Characterization of Serine 916 as an in Vivo Autophosphorylation Site for Protein Kinase D/Protein Kinase Cμ*

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Activation of the serine kinase protein kinase D (PKD)/PKCμ is controlled by the phosphorylation of two serine residues within its activation loop via a PKC-dependent signaling cascade. In this study we have identified the C-terminal serine 916 residue as an in vivo phosphorylation site within active PKD/PKCμ. An antibody that recognized PKD/PKCμ proteins specifically phosphorylated on the serine 916 residue was generated and used to show that phosphorylation of Ser-916 is induced by phorbol ester treatment of cells. Thus, the pS916 antibody is a useful tool to study the regulation of PKD/PKCμ activity in vivo. Antigen receptor ligation of T and B lymphocytes also induced phosphorylation of the serine 916 residue of PKD/PKCμ. Furthermore, the regulatory FcγRIIB receptor, which mediates vital negative feedback signals to the B cell antigen receptor complex, inhibited the antigen receptor-induced activation and serine 916 phosphorylation of PKD/PKCμ. The degree of serine 916 phosphorylation during lymphocyte activation and inhibition exactly correlated with the activation status of PKD/PKCμ. Moreover, using different mutants of PKD/PKCμ, we show that serine 916 is not trans-phosphorylated by an upstream kinase but is rather an autoprophosphorylation event that occurs following activation of PKD/PKCμ.

The protein kinase C (PKC) family of serine/threonine kinases has been implicated in a wide range of biological responses in a number of different cellular systems, including roles in the control of cell morphology, differentiation, and proliferation (1–5). There are multiple related PKC isoforms (5–8), which can be classified into three distinct subgroups on the basis of structural and regulatory differences: the conventional PKCs (α, β1, β2, and γ), which are regulated by calcium, diacylglycerol (DAG), and phospholipids; the novel PKCs (δ, ε, η, and θ), which are regulated by DAG and phospholipids; and the atypical PKCs (ζ and λ), whose regulation is less characterized but that have been proposed to be regulated by D-3 phosphoinositides (9). The DAG-regulated PKC isoforms bind phorbol esters and are the major cellular targets for this class of tumor promoter (10). All PKCs share a highly conserved catalytic domain, although each isoform has a different optimal substrate specificity (11), supporting the idea that each isoform has specific functions in vivo.

A recently described PKC-related serine/threonine protein kinase is protein kinase D (PKD), also named PKCμ (12, 13). PKD/PKCμ contains a cysteine-rich domain that binds DAG and phorbol esters but lacks the C2 calcium binding domain seen in the classical PKCs. In contrast to other PKCs, (including mammalian, Drosophila, and yeast isoforms), the N-terminal regulatory region of PKD/PKCμ contains a pleckstrin homology (PH) domain that regulates enzyme activity (14) and lacks a sequence with homology to a typical PKC autoinhibitory pseudosubstrate motif. Moreover, the PKD/PKCμ catalytic domain shows little similarity to the highly conserved regions of the kinase subdomains of the PKC family, instead showing distant homology to that of Ca2+-regulated kinases. Consistent with this, PKD/PKCμ shows optimal specificity for a unique peptide substrate unrelated to those identified for other PKC isoforms (11), and PKD/PKCμ does not phosphorylate a variety of substrates utilized by PKCs in vitro (12, 15).

In fibroblasts PKD/PKCμ has been shown to be activated by pharmacological agents such as phorbol esters and bryostatin 1 (15–17) or by physiological stimuli that elevate intracellular DAG levels, such as platelet-derived growth factor, angiotensin II, and neuropeptide agonists (18–20). In vitro, PKD/PKCμ can be activated through the binding of bioactive DAG or phorbol esters to the cysteine-rich domain in the presence of phosphatidyserine (12, 15). However, the in vivo activation of PKD/PKCμ is dependent on the phosphorylation of two activation loop sites, namely serine 744 and serine 748 (21). Mutational analysis indicates that phosphorylation of both residues is required and sufficient for activation of PKD/PKCμ. Several lines of evidence indicate that phosphorylation of these activation loop sites (and the subsequent induction of PKD/PKCμ catalytic activity) is not an autoprophosphorylation event but is mediated by a novel PKC-dependent signal transduction pathway (16, 20, 22). Thus PKC-specific inhibitors (with no direct activity toward PKD/PKCμ) prevent PKD/PKCμ phosphorylation and activation in response to phorbol esters or mitogens. In addition, the expression of constitutively activated mutants of PKC is sufficient to induce the phosphorylation and activation of PKD/PKCμ. In particular, the η and ε isoforms of PKC have been implicated in the regulation of PKD/PKCμ activity (16). Recent data indicating that PKCη can interact directly with the PH domain of PKD/PKCμ (23) indicates that a direct link between PKCs and PKD/PKCμ may exist.

Two-dimensional trypsin phosphopeptide mapping of activated PKD/PKCμ, in combination with sequence analysis, indicates that in addition to the two activation loop sites described above, there are other, as yet unknown phosphorylation sites present in PKD/PKCμ, including several potential auto-

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‡ The abbreviations used are: PKC, protein kinase C; PKD, protein kinase D; BCR, B cell antigen receptor complex; DAG, diacylglycerol; PAGE, polyacrylamide gel electrophoresis; PD Bu, phorbol 12,13-dibutyrate; PH domain, pleckstrin homology domain; ELISA, enzyme-linked immunosorbent assay.
phosphorylation sites (21). Recent work has identified two autophosphorylation motifs within the N-terminal regulatory domain of PKD/PKC\(\mu\) at residues Ser-205/208 and Ser-219/223 that appear to mediate the association of 14-3-3 proteins to PKD/PKC\(\mu\) (24). In addition, an autophosphorylation event within the regulatory region of PKD/PKC\(\mu\) appears to be involved in the association of lipid kinases with PKD/PKC\(\mu\) (25), although the site(s) involved have not been identified.

In this study we have identified the C-terminal serine 916 residue of PKD/PKC\(\mu\) as an in vivo phosphorylation site. Phosphorylation of the Ser-916 site correlated extremely well with PKD/PKC\(\mu\) catalytic activity and was induced by phorbol esters and by antigen receptor engagement in lymphocytes. Studies of Ser-916 phosphorylation in a set of constitutively active or kinase-dead mutants identified Ser-916 as an autophosphorylation site for PKD/PKC\(\mu\). An antibody specifically reactive against PKD/PKC\(\mu\) molecules phosphorylated on Ser-916 emerges as a useful tool to monitor PKD/PKC\(\mu\) activity in primary cells.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs—cDNA constructs containing various wild-type and mutant PKD/PKC\(\mu\) sequences have previously been described: pcDNA3-PKD/PKC\(\mu\) wild type; pcDNA3-PKD/PKC\(\mu\) ΔPH; pcDNAs-PKD/PKC\(\mu\) D733A and pcDNA3-PKD/PKC\(\mu\) S744E/S748E (14, 15, 21). A Myc-tagged PKD/PKC\(\mu\) cDNA construct was generated by ligating a double-stranded oligonucleotide encoding the Myc epitope (MEQLISEEDDL) in-frame to the N terminus of wild-type PKD/PKC\(\mu\). A PKD/PKC\(\mu\) mutant lacking the C-terminal 23 residues (PKD/PKC\(\mu\) ΔCT) was generated by introducing a double-stranded oligonucleotide encoding a stop codon in frame into the NheI site (underlined) of pcDNA3 Myc-PKD/PKC\(\mu\) (GCTAGCTGAGATCTAGAAGATCTGGATCC-CTAGC). A single point mutation at the Ser-916 residue was generated using a polymerase chain reaction-based technique. Briefly, a polymerase chain reaction fragment containing mutant nucleotides (in bold) encoding a serine to alanine substitution at the 916 site (PKD/PKC\(\mu\) S916A) was obtained using the following oligonucleotides: forward primer, 5'-AGTGCTAGACACCAAGCAGTCGCTGAGGCTGAAGAGAGAGATGAAAGCTCTGTCGGATCCGTAGC; reverse primer, 5'-CCCTCTAGACAGCTGGCTCCTCGCTGACGGTCTCAG-3'. The resulting polymerase chain reaction fragment was digested with NheI and Xbal restriction enzymes (underlined) and used to replace the original pcDNA3 Myc-PKD/PKC\(\mu\) NheI/Xbal fragment. All constructs were verified by restriction enzyme digestion and DNA sequencing.

**Cell Culture and Transient Transfection—** COS-7 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transient expression of PKD/PKC\(\mu\) constructs, 4 × 10^5/0.5 ml COS-7 cells were electroporated with 5 μg of cDNA constructs at 450 V and 250 microfarads, with the cells incubated on ice for 5 min before and following electroporation. COS-7 cells were plated on 3 × 6-cm dishes in complete medium and used for stimulation after 48 h. The BALB/c mouse B lymphoma A20 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol. For transient expression of PKD/PKC\(\mu\) constructs, 1.5 × 10^5/0.5 ml A20 cells were electroporated with 20 μg of cDNA at 310 V and 960 microfarads, resuspended in 5 ml of complete medium, and left overnight to recover before stimulation. Human peripheral blood-derived T lymphoblasts were generated and maintained as described previously (26). Cells were quiesced by washing three times in RPMI 1640 medium and culturing in RPMI 1640 medium supplemented with 10% fetal calf serum in the absence of interleukin-2 for 48 h before experiments.

**Cell Stimulation and Western Blot Analysis—** Cells were stimulated with either phorbol 12,13-dibutyrate (PDBu), rabbit anti-mouse F(ab')2 fragment, or rabbit anti-mouse IgG (Zymed Laboratories Inc.) before lysis for 20 min at 4 °C in a buffer containing 50 mM Tris/HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, and 1% Triton X-100. Lysates were then clarified by centrifugation at 14,000 rpm for 10 min at 4 °C, and proteins in the supernatant were then acetone-precipitated and resuspended in 2× SDS-PAGE sample buffer. Samples were resolved under reducing conditions by 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Western blot analysis was performed with an antibody raised against PKD/PKC\(\mu\)-pS916 (1:10,000 dilution of crude serum) or a pan antibody directed against the C-terminal residues 904–918 of PKD/PKC\(\mu\) (200 ng/ml). Immunoreactive bands were visualized by ECL.

**Generation of a Phosphoserine-specific PKD/PKC\(\mu\) Antibody—** A phosphopeptide corresponding to the C-terminal 15 amino acids (residues 904–919) of murine PKD/PKC\(\mu\) (Glu-Glu-Arg-Glu-Met-Lys-Lys-Leu-Ser-Glu-Arg-Val-Ser^915-Arg-Leu) was synthesized with serine 916 as a phosphorylated residue. The peptide was then coupled to keyhole limpet hemocyanin using glutaraldehyde and used to generate a rabbit antibody specific for PKD/PKC\(\mu\)-pS916 using standard immunization techniques. The resulting antibody was screened for antigen reactivity by ELISA and Western blot analysis.

**ELISA Assays—** Microtiter plates were coated with 2.5 pmol of various peptides overnight at 4 °C and subsequently blocked with gelatin. Plates were incubated for 1 h at room temperature with serial (2-fold) dilutions of antibody, washed in phosphate-buffered saline containing 0.05% Tween, and incubated for 1 h with donkey anti-rabbit–horseradish peroxidase (1:5000 dilution). Plates were washed, and immunooabsorbance was detected using ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) substrate (Roche Molecular Biochemicals), reading at an optical density of 405 nm.

**In Vitro Kinase Assay—** Endogenous PKD/PKC\(\mu\) was immunoprecipitated from lysates at 4 °C for 2 h with the PA-1 antibody (1:100 dilution), previously described (12), and recovered with protein A-Sepharose beads. Myc-tagged PKD/PKC\(\mu\) proteins were immunoprecipitated with a 9E10 monoclonal antibody covalently coupled to protein-G-Sepharose. Immunocomplexes were washed twice in lysis buffer and resuspended in (20 mM Tris/HC1, pH 7.4, 150 mM NaCl, 0.1% SDS). PKD/PKC\(\mu\) autophosphorylation was determined by incubating immunocomplexes with 20 μl of kinase buffer containing 100 μM [γ-^32P]ATP final concentration at 30 °C for 10 min. Reactions were terminated by the addition of 2× SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography. Exogenous substrate phosphorylation by PKD/PKC\(\mu\) was measured by the incorporation of [γ-^32P]ATP into synthetic peptides, as described previously (15). K_m was determined by plotting reciprocal values of phosphate incorporation into a synthetic peptide against reciprocal values of the peptide concentrations (Lineweaver-Burk plot). The intercept with the x axis of this double-reciprocal plot was used to calculate the K_m of PKD/PKC\(\mu\) for the peptide.

**Materials—** ECL reagents and [γ-^32P]ATP (370 MBq/ml) were from Amer sham Pharmacia Biotech. PDBs was from Sigma. Protein A-Sepharose was from Roche Molecular Biochemicals, and Protein G-Sepharose was supplied by the Imperial Cancer Research Fund (ICRF) Research Monoclonal Antibody Service as were monoclonal antibodies directed against the Myc epitope (9E10) and the T cell antigen receptor CD3 complex (UCHT1). Synthetic peptides and oligonucleotides were generated by the ICRF Peptide Synthesis Unit and the ICRF Oligonucleotide Synthesis Unit. Rabbit immunization was carried out by the ICRF Biological Service Unit. All other reagents were from standard suppliers or as indicated in the text.

**RESULTS AND DISCUSSION**

**Generation of a Phospho-specific Antibody Directed against the C Terminus of PKD/PKC\(\mu\)—** It has previously been shown that two serine residues within the activation loop of PKD/PKC\(\mu\) become hyperphosphorylated upon cell stimulation, controlling the catalytic activity of PKD/PKC\(\mu\) (21). However, PKD/PKC\(\mu\) is phosphorylated at multiple sites in vivo, including sites that are basally phosphorylated and also sites that are regulated either through transphosphorylation or autophosphorylation events after stimulation of PKD/PKC\(\mu\) activity (21). In an attempt to map these phosphorylation sites, we observed that the amino acid sequence surrounding a serine residue within the extreme carboxyl tail of PKD/PKC\(\mu\) (Ser-916) exhibited a high degree of homology to the optimal peptide substrate sequence for PKD/PKC\(\mu\) described by Nishikawa et al. (11), i.e. Leu-Xaa-Xaa-Arg-Xaa-Ser(P)-Xaa (Fig. 1A). This raised the possibility that serine 916 is an in vivo autophosphorylation site for this enzyme. This hypothesis was supported by previous observations that this region of PKD/PKC\(\mu\) is modified by post-translational events (e.g. phosphorylation) upon activation, blocking the recognition of active PKD/PKC\(\mu\)
The pS916 antibody specifically recognizes phorbol ester
activated PKD/PKCα expressed in COS-7 cells—To determine
whether the pS916 antibody could recognize PKD/PKCα
isolated from intact cells, we transfected COS-7 cells (which
express low levels of endogenous PKD/PKCα, see Ref. 14) with a
cDNA construct encoding wild-type Myc-tagged murine PKD/
PKCα. Cells were subsequently left unstimulated or were
treated with PDBu before lysis. The Western blot analysis
shown in Fig. 2A indicates that the pS916 antibody recognized
a single protein band migrating at the expected size for Myc-
tagged PKD/PKCα in the PDBu-treated COS-7 cells, both in
whole cell lysates and in Myc immunoprecipitates. In contrast,
the pS916 antibody only very weakly recognized PKD/PKCα
isolated from unstimulated COS-7 cells. Thus the pS916 anti-
body was able to specifically detect activated PKD/PKCα when
ectopically expressed in COS-7 cells.

To test the hypothesis that the phosphorylation of serine 916 is
mediated by an autophosphorylation event rather than being
transphosphorylated by a proximal serine kinase, we examined
whether serine 916 was phosphorylated in kinase-deficient
mutants of PKD. A catalytically inactive PKD/PKCα
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by antibodies directed against the C terminus of PKD/PKCα
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In initial experiments to test this hypothesis, we examined
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containing the C-terminal region surrounding the Ser-916 resi-
due in vitro. As shown in Fig. 1, B and C, active PKD/PKCα
immunoprecipitated from phorbol ester-treated COS-7 cells
(transiently transfected with wild-type Myc-PKD/PKCα) was
able to phosphorylate a synthetic peptide containing the C-terminal
region of PKD/PKCα in a time- and substrate concentration-
dependent manner in addition to phosphorylating the syntide-2
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Generation of phosphorylation state-specific antibodies (28)
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encompassing residues 904–918 (Glu-Glu-Arg-Glu-Met-Lys-
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phorbol ester-treated cells was efficiently phosphorylated on serine 916 (Fig. 2B). Mutation of the ATP binding site of PKD/PKC\(\mu\) (K618M) also results in a catalytically inactive PKD/PKC\(\mu\) mutant, indicating that Ser-916 is indeed phosphorylated in vivo through an autophosphorylation mechanism in vivo.

In addition, we determined whether Ser-916 was constitutively phosphorylated in vivo in two different mutationally active PKD/PKC\(\mu\) mutants, indicating that Ser-916 is indeed phosphorylated through an autophosphorylation mechanism in vivo.

The pS916 Antibody Recognizes Endogenous Active PKD/PKC\(\mu\) in Lymphocytes — The pattern of PKD/PKC\(\mu\) expression is ubiquitous but is highest in tissues of hematopoetic origin, including the thymus and peripheral blood lymphocytes (27). It has also been shown that PKD/PKC\(\mu\) expression is regulated by antigen receptors in both B and T lymphocytes (31), which prompted us to examine the phosphorylation status of the Ser-916 residue of the endogenous PKD/PKC\(\mu\) present in these cells.

In initial experiments, cells from a murine B lymphocyte cell line (A20) were left unstimulated or were treated with the phorbol ester PDBu before whole cell lysates were prepared and Western-blotted, first with the pS916 antibody and subsequently with a pan C-terminal PKD/PKC\(\mu\) antibody. The pS916 antibody showed no immunoreactivity with lysates prepared from unstimulated B cells but specifically recognized a single protein in PDBu-treated B cell extracts (Fig. 3A, left panel). Reprobing with the pan-PKD/PKC\(\mu\) antibody demonstrated that the pS916 immunoreactive protein had an identical electrophoretic mobility to phorbol ester-activated PKD/PKC\(\mu\) (i.e. 110–120 kDa), which itself migrates more slowly than nonactivated PKD/PKC\(\mu\) upon SDS-PAGE (Fig. 3A, right panel).

Because the pS916 antibody could recognize active PKD/PKC\(\mu\) from pharmacologically stimulated cells, we wanted to
determine whether the pS916 antibody could also recognize PKD/PKC\(\mu\) that had been activated by physiological stimuli. 

Previous studies have shown that triggering of the B cell antigen (BCR) complex activates PKD/PKC\(\mu\). We therefore examined the immunoreactivity of the pS916 antibody with PKD/PKC\(\mu\) under these conditions. As indicated in Fig. 3B, the pS916 antibody was strongly reactive with PKD/PKC\(\mu\) isolated from B cells activated by cross-linking the BCR with F(ab')2 anti-mouse IgG but not with PKD/PKC\(\mu\) from unstimulated cells. These results confirmed that the pS916 antibody could specifically recognize endogenous PKD/PKC\(\mu\) isolated from phorbol ester- and antigen receptor-stimulated B lymphocytes. Importantly, the pS916 antibody did not cross-react with additional cellular proteins including other members of the PKC superfamily that are ~80 kDa in size.

**Specificity of the pS916 Antibody for PKD/PKC\(\mu\) Proteins Phosphorylated on Ser-916 in B Lymphocytes**—Although the data presented in Figs. 1–3 revealed that the pS916 antibody was selectively reacting with phosphorylated active PKD/PKC\(\mu\), it did not prove that this antibody was recognizing PKD/PKC\(\mu\) molecules that were phosphorylated only on serine 916. Since active PKD/PKC\(\mu\) is phosphorylated on multiple serine residues in vivo (21) a possibility existed that the pS916 antibody could also bind to other PKD/PKC\(\mu\) phosphorylation sites. We therefore investigated the specificity of this antibody for the C-terminal Ser-916 residue of PKD/PKC\(\mu\). As shown in Fig. 4A, the reactivity of the pS916 antibody for PKD/PKC\(\mu\) isolated from PDBu-treated B cells was completely blocked by competition with the C-terminal pS916 immunizing peptide (Fig. 4A). In contrast, a phosphopeptide of the activation loop of PKD/PKC\(\mu\) (containing a pS744 residue, a site that has previously been shown to be phosphorylated in active PKD/PKC\(\mu\), see Ref. 21) could not block the interaction of the pS916 antibody with activated PKD/PKC\(\mu\) (Fig. 4A). The ELISA data presented in Fig. 1D indicated that the pS916 antibody could weakly cross-react with the nonphosphorylated C-terminal PKD/PKC\(\mu\) peptide. This was confirmed by the peptide competition experiments shown in Fig. 4A, where the nonphosphorylated C-terminal PKD/PKC\(\mu\) peptide could weakly compete for the binding of the pS916 antibody to activated PKD/PKC\(\mu\). A low level of weak reactivity of the pS916 antibody for PKD/PKC\(\mu\) isolated from quiescent cells was variably observed in Western blotting experiments (see Figs. 2A and 4A). The ELISA data presented in Fig. 1D and the competition of this band by the nonphosphorylated C-terminal peptide (Fig. 4A) suggests that this represents a weak cross-reactivity of the pS916 antiserum for unstimulated PKD/PKC\(\mu\) rather than basal phosphorylation of the serine 916 residue in nonstimulated PKD/PKC\(\mu\).

To further confirm the specificity of the pS916 antibody, we assessed its reactivity against two PKD/PKC\(\mu\) mutants: one containing a deletion of the C-terminal 23 residues (896–918) of PKD/PKC\(\mu\) (\(\Delta CT\)), thus lacking the serine 916 residue, and one containing a single amino acid substitution, where the serine at position 916 was replaced by a neutral nonphosphorylatable alanine residue (S916A). A20 B cells were transfected with one of the following expression constructs: Myc-PKD/PKC\(\mu\) wild type, Myc-PKD/PKC\(\mu\) \(\Delta CT\), or Myc-PKD/PKC\(\mu\) S916A. The expression of all three constructs was confirmed by Western blot analysis with a monoclonal 9E10 antibody, reactive with the Myc epitope tag (Fig. 4B). The PKD/PKC\(\mu\) \(\Delta CT\) and S916A mutants expressed well and exhibited a low basal catalytic activity in unstimulated B cells that was markedly increased upon stimulation with PDBu or after antigen receptor ligation, in a manner comparable to that of wild-type PKD/PKC\(\mu\) (Fig. 4B).

![Fig. 4. Specificity of the pS916 antibody.](image)

**FIG. 4. Specificity of the pS916 antibody.** A, lysates from unstimulated (\(\times 0\)) or PDBu-treated A20 B lymphocytes (2 or 10 min) were analyzed by SDS-PAGE and Western blotting with either the pS916 antibody alone or with pS916 antibody containing 5 \(\mu\)g/ml each of different competing peptides, as indicated (CT, C-terminal; kinase, kinase domain). Reprobing of the Western blots with a pan C-terminal PKD/PKC\(\mu\) antibody confirmed equivalent loading of protein samples. Data are representative of two independent experiments. Non-P, nonphosphorylated. B, A20 B lymphocytes were transiently transfected with either wild-type Myc-PKD/PKC\(\mu\) (WT), a Myc-PKD/PKC\(\mu\) mutant containing a single amino acid substitution at residue Ser-916 (S916A), or an Myc-PKD/PKC\(\mu\) C-terminal deletion mutant (\(\Delta CT\)). Cells were left unstimulated (–) or were treated with either 50 ng/ml PDBu for 10 min or 10 \(\mu\)g/ml F(ab')2 anti-mouse IgG for 2 min (+) as indicated. PKD/PKC\(\mu\) was immunoprecipitated using a 9E10 antibody directed against the Myc epitope tag, and in vitro kinase assays were performed measuring PKD/PKC\(\mu\) autophosphorylation (upper panel). Expression levels of the different constructs is shown (lower panel), as assessed by Western blotting whole cell lysates with the 9E10 antibody. IVK, in vitro kinase assay. C, in parallel experiments A20 B lymphocytes transiently expressing the Myc-PKD/PKC\(\mu\) CT mutants were left unstimulated (–) or were treated with either 50 ng/ml PDBu for 10 min or 10 \(\mu\)g/ml F(ab')2 anti-mouse IgG for 2 min (+) as indicated. Western blot analysis of whole cell lysates was performed with the pS916 antibody; second, with a pan C-terminal PKD/PKC\(\mu\) antibody; and finally with the 9E10 monoclonal antibody directed against the Myc epitope. Similar results were obtained in three individual experiments. Black arrows indicate overexpressed Myc-tagged PKD/PKC\(\mu\) proteins. White arrows indicate endogenous PKD/PKC\(\mu\).

The Myc-tagged PKD/PKC\(\mu\) proteins migrate more slowly than endogenous PKD/PKC\(\mu\) upon SDS-PAGE, allowing the discrimination of endogenous PKD/PKC\(\mu\) and ectopically expressed Myc-tagged PKD/PKC\(\mu\). Cell lysates were prepared from quiescent B cells or from B cells that had been stimulated...
by antigen receptor ligation or with PDBu. The pS916 antibody did not react with either the endogenous or Myc-tagged PKD/PKC\(\mu\) isoforms from quiescent cells but did react with endogenous PKD/PKC\(\mu\) isolated from antigen receptor or PDBu stimulated B cells (Fig. 4C). The pS916 antibody also reacted strongly with Myc-tagged wild type PKD/PKC\(\mu\) isolated from activated B cells but was unable to recognize either the ΔCT or S916A PKD/PKC\(\mu\) mutants isolated from activated cells (Fig. 4C). The loss of reactivity of the pS916 antibody for the C-terminal PKD/PKC\(\mu\) mutants confirmed that the pS916 antibody was indeed specifically recognizing phosphorylated Ser-916 residues within the C terminus of PKD/PKC\(\mu\) in antigen receptor- and phorbol ester-activated lymphocytes.

Phosphorylation of Ser-916 Is a Marker for PKD/PKC\(\mu\) Activity in Antigen Receptor-activated Lymphocytes—Once the specificity of the pS916 antibody had been confirmed, we went on to determine if serine 916 phosphorylation is an accurate marker for PKD/PKC\(\mu\) kinase activity in antigen receptor-activated lymphocytes. The kinetics of PKD/PKC\(\mu\) activity after cross-linking of the BCR complex, using F(ab)\(^2\) anti-mouse IgG, was measured by \emph{in vitro} kinase assays using autophosphorylation as a readout for activity. The data in Fig. 5A shows an autoradiograph of a representative experiment and phosphoimager analysis of 3 separate experiments indicates that there is a rapid, sustained 8–10-fold increase in PKD/PKC\(\mu\) catalytic activity after stimulation of the BCR complex. The increase in PKD catalytic activity seen in the \emph{in vitro} kinase assays (Fig. 5A, upper panel) was exactly paralleled by the increased immunoreactivity of PKD/PKC\(\mu\) with the pS916 antibody in Western blot analyses of whole cell lysates (Fig. 5A, middle panel).

Activation of the BCR is essential for the mammalian immune response, but during B cell activation negative regulatory signaling cascades are vital to balance this response and ensure homeostasis of the immune response. One important negative feedback mechanism that operates in B cells is mediated by the FcγRIIB, so that simultaneous occupancy of the FcγRIIB and the BCR attenuates BCR signaling events (32). The data in Fig. 5A show that stimulation of B cells with intact IgG (which cross-links the FcγRIIB into the BCR complex) had a marked inhibitory effect on the BCR-induced activation of PKD/PKC\(\mu\). Thus, rather than a rapid prolonged activation of PKD/PKC\(\mu\), a rapid but transient increase in PKD/PKC\(\mu\) catalytic activity was observed (Fig. 5A, upper panel). Strikingly this pattern of PKD/PKC\(\mu\) catalytic activity, as revealed using \emph{in vitro} kinase assays, was paralleled by transient immunoreactivity of PKD/PKC\(\mu\) with the pS916 antibody in Western blot analyses of total cell lysates. (Fig. 5A, middle panel). These data show that PKD/PKC\(\mu\) is regulated by both positive and negative signaling pathways in B lymphocytes and indicates that the pS916 antibody can be used to effectively monitor the status of PKD/PKC\(\mu\) catalytic activity in B lymphocytes.

The serine 916 residue is conserved between the murine and human homologues of PKD/PKC\(\mu\), and we therefore wanted to determine whether the pS916 antibody could also be used to monitor PKD/PKC\(\mu\) activity in Western blot analyses of primary human cells. The data in Fig. 5B shows that stimulation of human peripheral blood-derived T cells with the CD3 antibody UCHT1 (triggering the T cell antigen receptor complex) induced a rapid 5–7-fold increase in PKD/PKC\(\mu\) catalytic activity. The pS916 antibody was strongly reactive with active PKD/PKC\(\mu\) found in either T cell antigen receptor-activated lymphocytes or in T cells treated with phorbol esters. Collectively the results presented in Fig. 5 show that triggering of antigen receptors in both B and T lymphocytes results in the phosphorylation of serine 916 of PKD/PKC\(\mu\) \emph{in vivo}. Moreover, the pS916 antibody is a sensitive tool to monitor PKD/PKC\(\mu\) activity in lymphoid cell lines and in primary lymphocyte cultures.

Concluding Remarks—Previous work has shown that activation of PKD/PKC\(\mu\) results in the phosphorylation of this enzyme on multiple sites. In this study we have taken a site-specific phosphopeptide antibody approach to identify a phosphorylation site within the C-terminal region of PKD/PKC\(\mu\) at residue Ser-916. The pS916 antibody preferentially recognized active PKD/PKC\(\mu\), and mutational analysis confirmed that this phospho antibody was specific for the C-terminal Ser-916 site of PKD/PKC\(\mu\). The amino acid sequence surrounding the serine 916 residue shows high homology to the optimal peptide substrate sequence for PKD/PKC\(\mu\), which raised the possibility that serine 916 is an autophosphorylation site for this enzyme. \emph{In vitro} kinase assays established that PKD/PKC\(\mu\) could efficiently phosphorylate a synthetic peptide...
containing the Ser-916 residue. More importantly, the serine 916 residue was constitutively phosphorylated in activated PKD/PKC\(\mu\) mutants and could not be detected in kinase-deficient PKD/PKC\(\mu\) mutants, indicating that Ser-916 is an in vivo autophosphorylation site for active PKD/PKC\(\mu\).

Using the pS916 antibody, we have shown that phosphorylation of serine 916 occurs in response to both pharmacological (phorbol ester) and physiological (antigen receptor triggering) stimulation of lymphocytes. Rapid phosphorylation of Ser-916 occurs in both murine and human systems. Previous work has shown that antigen receptors are coupled to the activation of PKD/PKC\(\mu\) in primary peripheral blood-derived T lymphocytes. Hence, the pS916 antibody is a useful tool to study the regulation of PKD/PKC\(\mu\) activity in vivo.

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