ, relative to other PKC family members PKCζ (K_m, 6.89 μM) and PKCβII (K_m, 14.03 μM).

Furthermore, we examined hBVR peptide sequence requirements for PKCδ substrates, by substitution of the serine in the KRRNRLSHFK sequence, as well as the basic residues N-terminal to the potential Ser230 phosphorylation site (Fig. 1d). A serine → alanine replacement at Ser230 of the peptide (i.e. GLKNRNYLSHFK → GLKNRNLAFHFK) produced a peptide that was not a substrate for PKCδ. PKCδ specifically targets Ser230, a longer peptide containing two additional serines (corresponding to hBVR Ser235 and Ser237), and the S230A substitution was a poor PKCδ substrate (Fig. 1d), although there was some incorporation of 32P above basal levels, suggesting that one of the two Ser residues might be a kinetically unfavorable target. Similarly, the positively charged residues, N-terminal to the target serine, were also critical for phosphorylation of Ser230 in the peptide by PKC. The observed essential role of positively charged residues to render the peptide a suitable substrate is consistent with composition of the optimal substrate for PKCδ (45).

We had observed that in IGF-1-stimulated cells, hBVR stimulated PKCδ activity (7). Presently, we examined whether an external stimulus is required for hBVR-mediated enhancement of PKCδ activity, using a constitutively active form of PKCδ that was engineered by deleting 10 residues (amino acids 151–160) from the pseudosubstrate domain of the PKC (43). The results are shown in Fig. 1e. Co-expression of hBVR with the mutant...
PKC resulted in a near doubling of PKC kinase activity. This suggests that hBVR stimulation of PKCδ is independent of and/or synergizes the action of other mechanisms that activate the kinase. This observation further indicates that hBVR indeed interacts with and activates PKCδ; as reported before, hBVR does not phosphorylate PKCδ.

Detection of Peptide Phosphorylation by PKCδ Using Mass Spectrometry—We extended the above observations to mapping the modification site in the peptide using mass spectrometry. In the first experiment, a mixture of three peptides, GLKRNRYLSFHFK, ARRKKRGSFFYGG, and KYYCCSRK, was phosphorylated by PKCδ in vitro. The peptide mixtures yielded high intensity signals and chromatograms that were readily interpretable using LC-MS/MS analysis, as illustrated by GLKRNRYLSFHFK (Fig. 2). A peptide having an experimental mass of 1665.9 daltons was observed in the untreated mixture (Fig. 2A); this peptide was depleted in the PKCδ-treated sample, and a modified species with a mass of 1746.1 daltons was observed (Fig. 2B). The increase in mass is characteristic of an addition of a single phosphate group, and it was apparent that this species was not present in the untreated sample. The peptide is highly basic and is protonated in the LC-MS system; as shown in Fig. 2C, the predominant species was in a 4+ charge state, with lesser amounts of 3+ and 2+ states. As only one serine or tyrosine could be phosphorylated in this peptide, collision-induced dissociation and neutral loss analysis were used to distinguish the less stable phosphoserine from phosphotyrosine; the peptide mass was reduced by 98 daltons (Fig. 2D), characteristic of a β-elimination reaction involving phosphoserine. Moreover, a Mascot search and fragmentation spectra based on the LC-MS³ data of Fig. 2E indicated that the peptide contained phosphoserine rather than phosphotyrosine, as expected for the product of PKCδ activity. Similar analyses were applied to the other two peptides in the mixture, ARRKKRGSFFYGG and KYYCCSRK; these data are summarized in Table 1. Serine phosphorylation of both GLKRNRYLSFHFK and ARRKKRGSFFYGG was highly efficient; both were at least 90% phosphorylated by the PKC, based on loss of signal from the unmodified peptide. Analysis of KYCCSRK was complicated by its being predominantly triple protonated, resulting in an m/z <400, below the scan range of the mass spectrometer.

Detection of Phosphorylation of Intact hBVR by PKCδ Using Mass Spectrometry—The phosphorylation of hBVR was determined by using GST-hBVR in an in vitro kinase reaction. Table 2 lists the aggregate mass spectrometry data on kinased recombinant proteins from in vitro and in vivo preparations. GST-hBVR (2 μg) was incubated in a PKCδ-driven kinase reaction, essentially as described for the peptides, as above. LC-MS analysis of a chymotrypsin digestion covered 93% of the protein (Fig. 3a) and yielded a single phosphorylated peptide; the peptide was identified from LC-MS³ data as GVVVQVGRAGSKVRMDL, where the phosphoserine corresponds to Ser²3 of hBVR (Fig. 3b); the peak assignments are shown as supplemental Fig. 1. As noted in Fig. 1a, a peptide including this Ser²1 sequence was phosphorylated by PKCδ in vitro. Ser¹⁴⁹, although readily detected in both a chymotrypsin and trypsin digest, was only found in an unmodified state, suggesting that this position is not a significant substrate for PKCδ modification.
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**TABLE 1**

Mass spectrometry analysis of unmodified and phosphorylated peptides in untreated and kinase-treated samples.

A mixture of the peptides GLKRNRYLSFHFK, ARRKKGSFFYGG, and KYCCSRK was the substrate of a PKCα kinase reaction in vitro. Peptides recovered after removal of lipid from the reaction and from a mock-treated control were analyzed by LC/MS.

| Peptide | Ret. time | Charge state | Mass (experiment) | Mass (calculated) | Neutral loss at MS | Modification groups | Ion intensity |
|---------|-----------|--------------|-------------------|-------------------|--------------------|---------------------|--------------|
| GLKRNRYLSFHFK | 19 | 3+ | 1665.9 | 1664.9 | No | None | 4.9 |
| GLKRNRYLSFHFK | 18 | 3+ | 1746.1 | 1744.9 | −98 | PO4 at S9 | 0.04 |
| ARRKKGSFFYGG | 8 | 3+ | 1528.9 | 1528.8 | No | None | 14.6 |
| ARRKKGSFFYGG | 4 | 4+ | 1610.2 | 1608.8 | −98 | PO4 at Ser8 | 0.01 |
| KYCCSRK | 1.43 | 2+ | 1000.7 | 1000.5 | No | None | 0.09 |

a The position of the phosphorylated residue is indicated by *.
b Retention time is indicated in minutes.
c Mass is in daltons.
d Ion intensity signal is in millions.

**TABLE 2**

Mapping of phosphopeptides in BVR sequences from phosphorylated GST-BVR

Total spectral counts are shown by SC; underlining indicates potential phosphorylation sites that were not modified. A dash indicates that no spectra were found for this site or peptide. The 4th column represents spectral data from kinased GST-BVR that was treated with acetic anhydride prior to trypsin digestion, to block cleavage of lysine residues and to facilitate mapping of position Ser294.

| Peptide data summary | Chymotrypsin digest | Partial and complete trypsin treatment | Trypsin, after chemical block of lysine | Total |
|----------------------|----------------------|----------------------------------------|----------------------------------------|-------|
| Sequence coverage | 96% | 89% | 67% | 99% |
| Unique peptides | 144 peptides | 112 peptides | 70 peptides | 326 |
| Total spectral counts | 1541 SC | 2865 SC | 1298 SC | 5704 |
| Phosphopeptide spectra | 4 SC | 48 SC | 6 SC | 58 SC |
| Mapped phospho-positions | Ser294 | Ser230, Ser237 | Ser240 | 4 sites |
| Ser230 | GVVVGVGRAGSVRMRL | Not found | AGS5VRMR | 3 SC |
| Phosphopeptides | 4 SC | - | - | 7 |
| Unmodified peptide | 1 SC | - | - | 1 |
| Ser237 | RNHPSSAFL | NPPHSSAFLNLIGFSVR | NPPHSSAFLNLIGFSVR | 3 SC |
| Phosphopeptides | - | 97 SC | 61 SC | 178 |
| Unmodified peptide | 20 SC | 19 SC | 18 SC | 0 |
| Ser240 | KKEVVKDDLQGSL | G5LFTAGPLEEER | Not found | 0 |
| Phosphopeptides | - | - | - | 0 |
| Unmodified peptide | 40 SC | 52 SC | 52 SC | 92 |
| Ser240 | IEKGPGGLRNRYLSF | YLS5FHFK | YLS5FHFK | 3 SC |
| Phosphopeptides | 43 SC | 43 SC | 43 SC | 43 |
| Unmodified peptide | 113 SC | 113 SC | 113 SC | 118 |
| Ser230 | KGS/underlin| 160SCVNGVNNKIF | YLSFHFKSLS/RNLVPGVNNK | 5 SC |
| Phosphopeptides | 5 SC | - | - | 5 |
| Unmodified peptide | 46 SC | 46 SC | 46 SC | 75 |
| Ser294 | Not found | Not found | Not found | 0 |
| Phosphopeptides | - | - | - | 0 |
| Unmodified peptide | 91 SC | 91 SC | 91 SC | 91 |

a The position of the phosphate in sequence is indicated by *.
b This indicates positions with low Mascot Scores and spectral counts.

d This indicates positions with low Mascot Scores and spectral counts.
fected with pcDNA-PKCδ, starved, and treated with PMA. The cells were permeabilized to allow entry of the same peptides as used above, together with ATP (see under “Experimental Procedures”). At the conclusion of the reaction, soluble materials were recovered and processed by size-exclusion chromatography, to remove larger proteins. The recovered peptides were analyzed by LC-MS/MS, as described for the in vitro kinase reactions. LC-MS/MS analysis revealed that the peptide GLKRNRYLS*FHFK was predominantly phosphorylated, as the spectral count for the phosphorylated form was 6-fold greater than the unmodified peptide sequence (Table 3). The spectral signal was not as high as that seen in the in vitro assay, but it was significant. In addition, the phosphorylated peptide produced essentially the same neutral loss in the MS2 spectra and mapped the phosphorylation site to the same serine position in the MS3 spectra (Table 3). The mass spectrometry data therefore indicate that peptides introduced into the cell are phosphorylated with a similar specificity as in the in vitro PKC assay.

Identification of Potent hBVR-based PKCδ Inhibitor Peptides—Because hBVR protein is a substrate for PKCδ kinase activity and because, as established in the above experiments, the peptide GLKRNRYLSFHFK has amino acid sequence that is critical for PKC reactivity, the peptide sequence at either side of the target serine was dissected and analyzed in the tissue culture assay. Cells transfected with PKCδ expression vectors were serum-starved and treated with these myristoylated peptides for 2 h prior to treatment with PMA. PKCδ activ-

![FIGURE 3. Mass spectrometry mapping of PKCδ phosphorylation sites in hBVR.](image-url)
**TABLE 3**

| Reaction | Peptide | Mass (calculated) | Neutral loss at MS² | Modification groups | Spectral counts |
|----------|---------|-------------------|---------------------|---------------------|----------------|
| In vitro | GLKRNRYLSFHFK | 1665.9 | 1664.9 | No | None | 1 |
| In vitro | GLKRNRYLSFHFK | 1746.1 | 1744.9 | ~98 | PO₄ at Ser² | 66 |
| In situ | GLKRNRYLSFHFK | 1666.3 | 1664.9 | No | None | 2 |
| In situ | GLKRNRYLSFHFK | 1745.8 | 1744.9 | ~98 | PO₄ at Ser² | 12 |

² Mass is in daltons.

The position of the phosphorylated residue is indicated by *.

Product of m/z value times peptide charge state is shown.

Mass is in daltons.

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PKCδ activity was determined after immunoprecipitation of cell lysates with antibodies raised against the C terminus of the enzyme. Contrary to our expectation, treatment with the peptide KRNRYLSF led to significant inhibition of PKCδ activity (Fig. 4a). The inhibition was also observed for PKCδ obtained from cells expressing the constitutively active PKCδ. The hBVR-based SFHFKSGSL peptide also attenuated PMA-mediated stimulation of intact and constitutively active PKCδ. The findings that the peptides were effective inhibitors of the constitutively active kinase argue for a direct interaction of the peptide with the kinase.

**Only hBVR-based Peptide SFHFKSGSL Disrupts Cell Membrane Integrity**—The two hBVR-based PKCδ inhibitory peptides in the cell were tested for their effects on translocation of the PKC in response to PMA stimulation and cell membrane integrity using confocal microscopy. Experimental details are provided in the legend to Fig. 4b. In the presence of KRNRYLSF, PKCδ exhibited the expected response to PMA, which is localization in the cell membrane (Fig. 4b, panel i). This redistribution was similar to that observed in cells treated with PMA, where PKCδ was observed at the periphery of the cell (Fig. 4b, panel ii); in the absence of the phorbol ester, it was located in the Golgi apparatus (Fig. 4b, panel iii). A similar distribution was observed in cells treated with an unrelated peptide KKRLHC (Fig. 4b, panel iv). However, prior treatment with SFHFKSGSL, the sequence of which somewhat resembles PKCδ translocation inhibitory peptide, SFNSYELGSL (46), caused a more dramatic response, manifested by extensive membrane blebbing and contraction of cell size (Fig. 4b, panel v). The effect of the peptide appeared specific to cells expressing PKCδ upon exposure to PMA; notably, treatment with SFHFKSGSL did not appear to disrupt the integrity of cells expressing hBVR and treated with PMA (Fig. 4b, panel vi).

**Disruption of Elk1 Activation by the Inhibitory Peptide KRNRYLSF**—We next examined the consequences of KRNRYLSF inhibition on a PKCδ-dependent signaling function, using activation of ERK/Elk1- and NF-κB-dependent transcription of a luciferase reporter. In the first such experiment, cells were co-transfected with pcDNA-PKCδ and Elk1-luciferase reporter plasmids (“Experimental Procedures”) and serum-starved. They were then treated with myristoylated inhibitor peptides or with randomly selected inactive control peptide, KEVVGKD, for 2 h prior to treatment with PMA for 10 h, and additional peptide was added at 2-h intervals. Experimental details are provided under “Experimental Procedures.” As noted in Fig. 5a, PMA treatment resulted in a robust stimulation of Elk1 activity, which was attenuated in cells treated with either of the inhibitor peptides but not with the control. Similarly, in cells co-transfected with pcDNA-PKCδ and an NF-κB reporter, TNF-α-mediated activation of NF-κB was blocked by treatment with KRNRYLSF (Fig. 5b). In contrast, in cells treated with SFHFKSGSL, the expression was strikingly reduced to about 10% that of the untreated cells, an observation that is consistent with the likelihood that this peptide rapidly stimulated the onset of apoptosis and thus extensive cell loss. In the experiments shown in Fig. 5c, cells were co-transfected with constitutively active PKCδ and the reporter plasmids for Elk1 or NF-κB; in both instances, the peptide KRNRYLSF inhibited expression of the luciferase reporter gene. These observations support the above noted suggestion that the peptide directly inhibits PKCδ activity, as opposed to preventing its stimulus-dependent activation.

**hBVR Reductase Activity Is Increased in the Presence of PKCδ, and Biliverdin Blocks Activation of PKCδ by PMA in Cells**—Having established that hBVR is a substrate for PKCδ (7), we next examined the consequences of phosphorylation by PKCδ on the reductase activity of the enzyme; as noted earlier, this activity is dependent on hBVR phosphorylation (5). hBVR was used as a substrate by PKCδ and Biliverdin to bilirubin. The reductase activity was compared with that of the control assay system that did not contain PKCδ. As shown in Fig. 6a, there was a significant increase in the conversion rate by the reductase subsequent to phosphorylation by PKCδ. If the preliminary phosphorylation was carried out under conditions that favor hBVR kinase activity rather than PKCδ, there was no change in the reductase activity (Fig. 6b), indicating that the stimulation in activity observed in Fig. 6a was a consequence of hBVR phosphorylation by PKCδ. Next, to examine the potential consequences of increased activation of the reductase activity on PKCδ, we examined the effect on PKCδ autophosphorylation of biliverdin, the heme degradation product and hBVR substrate. The presence of biliverdin in the autophosphorylation reaction led to significant inhibition (Fig. 6c); the inhibition was independent of the order of addition of biliverdin and DTT. This suggested that biliverdin was not acting by interaction with sulphydryl groups in the PKC. A spectrophotometric analysis was used to test whether biliverdin binds covalently to PKCδ. The Soret band and α, β maxima in the absorption spectrum of biliverdin were not shifted in the presence of GST-PKCδ, suggesting that any interaction was transient (Fig. 6d). The biliverdin/GST-PKCδ was incubated at 25 °C for 5 min.
prior to recording the spectrum. The sample was scanned four times, over a period of 30 min., and there was no discernible difference among the spectra. Addition of SDS to the biliverdin/GST-PKC/H9254 sample followed by size-exclusion separation of GST-PKC/H9254 from low molecular weight components yielded a spectrum identical to that of SDS-treated GST-PKC/H9254, including the shifted ultraviolet absorbance peak, indicating that the inhibitor is removed by simple physical dissociation and is therefore not a consequence of covalent association of biliverdin with the PKC. This is in contrast to the observation of covalent binding of biliverdin to phytochrome proteins in Pseudomonas aeroginosa and Agrobacterium tumefaciens (44, 47).

**DISCUSSION**

Although hBVR is an activator of PKCδ (7), its substrate, biliverdin (the HO-1/HO-2 catalytic activity product), and two peptides, designed based on the primary structure of the hBVR protein, are potent inhibitors of the PKC. There are a number of ways to activate PKCδ (18, 48–51). The known mechanisms

**FIGURE 4.** hBVR-based peptides, KRNRYLSF and SFHFKSGSL, inhibit PKCδ activity; only the latter disrupts cell membrane integrity. a, peptides KRNRYLSF and SFHFKSGSL suppress PKCδ activity in cells. Cells were transfected with either pCDNA-PKCδ plasmid or the constitutively active pCDNA-PKCδ151–160 and treated with myristoylated KRNRYLSF or SFHFKSGSL for 2 h before treatment with PMA. Cells were processed, and PKCδ activity was measured as in Fig. 1a. b, hBVR-based peptide SFHFKSGSL disrupts cell membrane integrity in response to PMA. HeLa cells were transfected with pEGFP-PKCδ, pretreated with myristoylated KRNRYLSF (panel i), KKRILHC (panel iv), or SFHFKSGSL (panels v and vi) for 2 h, followed by treatment with 100 nM PMA for 15 min. Cells in panel iii were left untreated, and those in panel ii were treated with PMA alone. Cells in panel vi were co-transfected with pDsRed2-hBVR. Expressed proteins in live cells were imaged as described under “Experimental Procedures.” Scale bars, 10 μm.

**FIGURE 5.** Inhibition by hBVR-based peptides of PMA-dependent Elk1 and NF-κB induction. a, PKCδ inhibitory peptides attenuate activation of Elk1 signal transduction. Cells were co-transfected with pCDNA-PKCδ, the Elk1 luciferase reporters, and pCMV-βgal plasmids for 24 h. After overnight serum starvation, cells were treated for 2 h with the indicated myristoylated peptides (10 μM) and subsequently treated with 100 nM PMA for a further 10 h. Peptides were replenished at 2-h intervals. Luciferase activity was measured in cell lysates and normalized on the β-galactosidase control. *, p < 0.01 compared with untreated control. b, PKCδ inhibitory peptides attenuate activation of NF-κB signal transduction. Cells were co-transfected with pCDNA-PKCδ, pNF-κB, and pCMV-βgal plasmids and treated with peptides as in a. The regimen of treatment with TNF-α (20 ng/ml, final concentration) was similar to that described for PMA in a. Cell lysates were assayed for luciferase activity as above. c, KRNRLYLSF peptide also attenuates promoter activity induced by constitutively active PKCδ. Cells were co-transfected with pCDNA-PKCδ151–160, pCMV-βgal, and either the Elk1 or NF-κB reporters. Treatment with stimulants and analysis of promoter activity were the same as described in a and b.
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FIGURE 6. PKCδ activates the reductase activity of hBVR, and biliverdin suppresses the PKC activity. a, phosphorylation of hBVR by PKCδ accelerates the conversion of biliverdin to bilirubin by the enzyme. PKCδ was used to phosphorylate hBVR in vitro under PKCδ assay conditions. The reductase activity was measured as detailed in the text. b, phosphorylation of hBVR by PKCδ is essential for stimulation of the reductase. hBVR was incubated with PKCδ under hBVR kinase conditions prior to measurement of the reductase activity. c, biliverdin suppresses PKCδ autophosphorylation. Recombinant human PKCδ was preincubated in vitro either in standard kinase buffer containing DTT or in buffer lacking DTT but including the indicated concentrations of biliverdin. As indicated, biliverdin was added to the DTT-treated samples or DTT to those treated with biliverdin. The samples were then used in an in vitro kinase assay, and autophosphorylation of PKCδ was detected by gel electrophoresis and autoradiography. d, biliverdin (BV) interaction with PKCδ is noncovalent. The absorbance spectra, in PBS, pH 7.4, of biliverdin and of biliverdin together with GST-PKCδ were measured between 260 and 760 nm. The latter spectrum was measured after 5 min of incubation at 25 °C. The sample containing GST-PKCδ and biliverdin was adjusted to 1% SDS and fractionated by size-exclusion chromatography, and the spectrum of the high molecular weight (MW) fraction was determined. Details are provided in the text.

include phosphorylation of the C-terminal Ser645 and Ser664, the change in the conformation of the PKC that follows binding of second messengers, and proteolysis to remove the pseudosubstrate sequence and C1- and C2-like regulatory domains (2, 52, 53). Phosphorylation at Tyr311 and Tyr334 in response to ROS-generating stimuli is also linked to PKCδ activation (50, 54, 55). Because kinase-inactive hBVR can activate PKCδ (7), it is most likely that the mechanism of activation of PKCδ by hBVR involves a conformational change brought about by the protein/protein interaction.

Analysis of the primary structure of hBVR suggested multiple potential PKCδ interaction sites. As proposed previously, the hBVR D(δ)-Box-like motif 272KKRLHCLGL, which is essential for interaction with, and activation of, PKCδ (7), is a likely site of interaction with the sequence RLGVTGNIHKPFK in the catalytic domain of PKCδ. This interaction is likely to change the PKC kinase domain structure to a more active form. The association of the two proteins can be considered to predispose them to additional forms of binding and interaction. For instance, the sequence in PKCδ SFNSYELGSL that mediates annexin binding (56) is located in the C2-like domain; in nonactivated PKCδ this sequence is associated with the IVLM-RAAEYPVSE sequence to maintain the kinase in an inactive conformation. We postulate that the hBVR SFHFKGSL sequence, which closely resembles the PKCδ motif SFNSYELGSL, could compete with that motif for binding to IVLM-RAAEYPVSE, thereby changing the conformation of the PKC regulatory domain. The combination of the two interactions, which are depicted in Fig. 7, could result in enhanced activity of pseudosubstrate-deleted PKCδ, which is shown in Fig. 1e. Moreover, based on our previous study with another hBVR-interactive kinase, Goodpasture antigen-binding protein, an atypical protein kinase (57, 58), it is plausible that the C-terminal segment of hBVR is involved in interaction with the Zn2+-binding sites in the C1 domain of PKCδ. The Zn2+-binding domain of hBVR 280H(CX)10CC is in part contained in the D(δ)-Box-like motif (17). Zn2+, as does Ca2+, targets PKCs to the cell membrane (59); it is conceivable that the metal ion may be involved in membrane translocation of an hBVR-PKCδ complex mediated by their respective Zn2+-binding domains. The combination of interactions would be expected to maintain the conformation of the PKC in a more open form during activation in response to PMA or IGF-1, preventing binding of the pseudosubstrate to the active site.

The Ser/Thr residues in RXX(S/T) and its related motif RXRXX(S/T) are phosphorylation targets of PKCδ, as well as CaMK2 and PKB/Akt (34, 36, 37). The identified substrate peptide, GLKRNRRYLSFHFK, presents a new type of substrate for the PKC, and it shares with the previously identified substrates,
myristoylated alanine-rich C kinase substrate (KKKRFSDKKSKFLSG) (60) and the commercially available peptide (ARKRKKGSFFYGGL), the density and distribution of positively charged residues. This peptide also stands in contrast to peptides derived from PKC regulatory sequences, such as those based on the pseudosubstrate sequence or the ones that resemble regions in the receptor for activated C-kinase-1 (RACK1). The pseudosubstrate sequences of PKCs are a potent inhibitor of the kinase from which they are derived (52). Also, a peptide based on the PKC\(\beta\) pseudo-RACK sequence activates the kinase (61). Here, multiple types of analyses, mass spectrometry analysis of the substrate peptide and that of the intact hBVR protein as well as in vitro and in situ kinase assays, revealed that the serine residue in the RXXS motif contained in the substrate peptide, which corresponds to Ser\(^{230}\) of hBVR, is a high affinity acceptor of the PKC\(\delta\) phosphotransferase activity. A synthetic peptide that lacked the arginine and lysine residues, but contained hydrophobic residues downstream of serine, GLAANAYLSFHFK, was not an effective substrate in vitro for the PKC, indicating that the sequence and the composition of the peptide, as a whole, are required for its serving as a substrate for PKC\(\delta\) activity. The specificity of Ser\(^{230}\) for phosphorylation by PKC\(\delta\) was suggested by the finding that the residue in the intact protein was not phosphorylated by kinase-inactive PKC\(\delta\). Collectively, the data permit consideration that the peptide GLKRNYLSFHFK has the potential value for experimental/therapeutic/clinical evaluation of PKC\(\delta\) activity.

Dissection of the substrate peptide composition resulted in an unexpected finding that the two related peptides, KRNYLFSF and SFHKSGSGL, were potent inhibitors in cells toward both PMA-activated PKC\(\delta\) and a constitutively active pseudo-substrate-deleted mutant protein. The SFHKSGSGL peptide is suggested to be an inhibitor of PKC\(\delta\) binding to hBVR, which is in line with the site of action proposed above. Notably, the SFHKSGSGL peptide, to a certain extent, resembles the PKC\(\delta\) translocation inhibitor SFNSYELGSL (46).

KRNYLFSF did not cause morphological disruption of the cell membrane integrity and did not affect the membrane translocation of PKC\(\delta\). Because the SFHKSGSGL peptide promotes a morphological change in the membrane, visualized as blebbing, it is likely that inhibition of the PKC\(\delta\) activity is, in part, a manifestation of disrupted cell integrity. Blebbing is an early event in apoptosis (62). Accordingly, the decreased activity of the kinase in cells in the presence of this peptide may, in part, be a consequence of the onset of apoptosis. The effects appear to be specific to cell culture conditions with the combination of PKC\(\delta\)/PMA/SFHFKSGSGL, as it was neither observed in cells expressing hBVR and treated with PMA together with the peptide nor with the combination of PKC\(\delta\)/PMA/KRRNYLFSF or KRRRLHLC. We propose the following chain of events underlies the SFHKSGSGL peptide-mediated membrane blebbing. In response to prolonged treatment with PMA or oxidative stress caused by ionizing radiation, activated PKC\(\delta\) can be cleaved, presumably by caspase-3, and the released catalytic domain is translocated to the nucleus (21, 63), although there is a lag of several hours between treatment and PKC\(\delta\) cleavage. Nuclear PKC\(\delta\) phosphatases and thereby inactivates both the DNA damage checkpoint protein hRad9 and DNA-dependent protein kinase; the latter is essential for double strand break repair (64, 65). In addition, PKC\(\delta\)-dependent phosphorylation of lamin-B initiates its degradation and thus compromises the integrity of the nucleus (66). The chain of events is depicted in Fig. 8. The short term exposure of PKC\(\delta\) to SFHKSGSGL prior to PMA may predispose the cell to processes that trigger caspase-3 cleavage of the PKC.

The transactivation of hBVR and PKC\(\delta\) in the cell signaling network and pathophysiological conditions that are associated with disorders of PKC\(\delta\) activity are likely to be of biological relevance. The significance of hBVR phosphorylation by PKC\(\delta\) can be viewed in the context of its role in the cellular defense mechanisms against free radicals. As noted above, activation of hBVR can significantly influence those cellular functions that extend beyond its role in the cellular defense mechanisms (5, 6, 12, 14, 25, 42, 67).

Furthermore, it is not unreasonable to ponder whether in PKC\(\delta\) deficiency-related disorders, such as the autoimmune disease lupus (68), there is an associated defect in hBVR expression. However, there are those instances in which excessive activation and expression of PKC\(\delta\) result in the undesirable outcome of sustaining cell survival in certain types of tumors, such as non-small cell lung cancer cells, by promoting chemotherapeutic resistance (69). Another example is human breast tumor cells, in which the PKC functions as a survival factor (69, 70). Clearly, in such instances, a therapeutic approach based on blunting PKC\(\delta\) activity would be expedient. The cell morphology nondisruptive hBVR-based inhibitory peptide may be a good candidate for this purpose. The peptides offer an intriguing possibility of their application to initiate cell death or to halt cell growth, the outcomes that are sought in the treatment of cancer and inhibiting tumorigenesis. The previous findings that hBVR and biliverdin activate and inhibit NF-κB, respectively (71), together with the recently reported observation that silencing hBVR is a highly effective inhibitor of PKC\(\delta\) transcriptional activation of NF-κB as well as Elk1 (7), are supportive of the potential applicability of hBVR-based therapeutics in blunting PKC\(\delta\) activity. This assertion is further supported by the finding that biliverdin and the hBVR-based peptides, at low concentrations (2–10 \(\mu\)M), were very effective inhibitors of PKC\(\delta\). The inhibitory action of biliverdin on PKC\(\delta\), which we show here to be due to noncovalent interaction between the bile pigment and the PKC, can be distinguished from its function as a chromophore in bacterial phytochromes, such as those of _A. tumefaciens_ and _P. aeruginosa_ (44, 47). The observed noncovalent association would lend itself more to regulatory significance by being a reversible event. Hence, the inhibition could be reversed by activated hBVR. Collectively, the findings of this study and previously published observations allow us to postulate the occurrence of a regulatory loop between hBVR and PKC\(\delta\) with opposing effects on cell survival and apoptosis (Fig. 8). In cells stimulated with PMA, insulin/IGF-1, or TNF-α, PKC and hBVR transactivate; and activation of hBVR mediates a two-pronged process, removal of the inhibitory biliverdin and HO-1 gene expression (25, 33). hBVR also stabilizes the HO-2 mRNA and protein, in the latter case by preventing proteasomal degradation (32). Because there are overlaps in the type of stimuli and downstream targets of hBVR and PKC\(\delta\), it is likely that the
outcome of their transactivation transcends their individual signaling activities. Observations with the inhibitory peptides permit the suggestion that the two peptides, particularly KRN-RYLSF, potentially could be useful for development of a new generation of PKC\textsubscript{H9254} inhibitors. It is also reasonable to suggest that both inhibitory peptides might disrupt substrate binding by blocking the access of the substrate to the catalytic site, a variation on the manner by which the pseudosubstrate hinders PKC kinase activity (52). The ability of the hBVR-based peptides to inhibit PKC\textsubscript{H9254} activity is not unprecedented, as inhibitor peptides targeting other regions of PKC\textsubscript{H9254} have been characterized (56). However, what is unique to the presently identified inhibitory peptides is that they were not experimentally designed in the laboratory (52, 61), rather are integral segments of the interacting protein hBVR.

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**REFERENCES**

1. Steinberg, S. F. (2008) Structural basis of protein kinase C isoform function. *Physiol. Rev.* **88**, 1341–1378
2. Newton, A. C. (2010) Protein kinase C. Posed to signal. *Am. J. Physiol. Endocrinol. Metab.* **298**, E395–E402
3. Griner, E. M., and Kazanietz, M. G. (2007) Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Cancer* **7**, 281–294
4. Kapitulnik, J., and Maines, M. D. (2009) Pleiotropic functions of biliverdin reductase. Cellular signaling and generation of cytoprotective and cytotoxic bilirubin. *Trends Pharmacol. Sci.* **30**, 129–137
5. Salim, M., Brown-Kipphut, B. A., and Maines, M. D. (2001) Human biliv...
hBVR and Its Fragments Regulate PKCβ Activity

Sci. U.S.A. 105, 6870–6875
25. Miralem, T., Hu, Z., Torno, M. D., Lelli, K. M., and Maines, M. D. (2005) Small interference RNA-mediated gene silencing of human biliverdin reductase, but not that of heme oxygenase-1, attenuates arsenite-mediated induction of the oxygenase and increases apoptosis in 293A kidney cells. J. Biol. Chem. 280, 17084–17092
26. McDonagh, A. F. (2001) Turning green to gold. Nat. Struct. Biol. 8, 198–200
27. Mancuso, C., Pani, G., and Calabrese, V. (2006) Bilirubin. An endogenous scavenger of nitric oxide and reactive nitrogen species. Redox. Rep. 11, 207–213
28. Ryter, S. W., Morse, D., and Choi, A. M. (2007) Carbon monoxide and bilirubin. Potential therapies for pulmonary/vascular injury and disease. Am. J. Respir. Cell Mol. Biol. 36, 175–182
29. Maghzal, G. J., Leck, M. C., Collinson, E., Li, C., and Stocker, R. (2009) Limited role for the bilirubin-biliverdin redox amplification cycle in the cellular antioxidant protection by biliverdin reductase. J. Biol. Chem. 284, 29251–29259
30. Siedlik, T. W., Saleh, M., Higginson, D. S., Paul, B. D., Juluri, K. R., and Snyder, S. H. (2009) Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Proc. Natl. Acad. Sci. U.S.A. 106, 5171–5176
31. Ewing, J. F., and Maines, M. D. (1991) Rapid induction of heme oxygenase 1 mRNA and protein by hyperthermia in rat brain. Heme oxygenase 2 is not a heat shock protein. Proc. Natl. Acad. Sci. U.S.A. 88, 5364–5368
32. Ding, B., Gibbs, P. E., Brooks, P. S., and Maines, M. D. (2011) The coordinated increased expression of biliverdin reductase and heme oxygenase-2 promotes cardiomyocyte survival. A reductase-based peptide counters β-adrenergic receptor ligand-mediated cardiac dysfunction. FASEB J. 25, 301–313
33. Kravets, A., Hu, Z., Miralem, T., Torno, M. D., and Maines, M. D. (2004) Biliverdin reductase, a novel regulator for induction of activating transcription factor-2 and heme oxygenase-1. J. Biol. Chem. 279, 19916–19923
34. Harms, S. K., Quinn, A. M., and Hunter, T. (1988) The protein kinase family. Conserved features and deduced phylogeny of the catalytic domains. Science 241, 42–52
35. Hunter, T. (2000) Signaling. 2000 and beyond. Cell 100, 113–127
36. Hutti, J. E., Jarrell, E. T., Chang, J. D., Abbott, D. W., Storz, P., Toker, A., Cantley, L. C., and Turk, B. E. (2004) A rapid method for determining protein kinase phosphorylation specificity. Nat. Methods 1, 27–29
37. Uberson, J. A., and Ferrell, J. E., Jr. (2007) Mechanisms of specificity in protein phosphorylation. Nat. Rev. Mol. Cell Biol. 8, 530–541
38. Pawson, T., and Scott, J. D. (1997) Signaling through scaffold, anchoring, and adaptor proteins. Science 278, 2075–2080
39. Baranano, D. E., Rao, M., Ferris, C. D., and Snyder, S. H. (2002) Biliverdin reductase. A major physiologic cytoprotectant. Proc. Natl. Acad. Sci. U.S.A. 99, 16093–16098
40. Immenschuh, S., Faehmi, H. D., and Baumgart-Vogt, E. (2005) Complementary regulation of heme oxygenase-1 and peroxiredoxin 1 gene expression by oxidative stress in the liver. Cell. Mol. Biol. 51, 471–477
41. Maines, M. D., and Trakshel, G. M. (1993) Purification and characterization of human biliverdin reductase. Arch. Biochem. Biophys. 300, 320–326
42. Maines, M. D. (2007) Biliverdin reductase. PKC interaction at the cross-talk of MAPK and PI3K signaling pathways. Antioxid. Redox. Signal. 9, 2187–2195
43. Zhao, J., Renner, O., Wightman, L., Sugden, P. H., Stewart, L., Miller, A. D., Latchman, D. S., and Marber, M. S. (1998) The expression of constitutively active isoforms of protein kinase C to investigate preconditioning. J. Biol. Chem. 273, 23072–23079
44. Lamparter, T., Michael, N., Caspani, O., Miyata, T., Shirai, K., and Inomata, K. (2003) Biliverdin binds covalently to agrobacterium phytochrome Agp1 via its ring A vinyl side chain. J. Biol. Chem. 278, 33786–33792
45. Nishikawa, K., Toker, A., Johanns, F. J., Songyang, Z., and Cantley, L. C. (1997) Determination of the specific substrate sequence motifs of protein kinase C isoforms. J. Biol. Chem. 272, 952–960
46. Ingaki, K., Chen, L., Ikeno, F., Lee, F. H., Imahashi, K., Bouley, D. M.,
hBVR and Its Fragments Regulate PKCδ Activity

Rezaee, M., Yock, P. G., Murphy, E., and Mochly-Rosen, D. (2003) Inhibition of β-protein kinase C protects against reperfusion injury of the ischemic heart in vivo. *Circulation* **108**, 2304–2307

Tasler, R., Moises, T., and Frankenberg-Dinkel, N. (2005) Biochemical and spectroscopic characterization of the bacterial phytochrome of *Psedomonas aeruginosa*. *FEBS J.* **272**, 1973–1976

Hannun, Y. A., and Bell, R. M. (1990) Rat brain protein kinase C. Kinetic analysis of substrate dependence, allosteric regulation, and autophosphorylation. *J. Biol. Chem.* **265**, 2962–2972

Jaken, S., and Parker, P. J. (2000) Protein kinase C binding partners. *BioEssays* **22**, 245–254

Kikkawa, U., Matsuoka, H., and Yamamoto, T. (2002) Protein kinase Cδ (PKCδ). Activation mechanisms and functions. *J. Biochem.* **132**, 831–839

Newton, A. C. (1995) Protein kinase C. Seeing two domains.

Ron, D., and Mochly-Rosen, D. (1995) An autoregulatory region in protein kinase C. The pseudoanchoring site.

Kheifets, V., Bright, R., Inagaki, K., Schechtman, D., and Mochly-Rosen, D. (1998) Inactivation of DNA-dependent protein kinase by protein kinase Cδ-annexin V interaction. A required step in PKCδ-mediated mast cell activation. *J. Immunol.* **162**, 12934–12940

Kiskinis, E., Kikkawa, U., Nishida, K., and Hirano, T. (2006) Zinc is required for promotion of apoptosis in the HaCaT cell line. *J. Biol. Chem.* **281**, 12551–12558

Kim, Y. K., Seo, D. W., Kang, D. W., Lee, H. Y., Han, J. W., and Kim, S. N. (2006) Human biliverdin reductase suppresses Goodpasture antigen-binding protein (GPBP) kinase activity. The reductase regulates tumor necrosis factor-α-NF-κB-dependent GPBP expression. *J. Biol. Chem.* **281**, 12551–12558

Kabu, K., Yamasaki, S., Kamimura, D., Ito, Y., Hasegawa, A., Sato, E., Kitamura, H., Nishida, K., and Hirano, T. (2006) Zinc is required for FceRI-mediated mast cell activation. *J. Immunol.* **177**, 1296–1305

Liu, Y., Belkina, N. V., Graham, C., and Shaw, S. (2006) Independence of protein kinase Cδ activity from activation loop phosphorylation. Structural basis and altered functions in cells. *J. Biol. Chem.* **281**, 12102–12111

Ron, D., and Mochly-Rosen, D. (1995) An autoregulatory region in protein kinase C. The pseudoanchoring site. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 492–496

Hampton, M. B., and Orrenius, S. (1998) Redox regulation of apoptotic cell death in the immune system. *Toxicol. Lett.* **102**, 355–358

Emoto, Y., Manome, Y., Meinhardt, G., Kusii, H., Kharbanda, S., Robertson, M., Gayeur, T., Wong, W. W., Kamen, R., and Weichselbaum, R. (1995) Proteolytic activation of protein kinase Cδ by an ICE-like protease in apoptotic cells. *EMBO J.* **14**, 6148–6156

Bharti, A., Kraeft, S. K., Gouder, M., Pandey, P., Jin, S., Yuan, Z. M., Lees-Miller, S. P., Weichselbaum, R., Weaver, D., Chen, L. B., Kufe, D., and Kharbanda, S. (1998) Inactivation of DNA-dependent protein kinase by protein kinase Cδ. Implications for apoptosis. *Mol. Cell. Biol.* **18**, 6719–6728

Yoshida, K., Wang, H. G., Miki, Y., and Kufe, D. (2003) Protein kinase Cδ is responsible for constitutive and DNA damage-induced phosphorylation of Rad9. *EMBO J.* **22**, 1431–1441

Cross, T., Griffiths, G., Deacon, E., Sallis, R., Gough, M., Watters, D., and Lord, J. M. (2000) PKCδ is an apoptotic lamin kinase. * Oncogene* **19**, 2331–2337

Wegiel, B., Baty, C. J., Gallo, D., Csizmadia, E., Scott, J. R., Akhavan, A., Chin, B. Y., Kaczmarek, E., Alam, J., Bach, F. H., Zuckerbraun, B. S., and Otterbein, L. E. (2009) Cell surface biliverdin reductase mediates biliverdin-induced anti-inflammatory effects via phosphatidylinositol 3-kinase and Akt. *J. Biol. Chem.* **284**, 21369–21378

Gorelik, G., Fang, J. Y., Wu, A., Sawalha, A. H., and Richardson, B. (2007) Impaired T cell protein kinase Cδ delta activation decreases ERK pathway signaling in idiopathic and hyaluridine-induced lupus. *J. Immunol.* **179**, 5553–5563

Clark, A. S., West, K. A., Blumberg, P. M., and Dennis, P. A. (2003) Altered protein kinase C (PKC) isoforms in non-small cell lung cancer cells. PKCδ promotes cellular survival and chemotherapeutic resistance. *Cancer Res.* **63**, 780–786

McCracken, M. A., Miraglia, L. J., McKay, R. A., and Strobl, J. S. (2003) Protein kinase Cδ is a prosurvival factor in human breast tumor cell lines. *Mol. Cancer Ther.* **2**, 273–281

Gibbs, P. E., and Maines, M. D. (2007) Biliverdin inhibits activation of NF-κB. Reversal of inhibition by human biliverdin reductase. *Int. J. Cancer* **121**, 2567–2574

Konishi, H., Tanaka, M., Takemura, Y., Matsuhashi, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. (1997) Activation of protein kinase Cδ by tyrosine phosphorylation in response to H2O2. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11233–11237

Konishi, H., Yamauchi, E., Taniguchi, H., Yamamoto, T., Matsuhashi, H., Takemura, Y., Ohmoe, K., Kikkawa, U., and Nishizuka, Y. (2001) Phosphorylation sites of protein kinase Cδ in H2O2-treated cells and its activation by tyrosine kinase in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 6587–6592

Sun, X., Wu, F., Datta, R., Kharbanda, S., and Kufe, D. (2000) Interaction between protein kinase Cδ and the c-Ab1 tyrosine kinase in the cellular response to oxidative stress. *J. Biol. Chem.* **275**, 7470–7473

Kumar, S., Bharti, A., Mishra, N. C., Raina, D., Kharbanda, S., Saxena, S., and Kufe, D. (2001) Targeting of the c-Ab1 tyrosine kinase to mitochondria in the necrotic cell death response to oxidative stress. *J. Biol. Chem.* **276**, 17281–17285

Denning, M. F., Wang, Y., Tibudan, S., Alkan, S., Nickoloff, B. J., and Qin, J. Z. (2002) Caspase activation and disruption of mitochondrial membrane potential during UV radiation-induced apoptosis of human keratinocytes requires activation of protein kinase C. *Cell Death Diff.* **9**, 40–52

Matassa, A. A., Carpenter, L., Biden, T. J., Humphries, M. J., and Reyland, N. (2007) Involvement of HDAC1 and the PI3K/PKC signaling pathways in NF-κB activation by the HDAC inhibitor apicidin. *Biochem. Biophys. Res. Commun.* **347**, 1088–1093

Kim, Y. K., Seo, D. W., Kang, D. W., Lee, H. Y., Han, J. W., and Kim, S. N. (2006) NF-κB is required for UV-induced JNK activation via induction of PKCδ. *Mol. Cell* **21**, 467–480

Kwon, M. J., Yao, Y., Walter, M. J., Holtzman, M. I., and Chang, C. H. (2007) Role of PKCδ in IFNγ-inducible CIITA gene expression. *Mol. Immunol.* **44**, 2841–2849

Majewski, M., Bose, T. O., Sillé, F. C., Pollington, A. M., Fiebiger, E., and Boes, M. (2007) Protein kinase Cδ stimulates antigen presentation by class II MHC in murine dendritic cells. *Int. Immunol.* **19**, 719–732

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