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Additions and Corrections

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Protein kinase C βII regulates Akt phosphorylation on Ser-473 in a cell type- and stimulus-specific fashion.

Yuko Kawakami, Hajime Nishimoto, Jiro Kitaura, Mari Maeda-Yamamoto, Roberta M. Kato, Dan R. Littman, Michael Leitges, David J. Rawlings, and Toshiaki Kawakami

Dr. Michael Leitges was inadvertently omitted from the author list. His affiliation is the Max Planck Institute for Experimental Endocrinology, 30625 Hannover, Germany. The correct author list is shown above.

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The glutamine residue of the conserved GGQ motif in *Saccharomyces cerevisiae* release factor eRF1 is methylated by the product of the *YDR140w* gene.

Valérie Heurgué-Hamard, Stéphanie Champ, Liliana Mora, Tatiana Merkulova-Rainon, Lev L. Kisselev, and Richard H. Buckingham

Dr. Merkulova-Rainon's last name was misspelled. The correct spelling is shown above.

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Protein Kinase C βII Regulates Akt Phosphorylation on Ser-473 in a Cell Type- and Stimulus-specific Fashion*[S]♦

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Akt (= protein kinase B), a subfamily of the AGC serine/threonine kinases, plays critical roles in survival, proliferation, glucose metabolism, and other cellular functions. Akt activation requires the recruitment of the enzyme to the plasma membrane by interacting with membrane-bound lipid products of phosphatidylinositol 3-kinase. Membrane-bound Akt is then phosphorylated at two sites for its full activation; Thr-308 in the activation loop of the kinase domain is phosphorylated by 3-phosphoinositide-dependent kinase-1 (PDK1) and Ser-473 in the C-terminal hydrophobic motif by a putative kinase PDK2. The identity of PDK2 has been elusive. Here we present evidence that conventional isoforms of protein kinase C (PKC), particularly PKCβII, can regulate Akt activity by directly phosphorylating Ser-473 *in vitro* and in IgE/antigen-stimulated mast cells. By contrast, PKCβ is not required for Ser-473 phosphorylation in mast cells stimulated with stem cell factor or interleukin-3, in serum-stimulated fibroblasts, or in antigen receptor-stimulated T or B lymphocytes. Therefore, PKCβII appears to work as a cell type- and stimulus-specific PDK2.

Akt is a subfamily of the AGC serine/threonine kinases consisting of Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. These kinases have an N-terminal pleckstrin homology domain followed by a C-terminal kinase catalytic domain. Numerous studies implicate Akt in survival, proliferation, and glucose metabolism (reviewed in Refs. 1–3). Akt activation requires the recruitment of the enzyme to the plasma membrane through the interaction of the pleckstrin homology domain with plasma membrane-bound lipid products of phosphatidylinositol 3-kinase, *i.e.* phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (4–7). Membrane-bound Akt is then phosphorylated at two sites for full activation (8); Thr-308 in the activation loop of the kinase domain is phosphorylated by PDK1[1] (5, 7, 9) and Ser-473 in the C-terminal hydrophobic motif by a putative kinase PDK2. The identity of PDK2 has been controversial (10), although several PDK2 candidates including integrin-linked kinase (ILK) (11, 12) and autophosphorylation (13) have been proposed.

PKC is also a subfamily of the AGC serine/threonine kinases, which are involved in proliferation, differentiation, metabolism, and cell-type specific functions (14, 15). Based on structural features and cofactor requirements, PKC isoforms are classified into three categories; conventional or classical isoforms (cPKCs: α, βI, βII, and γ) depend on diacylglycerol (DAG) and Ca^{2+} for their activation, novel isoforms (nPKCs: δ, ϵ, η, and θ) depend on DAG, but not Ca^{2+}, and atypical isoforms (atypical PKC isoforms: ζ and λ/ι) do not require either DAG or Ca^{2+}.

Cross-linking of high-affinity IgE receptors (FcϵRI) expressed on the surface of mast cells activates Akt (16) and various PKC isoforms including PKCβI and PKCβII (17). Importantly, Akt in mast cells is involved in FcϵRI-induced production of cytokines such as IL-2 through the activation of such transcription factors as NF-κB, NF-AT, and AP-1 (16), and PKCβ is also required for FcϵRI-induced degranulation and cytokine production (18). In the present study we have found that cPKC isoforms, particularly PKCβII, can regulate Akt activity by directly phosphorylating the critical residue Ser-473 *in vitro* and in FcϵRI-stimulated mast cells. By contrast, PKCβ is not required for Ser-473 phosphorylation in mast cells stimulated with stem cell factor (SCF) or IL-3, in serum-stimulated fibroblasts, or in antigen receptor-stimulated T or B cells. Therefore, PKCβII functions as a cell type- and stimulus-specific PDK2.

EXPERIMENTAL PROCEDURES

Antibodies and Recombinant Proteins—Anti-FcϵRI β subunit monoclonal antibody JRK was originally donated by Dr. Juan Rivera, National Institutes of Health. Commercial sources of antibodies used were: anti-Akt (C-20), anti-PKCα (C-20), anti-PKCβI (C-16), anti-PKCβII (C-18), anti-PKCϵ (C-15), anti-PKCθ (C-19), anti-PKCζ (C-20), and anti-Vav (C-14) from Santa Cruz Biotechnology; anti-phospho-PKC (pan), a gift from Dr. Michael Comb; anti-Akt(pThr308), Akt(pS473), anti-BAD, and anti-GSK-3α(pS21) from Cell Signaling Technology; anti-HA (12CA5) from Roche Applied Science; anti-ILK and anti-c-Raf1 from Upstate Cell Signaling Solutions; and anti-FLAG from Kodak Scientific Imaging Systems. Recombinant PKCα, PKCβI, PKCβII, and PKCθ were from Invitrogen, and recombinant Akt was from Upstate Cell Signaling Solutions.

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♦ This article was selected as a Paper of the Week.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data and Figs. S1–S4.

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[1] The abbreviations used are: PDK, 3-phosphoinositide-dependent kinase; BMMC, bone marrow-derived mast cells; DNP, dinitrophenyl; FcϵRI, high-affinity IgE receptor; FLMC, fetal liver-derived mast cells; ILK, integrin-linked kinase; KD, kinase-dead; PKC, protein kinase C; cPKC, conventional or classical PKC isoforms; nPKC, novel PKC isoforms; PMA, phorbol 12-myristate 13-acetate; SCF, stem cell factor; wt, wild-type; IL, interleukin; HA, hemagglutinin.

Cells and Stimulation—Bone marrow cells derived from wild-type (wt), *PKCβ*−/− (19), *PKCε*−/− (20), and *PKCθ*−/− (21) mice were cultured in IL-3-containing medium for 4–6 weeks to generate >95% pure populations of mast cells (BMMC). Fetal liver cells were cultured in medium containing IL-3 and mouse recombinant SCF for 4–6 weeks to generate similarly pure populations of mast cells (FLMC). Mast cells cultured in SCF for more than a week are labeled as SCF-BMMC or SCF-FLMC. Mast cells were sensitized overnight with anti-dinitrophenyl (DNP) IgE monoclonal antibody and stimulated with the antigen, DNP-human serum albumin conjugates, unless otherwise mentioned. Fibroblasts were cultured from 15–18 day embryos of *PKCβ*+/+, *PKCβ*+/−, and *PKCβ*−/− genotypes as described previously (22).

Retroviral transduction of *PKCβ*−/− mast cells was done as described previously (23). Briefly, pMX-puro plasmids harboring wt or mutant PKCβI, PKCβII, or HA-tagged Akt cDNAs were transfected into packaging cells to generate recombinant retroviruses. BMMC in culture medium containing IL-3 and SCF were infected with the viruses. Mass populations of puromycin-resistant cells were used for FcεRI stimulation. PKCα knock-down constructs consisted of pSUPER.retro (OligoEngine) vectors containing the oligonucleotides that could be double-stranded with a loop-out. These vectors were transfected into NIH/3T3 cells to test their ability to suppress PKCα expression. The best vector was selected by immunoblotting and used to generate recombinant retrovirus, and the virus was used to infect wt and *PKCβ*−/− BMMC.

Immunoblotting Analysis—Subcellular fractionation was performed as described previously (24). Otherwise, cells were lysed in 1% Nonidet P-40-containing lysis buffer. Proteins in cleared cell lysates or subcellular fractions were either immunoprecipitated before, or directly analyzed by, SDS-PAGE followed by electroblotting onto polyvinylidene difluoride membranes (Millipore).

In Vitro Kinase Assays—Protein kinases either in immunoprecipitated or purified forms were incubated with synthetic peptide or recombinant protein substrate in the presence of [γ-^{32}P]ATP. Reaction products were analyzed by SDS-PAGE and autoradiography. Alternatively, reaction mixtures were spotted onto P81 phosphocellulose filters, and the radioactivity of filters after washing in phosphoric acid was quantified. Synthetic peptides used in *in vitro* kinase assays were prepared in-house or purchased from A & A Laboratories (S473tide and T308tide) or from Upstate (Crosstide), and their sequences are as follows: 1) ECVDSERRPHFPQFSYSASSTA (the underlined S indicates Ser-473, in single-letter amino acid code), used in Ser-473 phosphorylation experiments (Fig. 2*a*); 2) S473tide for Ser-473 phosphorylation: RRPHFPQFSYSA (Ser-473 is underlined); 3) T308tide for Thr-308 phosphorylation: KTFCGTPEYLAPEVRR (Thr-308 is underlined); Crosstide for Akt assay: GRPTSSFAEG.

Measurement of Histamine and Cytokines—IgE-sensitized cells were stimulated with antigen. Histamine secreted for 45 min was measured by on-column high performance liquid chromatography. IL-2 and tumor necrosis factor-α secreted for 20 h were quantified by enzyme-linked immunosorbent assay kits (Pharmingen).

RESULTS

Akt Is Phosphorylated in Response to Phorbol Ester and Interacts Physically with cPKC Isoforms in Mast Cells—We first tested whether Akt phosphorylation can be induced by phorbol ester, a DAG analogue. Immunoblot analysis of BMMC derived from wt mice demonstrated that phorbol 12-myristate 13-acetate (PMA) rapidly induces Akt phosphorylation on Ser-473 (Fig. 1*a*), suggesting that PMA-responsive PKC (conventional and/or novel) isoforms can activate Akt. Interestingly, Ser-473 phosphorylation was substantially reduced in BMMC from *PKCβ*−/− mice. We then assessed whether Akt is physically associated with such PKCs. Immunoblotting of Akt immunoprecipitates showed that cPKCs (*i.e.* α, βI, and βII. γ is not expressed in BMMC) were coprecipitated with Akt, and PKCβII association was particularly robust and inducible by FcεRI stimulation with IgE and antigen (Fig. 1*b*). Conversely, Akt was detected in immune complexes precipitated by anti-PKCα, anti-PKCβI, or anti-PKCβII (data not shown), and these interactions were direct (supplementary Fig. S1). A small amount of ILK was also coimmunoprecipitated with Akt. By contrast, PKCε, PKCθ, PKCζ, or c-Raf1 was not detected in Akt immunoprecipitates, although these kinases were abundantly expressed in mast cells.

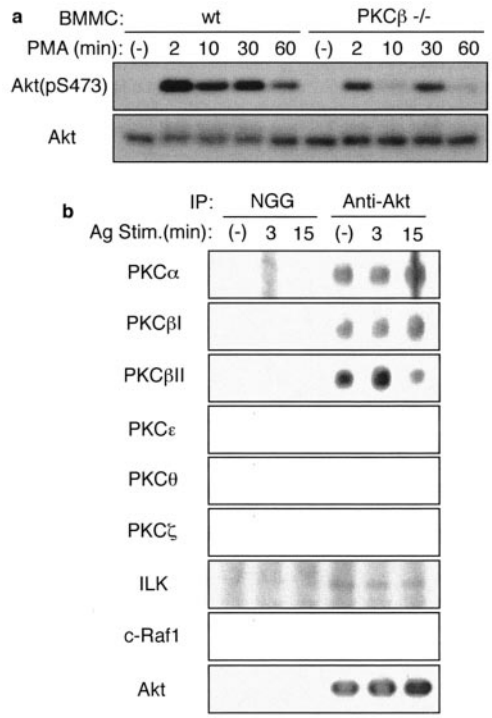

FIG. 1. **Akt is phosphorylated by phorbol ester and interacts physically with cPKC isoforms in mast cells.** *a*, wt and *PKCβ*−/− BMMC were stimulated by 100 nM PMA for the indicated periods. Cell lysates were analyzed by immunoblotting with anti-Akt(pS473). The blots were reprobed with anti-Akt. *b*, MCP-5 mouse mast cells were sensitized overnight with anti-DNP IgE and stimulated by 100 ng/ml DNP-human serum albumin for the indicated periods. Cleared cell lysates were immunoprecipitated with goat anti-Akt antibody or normal goat IgG (NGG) and immune complexes analyzed by immunoblotting with antibodies for the indicated proteins. The blots were stripped and reprobed with anti-Akt. *Ag*, antigen.

cPKC Isoforms Can Phosphorylate Akt on Ser-473 in Vitro—Given the physical association between Akt and cPKCs, we examined whether Akt serves as a phosphorylation target for cPKC isoforms. First, we tested whether synthetic peptides corresponding to the Akt C-terminal 22 residues encompassing Ser-473 can be phosphorylated by recombinant PKCα, PKCβI, and PKCβII. The non-phosphorylated (*NP*) peptide (or S473tide) was phosphorylated by these kinases with an order of PKCα ≥ PKCβII > PKCβI in potency (Fig. 2*a*), while the Ser-473-phosphorylated (pS) peptide was not phosphorylated by any of these isoforms despite the presence in this peptide of four Ser and one Thr residues as potential phosphorylation sites. We then tested whether these cPKCs can phosphorylate Akt. As shown in Fig. 2*b*, Akt was not phosphorylated on Thr-308 by these PKC isoforms, although the latter enzymes phosphorylated themselves on Thr-497 (PKCα) or Thr-500 (PKCβI and PKCβII) in their activation loop under the same conditions. Strikingly, Akt Ser-473 was phosphorylated by PKCα and PKCβII in a concentration-dependent manner (Fig. 2*b*). Ser-473 phosphorylation by PKCβI was weaker and saturated at lower levels than that by PKCα or PKCβII and did not correlate with its autophosphorylating potency. These results indicate that the cPKCs, particularly PKCα and PKCβII, have a vigorous PDK2 activity *in vitro* and they can phosphorylate Akt on Ser-473 without prior phosphorylation on Thr-308, consistent with previous studies showing that Ser-473 phosphorylation is independent of Thr-308 phosphorylation (8, 25, 26).

Akt Catalytic Activity and Ser-473 Phosphorylation Are Reduced in FcεRI-stimulated PKCβ−/− *Mast Cells*—BMMC are immature mast cells widely used for studies of FcεRI signal

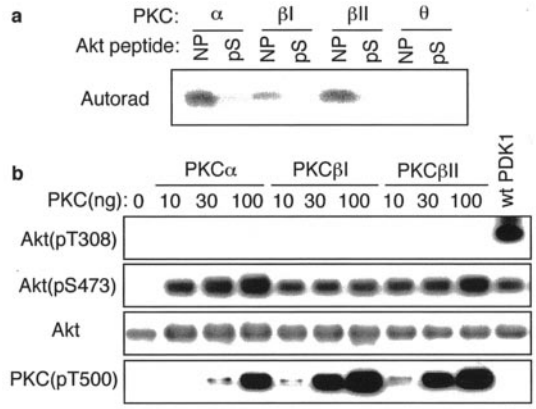

FIG. 2. **PMA-responsive cPKCs can phosphorylate on Ser-473 *in vitro*.** *a*, recombinant PKCα, PKCβI, PKCβII, and PKCθ (0.1 μg per reaction) were incubated with 2 μg each of synthetic non-phosphorylated (NP) or Ser-473-phosphorylated (pS) Akt C-terminal peptides in the presence of [γ-^{32}P]ATP. Reaction products were analyzed by SDS-PAGE and autoradiography. *b*, unactivated recombinant Akt (0.2 μg per reaction) was incubated with the indicated amounts of recombinant PKCα, PKCβI, or PKCβII under the kinase assay conditions in the presence of cold ATP. Reaction products were analyzed by immunoblotting with anti-Akt(pT308) and anti-Akt(pS473). The same blot was reprobed with anti-PKCβ (pT500) that reacts with Thr-497-phosphorylated PKCα and Thr-500-phosphorylated PKCβI and PKCβII.

transduction. Cell surface expression of FcϵRI and c-Kit was comparable between wt and *PKCβ−/−* BMMC, and cell viability was not affected by PKCβ deficiency (data not shown). Antigen stimulation of IgE-sensitized wt BMMC induced Ser-473 phosphorylation (Fig. 3*a*). Remarkably, Akt Ser-473 phosphorylation levels were lower in unstimulated *PKCβ−/−* BMMC than in unstimulated wt cells, and IgE/antigen-induced peak Ser-473 phosphorylation levels were also drastically (50–80%) reduced in *PKCβ−/−* BMMC at all antigen concentrations tested. Similar, but more dramatic, reduction was observed in IgE/antigen-stimulated *PKCβ−/−* cells (Fig. 3*a*, *lower panel* (the SCF-FLMC part), and data not shown) when BMMC and FLMC that had been cultured in the presence of SCF were used. SCF induces maturation of immature mast cells. Consistent with a significant role for Ser-473 phosphorylation in Akt activation, Akt activation induced by IgE/antigen stimulation, as assessed by its ability to phosphorylate its substrate, BAD or Crosstide, was also reduced in *PKCβ−/−* BMMC (Fig. 3, *b* and *c*). By contrast, FcϵRI-induced Ser-473 phosphorylation was not reduced in *PKCε−/−* or *PKCθ−/−* BMMC compared with their wt controls (supplementary Fig. S2), although these nPKC isoforms are expressed in BMMC. These results, together with the reduced Ser-473 phosphorylation in PMA-stimulated *PKCβ−/−* BMMC (Fig. 1*a*), demonstrate the selective ability of PKCβ relative to the nPKC isoforms to regulate Ser-473 phosphorylation in mast cells. Consistent with previous results that Ser-21 of GSK-3α is a phosphorylation target of Akt (27, 28), Ser-21 phosphorylation was substantially reduced in *PKCβ−/−* BMMC (Fig. 3*d*).

Reconstitution with PKCβII, but Not PKCβI, Can Restore Fcε RI-induced Akt Ser-473 Phosphorylation and IL-2 Production in PKCβ-deficient Mast Cells—PKCβI and PKCβII were generated by alternative splicing (29), and *PKCβ−/−* mice lack the expression of both isoforms. To test whether defects in Akt phosphorylation and activity in *PKCβ−/−* BMMC are direct effects of the deficiency of PKCβI and/or PKCβII, we reconstituted these cells with PKCβI or PKCβII by retroviral transduction. Under the current transduction experiments, which require SCF to drive cell cycling to promote retroviral integration in BMMC leading to further differentiation of mast cells, Akt phosphorylation on Thr-308 was minimally affected by PKCβ deficiency (see the rightmost eight lanes in Fig. 4*a*), although reconstitution with either PKCβI or PKCβII enhanced Thr-308 phosphorylation in *PKCβ−/−* cells. The increased Thr-308 phosphorylation seemed to be due to overexpression of PKCβI or PKCβII. Indeed, immunoblot analysis using recombinant PKCβI and PKCβII as a reference revealed overexpression of PKCβI and PKCβII in the reconstituted cells (wt PKCβI, 200 ng/10^6 cells; kinase-dead (KD) PKCβI, 50 ng; wt PKCβII, 250 ng; KD PKCβII, 19 ng in reconstituted cells *versus* PKCβI, 1.25 ng; PKCβII, 50 ng in non-transfected wt BMMC). Reconstitution with wt PKCβII restored Ser-473 phosphorylation to a full extent, while reconstitution with wt PKCβI at comparable expression levels failed to do so (Fig. 4*b*). By contrast, expression of KD mutants of PKCβI or PKCβII in *PKCβ−/−* cells failed to reconstitute Akt phosphorylation, indicating that the catalytic activity of PKCβII is required for Ser-473 phosphorylation. Taking into consideration our immunoblotting data that the BMMC used express 40-fold more PKCβII (50 ng/10^6 cells) than PKCβI (1.25 ng/10^6 cells), these results (Fig. 4*b*) and the stronger *in vitro* Ser-473 phosphorylating activity of PKCβII *versus* PKCβI (Fig. 2, *a* and *b*) suggest that Ser-473 phosphorylation and hence Akt catalytic activity are mainly under control of the enzymatic activity of PKCβII in FcϵRI-stimulated BMMC. Another potential interpretation of the reconstitution experiments, which does not contradict the notion of PKCβII-dependent Akt regulation is that PKCβ expression permits mast cell precursors to respond better to SCF or IL-3. However, there is no indication that PKCβ is involved in SCF or IL-3 signaling (Ref. 18 and data not shown).

Although a reduction in Thr-308 phosphorylation in *PKCβ−/−* cells was much smaller than that in Ser-473 phosphorylation, there is a possibility that autophosphorylation of Akt contributes to Ser-473 phosphorylation (13) in mast cells. To assess this possibility, we sought to measure Ser-473 phosphorylating activity of PKCβII under conditions that minimize the autophosphorylation of the transduced Akt. We transduced wt and *PKCβ−/−* BMMC with a HA-tagged KD (K179M) Akt mutant. For a comparison, HA-tagged wt Akt and T308A mutant Akt were also transduced. Ser-473 phosphorylation of HA-tagged wt and T308A was reduced in *PKCβ−/−* cells compared with wt cells (Fig. 4*c*), just like that of endogenous Akt protein in non-transfected cells. Importantly, Ser-473 phosphorylation of HA-tagged K179M Akt was robustly induced in wt cells upon FcϵRI stimulation (Fig. 4*c*), ruling out the possibility that Ser-473 phosphorylation is a result of mainly autophosphorylation. By contrast, Ser-473 phosphorylation of HA-tagged K179M Akt was almost abrogated in *PKCβ−/−* cells (Fig. 4*c*). These results indicate that Ser-473 is predominantly phosphorylated by PKCβII in FcϵRI-stimulated mast cells. Experiments using protein phosphatase inhibitors indicate that dephosphorylation at Ser-473 is not regulated by PKCβ (supplementary Fig. S3a).

Because the evidence described above indicates that PKCβII is a major PDK2 in FcϵRI-stimulated mast cells, we determined kinetic parameters of S473tide phosphorylation by PKCβII. The K_m and V_{max} values were in a similar range as those for T308tide phosphorylation by PDK1 (K_m = 873.6 ± 250.7 μM; V_{max} = 77,286 ± 7,767 μmol/min for S473tide phosphorylation by PKCβII *versus* K_m = 933.1 ± 261.9 μM; V_{max} = 20,100 ± 2,027 μmol/min for T308tide phosphorylation by PDK1), supporting the physiological relevance of our *in vitro* data. To further address the physiological relevance of PKCβII-mediated Akt regulation, *PKCβ−/−* BMMC transduced with PKCβI or PKCβII were stimulated with IgE and antigen. The cells reconstituted with either PKCβI or PKCβII released histamine as well as did the wt BMMC (Fig. 5*a*). Reconstitution with wt

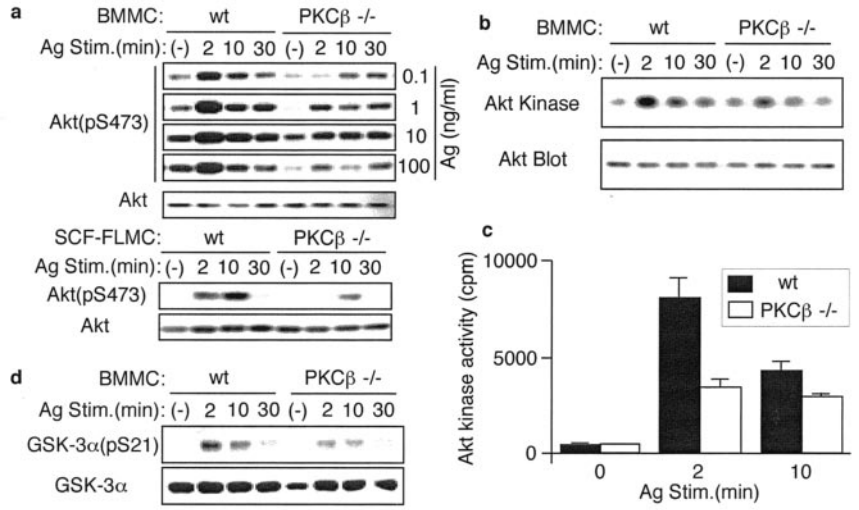

FIG. 3. **Ser-473 phosphorylation and catalytic activity of Akt are dependent on PKCβ.** wt and *PKCβ*−/− BMMC or SCF-FLMC were sensitized overnight with anti-DNP IgE and stimulated by the indicated concentrations (*a*) or otherwise 100 ng/ml DNP-human serum albumin for the indicated periods. Cell lysates were analyzed by immunoblotting with anti-Akt(pS473) (*a*) or anti-GSK-3α (pS21) (*d*) antibody. The blots were reprobed with anti-Akt (*a*) or anti-GSK-3α (*d*). *b* and *c*, anti-Akt immunoprecipitates from IgE/antigen-stimulated BMMC were subjected to kinase assays using recombinant BAD (*b*) or Crosstide (*c*) as substrate. *Ag*, antigen.

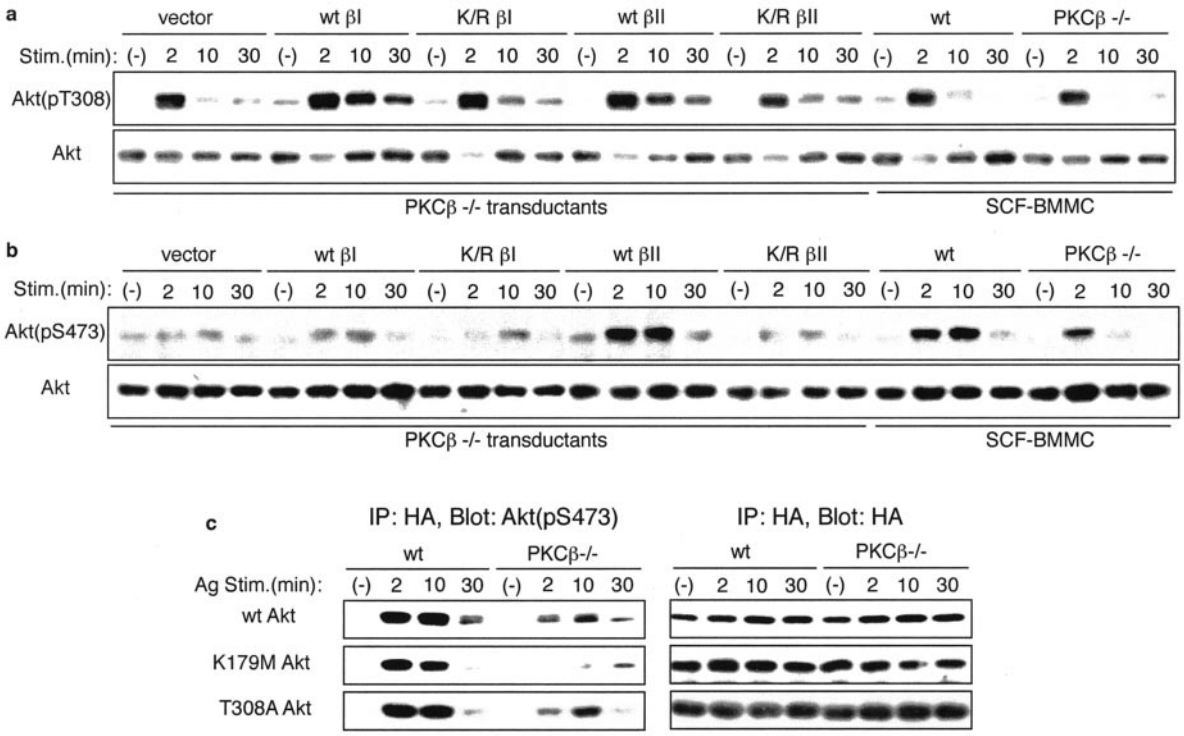

FIG. 4. **Akt Ser-473 phosphorylation is largely dependent on PKCβII in FcεRI-stimulated mast cells.** *a* and *b*, *PKCβ*−/− BMMC transduced with wt or KD (*K*/*R*) PKCβI or PKCβII were analyzed by immunoblotting consecutively with anti-Akt(pT308) (*a*) or anti-Akt(pS473) (*b*) and anti-Akt. Wt and *PKCβ*−/− BMMC cultured in the presence of SCF for >2 weeks were analyzed in parallel (*a* and *b*). Representative results from three sets of transduced cells are shown. *c*, wt and *PKCβ*−/− BMMC transduced with HA-tagged wt, KD (K179M), or T308A Akt were stimulated with IgE and antigen. Cells were analyzed for Ser-473 phosphorylation of HA-tagged Akt. The blots were reprobed with anti-HA. A representative result of three experiments is shown. *Ag*, antigen.

PKCβII robustly restored IL-2 production in *PKCβ*−/− BMMC, whereas that with PKCβI did not (Fig. 5*b*). Consistent with the role of Akt in FcεRI-induced IL-2 production (16), these results indicate that PKCβII-dependent Akt activation is crucial for FcεRI-induced IL-2 production.

PKCα Is Not a Major Regulator of Akt Ser-473 Phosphorylation in FcεRI-stimulated Mast Cells—Given the strong *in vitro* PDK2 activity of PKCα (Fig. 2, *a* and *b*) and without access to *PKCα*−/− mice, we tested effects of small interfering RNA-mediated PKCα knock-down on Akt phosphorylation. PKCα knock-down did not significantly affect Ser-473 phosphorylation in wt or *PKCβ*−/− BMMC (supplementary Fig. S4a). Furthermore, Gö-6976 (cPKC inhibitor) treatment of *PKCβ*−/− BMMC minimally affected Ser-473 phosphorylation, whereas the same treatment substantially reduced it in wt BMMC (supplementary Fig. S4b). These results are consistent with the predominant role of PKCβII as the PDK2 among the cPKCs expressed in FcεRI-stimulated mast cells.

PKCβ-mediated Akt Ser-473 Phosphorylation Is Dependent on Stimulus and Cell Type—To extend our inquiry into the role of PKCβII as the regulator of Ser-473 phosphorylation, we next stimulated mast cells with SCF or IL-3, two major mast cell growth factors. In contrast with FcεRI stimulation (Fig. 3*a*), SCF and IL-3 induced robust Ser-473 phosphorylation in *PKCβ*−/− cells similar to that observed in wt cells (Fig. 6*a*). When splenic T and B cells were stimulated with anti-CD3/anti-CD28 and anti-μ monoclonal antibodies, respectively, Akt was phosphorylated on Ser-473 at similar levels in wt and

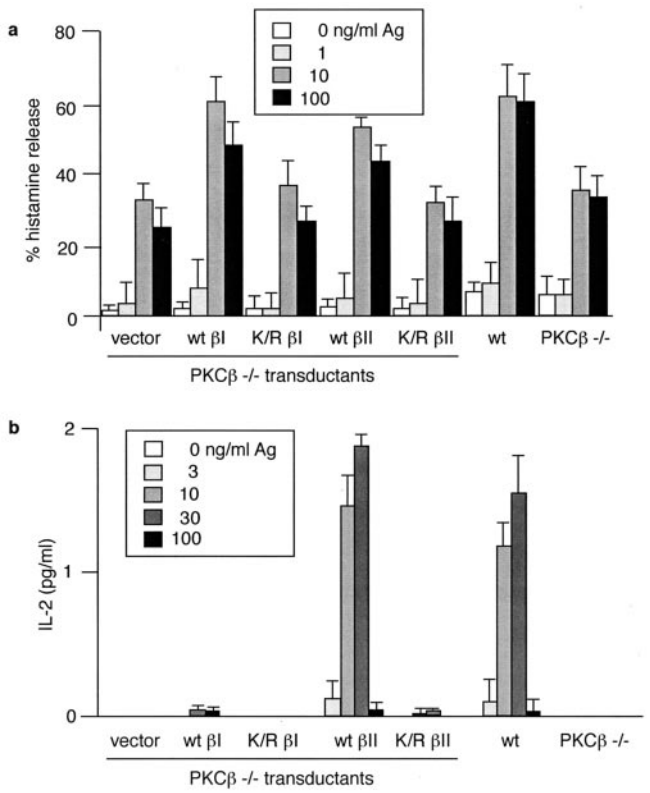

FIG. 5. **Reconstitution with PKCβII can restore degranulation and IL-2 secretion in IgE/antigen-stimulated *PKCβ−/−* mast cells.** *PKCβ−/−* BMMC were transfected with retroviral vectors encoding wild-type or KD (*K*/*R*) PKCβI or PKCβII. Drug-selected mass cultured transfectants were stimulated with IgE and antigen. Culture supernatants of IgE-sensitized transfectants stimulated with the indicated concentrations of antigen for 45 min (*a*) and 20 h (*b*) were used to measure histamine (*a*) and IL-2 (*b*). Representative results from three sets of transfectants are shown. The other two sets of transfectants exhibited similar results.

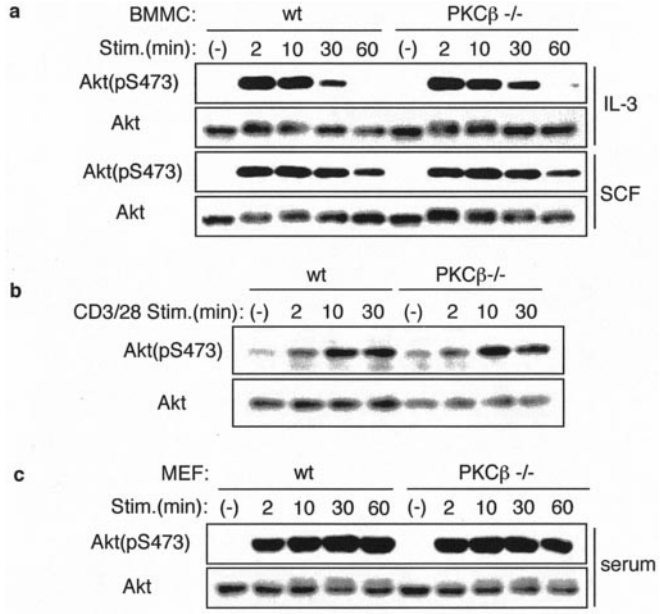

FIG. 6. **Akt Ser-473 phosphorylation does not require PKCβ in IL-3- or SCF-stimulated mast cells or other cellular situations.** *a*, growth factor-starved wt and *PKCβ−/−* BMMC were incubated with 100 ng/ml recombinant mouse IL-3 (*upper two panels*) or 100 ng/ml recombinant mouse SCF (*lower two panels*) for the indicated periods. *b*, splenic T cells from wt and *PKCβ−/−* mice were stimulated by anti-CD3 and anti-CD28. *c*, embryonic fibroblasts from wt and *PKCβ−/−* mice (MEF) were stimulated with 20% fetal calf serum for the indicated periods. Cell lysates were analyzed for Ser-473 phosphorylation and Akt amounts.

PKCβ−/− mice (Fig. 6*b* and data not shown). When mouse embryonic fibroblasts were stimulated with serum, we observed similarly robust Ser-473 phosphorylation in wt and *PKCβ−/−* cells (Fig. 6*c*). These results indicate that there must exist multiple kinases that have PDK2 or Ser-473 kinase activity.

DISCUSSION

Unlike PDK1 that was extensively characterized, the identity of PDK2 has been enigmatic. Three classes of PDK2 candidates have been proposed (10): 1) several distinct kinases including ILK, 2) peptide-induced conversion of PDK1 to PDK2 (30), and 3) Ser-473 autophosphorylation following Thr-308 phosphorylation (13). However, none of these hypotheses could fully explain the PDK2 activity in several cellular systems. Recent reports suggested that a constitutively active PDK2 distinct from ILK, PDK1, or Akt is localized to the plasma membrane (4, 26, 31) and is resistant to staurosporine (32, 33). In one study, PDK2 activity was found in a complex with PKCζ (33). By contrast, Ser-473 phosphorylation in FcεRI-stimulated mast cells was staurosporine-sensitive (supplementary Fig. S3b), and PKCζ was not found in Akt immune complexes from mast cell lysates (Fig. 1*b*). Our data also demonstrate that defects in Ser-473 phosphorylation in PKCβ-deficient mast cells are partial (up to 80% reduction) and depend on cell types and stimuli. These studies collectively point to the existence of multiple PDK2 enzymes.

Our data in favor of the hypothesis that PKCβII serves as a major PDK2 in FcεRI-stimulated mast cells are extensive. First, PMA could induce Akt phosphorylation on Ser-473. Second, PKCβII was physically associated with Akt, and this association was direct and augmented upon FcεRI stimulation. Third, consistent with previous studies that Ser-473 phosphorylation takes place at the plasma membrane (7), a substantial fraction of PKCβII was membrane-associated in mast cells. Fourth, PKCβII could phosphorylate Akt on Ser-473, and enzymatic kinetics of S473tide phoshorylation by PKCβII were similar to those of T308tide phosphorylation by PDK1. Fifth, Ser-473 phosphorylation was impaired in FcεRI-stimulated *PKCβ−/−* mast cells and this phosphorylation was restored by reconstitution with PKCβII. Sixth, Ser-473 phosphorylation of the HA-tagged KD Akt was almost abrogated in FcεRI-stimulated *PKCβ−/−* mast cells (Fig. 4*c*), whereas it was nearly intact in wt cells, excluding the possibility of Akt autophosphorylation as the major mechanism for Ser-473 phosphorylation. A residual and delayed Ser-473 phosphorylation of HA-tagged KD Akt in FcεRI-stimulated *PKCβ−/−* cells (Fig. 4*c*) could be due to other PDK2 activities or a residual autophosphorylating activity of the mutant Akt, although the latter possibility is less likely given the lack of kinase activity of K179M Akt (34).

Our *in vitro* results indicate that PKCα has a PDK2 activity. However, the extremely low Ser-473 phosphorylation of HA-tagged KD Akt in *PKCβ−/−* mast cells and the results with PKCα small interfering RNA and Gö-6976 indicate that the role of PKCα (40 ng/10^6 cells) as PDK2 is at best minor in FcεRI-stimulated mast cells. This could be due to a much lower ratio of membrane *versus* cytosolic PKCα (~0.5%) compared with that of PKCβII (20–70%; 50 ng/10^6 cells) (Ref. 23 and data not shown). Deficiency of PKCθ (8 ng/10^6 cells) or PKCε (0.05 ng/10^6 cells) did not affect Ser-473 phosphorylation. Expression levels of the other novel or atypical PKC isoforms in BMMC are less than 1 ng per 10^6 cells (data not shown). Another kinase with PDK2 activity in mast cells might be ILK, which was detected at a low level in Akt immunoprecipitates. However,

ILK activity was not reduced in *PKCβ−/−* mast cells (data not shown). Overall, these data favor the hypothesis that PKCβII is a major PDK2 in FcεRI-activated mast cells.

This study demonstrates that Akt Ser-473 phosphorylation is regulated by PKCβII in a cell type/stimulus-specific manner and suggests that there are multiple Ser-473 kinases that are used in a cell type/stimulus-specific manner. This is in stark contrast with Thr-308 phosphorylation, which is executed by an evolutionally conserved kinase PDK1 regardless of stimuli that elicit phosphatidylinositol 3-kinase activation. These results may provide a foundation to search for compounds that inhibit PKCβII or other kinase(s) that function as a cell type/stimulus-specific PDK2. Akt is implicated in the pathogenesis of various cancers and diabetes. Inhibition of a cell type/stimulus-specific regulator of Akt rather than that of the phosphatidylinositol 3-kinase/PDK1 arm of Akt activators may broaden our choices of strategies to treat these diseases.

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