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PKCδ and ζ Mediate IL-4/IL-13-Induced Germline ε Transcription in Human B Cells: A Putative Regulation via PU.1 Phosphorylation

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We have investigated the role of PKC isozymes in the function of IL-4 and IL-13 in human B cells. In a Burkitt's B lymphoma cell line, DND39, IL-4 induced the translocation of PKCδ and ζ from the cytosol to the membrane fraction. The activation of germline ε promoter by IL-4 was abrogated not only by the expression of dominant negative mutants of PKCδ and ζ but also by isozyme-selective PKC inhibitors, rottlerin and PKCζ pseudosubstrate peptide. These inhibitors also suppressed IL-4/IL-13-induced germline ε transcription in the IL-13Rα1-transfected DND39 cells as well as in normal human B cells, but had no influence on the induction of CD23b in the latter cells. As a downstream event of PKC, we found threonine phosphorylation of PU.1 in IL-4-stimulated DND39 cells. This phosphorylation was suppressed by the PKC inhibitors, although STAT6 activation was unaffected. These results suggest that, in human B cells, IL-4/IL-13 utilize PKCδ and ζ for the STAT6-independent signaling pathway and thereby modulate the transcriptional activity of PU.1.

Key Words: **PKC; IL-4; IL-13; PU.1; germline ε transcript; CD23.**

Interleukin-4 (IL-4) is a multifunctional cytokine produced mainly by Th2 cells, mast cells, and basophils. The biologic responses to IL-4 are dependent on the types and the differentiation stages of the cells. In human B cells, IL-4 induces class switching to IgE and IgG4, as well as the enhanced expression of CD23, IL-4R, and MHC class II (1). IgE class switching is initiated by IL-4-induced expression of germline ε transcript (2, 3). These activities of IL-4 are shared by another cytokine, IL-13, because both cytokines utilize the IL-4Rα chain as a common receptor component (4–6). Two types of the IL-4R have been elucidated; type I receptor is composed of IL-4Rα chain and commonγ (γc) chain, while type II receptor, composed of IL-4Rα chain and IL-13Rα1 chain, is also the functional receptor for IL-13. Unlike the ubiquitously expressed IL-4Rα, the IL-13Rα1 is detected in limited types of cells. T cells express only type I receptor, while fibroblast and endothelial cells express only type II receptor, and human B cells and monocytes express both types of IL-4R (7, 8).

The pleiotropic activities of IL-4 and IL-13 are ascribed to the ability of these cytokines to activate several signaling pathways in the cell. The IL-4Rα and γc associate with Janus kinase-1 (JAK1) and JAK3, respectively, while Tyk2 associates with IL-13Rα1 (9–12). Ligand binding activates these JAKs, which in turn activates signal transducer and activator of transcription-6 (STAT6) (13). In addition to JAK/STAT pathway, the IL-4Rα forms a complex with adaptor molecules including insulin receptor substrate-1 (IRS-1), IRS-2, Shc, and FRIP as well as with Fes kinase, and these molecules transmit the downstream signaling (14–18). Among them, the activation of phosphatidylinositol-3 kinase (PI3K) has been most extensively studied (19–21), but other downstream events are not well understood.

We have previously reported that IL-4 induces the translocation of PKCζ from the cytosol to the membrane fraction in a human B cell line (21). Other groups also reported that IL-4 induces the translocation of PKC in human monocytes and the increase of enzymatic activity of PKCζ in a murine T cell line (22, 23). Involvement of PKC in the biological function of IL-4 was suggested by the capability of PMA, a synthetic activator of PKC, to augment the IL-4-induced germline ε transcription, although PMA alone is unable to induce it (24). PKC isozymes can be classified into four groups according to endogenous and exogeneous acti-

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vators: (i) conventional PKC (α, β1, β2, and γ), which depend on both Ca^{2+} and diacylglycerol (DAG); (ii) novel PKC (δ, ϵ, θ, and η), which are Ca^{2+}-independent and regulated by DAG; (iii) atypical PKC (ζ and ι/λ), which do not require Ca^{2+} or DAG; and 4) PKCμ which has a putative transmembrane domain (25). To date, only a little information is available on the isozyme specificity of PKC activation by IL-4. As the changes in intracellular Ca^{2+} levels in response to IL-4 are not reproducible in human B cells (26, 27), it is likely that Ca^{2+}-independent isozymes are involved in its biological function.

In this study, we investigated a role of PKC in the signaling of IL-4 and IL-13 by using dominant-negative mutant of PKC and isozyme-selective PKC inhibitors. Here, we demonstrate that IL-4 and IL-13 utilize PKCδ and ζ in the induction of germline ϵ transcription, but not that of CD23b in human B cells. Moreover, PU.1 phosphorylation was observed as a possible downstream event of PKC.

MATERIALS AND METHODS

Cells. The human Burkitt's lymphoma cell line DND39 was kindly provided by Fujisaki Cell Center (Hayashibara Biochemical Laboratories Inc., Okayama, Japan) and was maintained in RPMI1640 medium supplemented with 10% FCS. Establishment of DND39/Gϵ/IL-13Rα1, stable transfectant of both IL-13Rα1 and germline ϵ reporter construct, was previously reported (12). Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation from heparinized venous blood of healthy adults who had given informed consent to participate in the study. B cells were negatively isolated from PBMC using magnetic beads coating with antibodies against $CD2^+$, $CD14^+$, and $CD56^+$ (Dynal, Oslo, Norway). Purified B cells contained >98% $CD19^+$ cells as determined by flow cytometry and were cultured in RPMI1640 medium supplemented with 10% FCS.

Reagents. Recombinant human IL-4 and IL-13 were purchased from Genzyme (Cambridge, MA). Rottlerin and biotinylated monoclonal antibodies against phosphoserine (PSR-45) and phosphothreonine (PTR-8) were obtained from Sigma (St. Louis, MO). Myristoylated PKCζ peptide inhibitor was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Rabbit polyclonal anti-phosphothreonine antibody was obtained from Upstate Biotechnology (South San Francisco, CA). Monoclonal antibodies specific for each PKC isozyme were obtained from Transduction Laboratories (Lexington, KY) and the following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-STAT6 (S-20), anti-NF-κB p50 (C-19), anti-NF-κB p65 (C-20), anti-PU.1 (T-21), anti-C/EBPβ(C-19).

Plasmids. Wild-type murine PKCζ cDNA and human PKCδ cDNA were obtained from the American Type Culture Collection (Rockville, MD), and were cloned into pTARGET expression vector (Promega, Madison, WI). Dominant-negative forms of PKCζ (K281A) and PKCδ (K378A) were constructed by site-directed mutagenesis using the GeneEditor *in vitro* site-directed mutagenesis system (Promega). To construct human germline ϵ reporter plasmid, a 251-bp fragment (−157/+94) that encompassed the sequence shown in Fig. 5 was cloned into pGL3-basic plasmid (Promega) as described previously (18).

Preparation of cytosolic and membrane fractions and nuclear extracts. To prepare cytosol and membrane fractions, the cells were resuspended in lysis buffer (20 mM Tris–HCl, pH 7.4, containing 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and were lysed by freeze–thawing followed by sonication on ice. They were centrifuged at 100,000*g* for 1 h and the supernatant was saved as the cytosolic fraction. The pellet was resolved in lysis buffer containing 1% NP-40, centrifuged again at 100,000*g* for 1 h and the supernatant was collected as the membrane fraction. Nuclear extracts were prepared according to the method of Andrews *et al.* (28) in the presence of 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 5 mM NaF and protease inhibitors. Protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL).

Immunoprecipitation and Western blotting. To immunoprecipitate PU.1 and C/EBPβ, nuclear extracts were diluted with 3-vol of lysis buffer, precleared with protein A-Sepharose (Pharmacia, Uppsala, Sweden) at 4°C for 1 h, and incubated with appropriate antibody at 4°C for 4 h. Immunocomplexes were precipitated with protein A-Sepharose and washed with lysis buffer containing 1% NP-40. For Western blotting, cytosolic, and membrane fraction (50–100 μg) and immunoprecipitates were resolved on SDS–PAGE, and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Proteins were probed with appropriate antibodies and visualized by enhanced chemiluminescence (ECL, Amersham).

Luciferase assay. For transient transfection assay, 2×10^6 DND39 cells were suspended in Iscov's modified Dulbecco's medium (GIBCO BRL, Grand Island, NY) containing 5% FCS and seeded per well of a 6-well plate. The cells were transiently transfected with germline ϵ reporter plasmid (0.5 μg) plus the indicated amounts of PKC expression or empty plasmid using Effectene transfection reagent (Qiagen) according to the manufacturer's instruction (DNA: Effectene ratio of 1:10). To control for transfection efficiencies, 40 ng of pRL-SV40 (Promega) encoding sea pansy luciferase was cotransfected. After 24 h of transfection, the cells were further incubated with or without IL-4 for 24 h and harvested for luciferase assay using Dual-Luciferase reporter assay system (Promega).

RT-PCR analysis. Total RNA was extracted from purified B cells after stimulation with IL-4 for 24 h using RNeasy total RNA system (Qiagen). Reverse transcription was performed using Superscript II reverse transcriptase (GIBCO BRL), and the PCR was performed using Pyrobest DNA polymerase (Takara Biomedicals, Shiga, Japan). The primer sequences were as follows: germline ϵ transcript, sense ATCCACAGGCACCAAATGGACGACC and antisense GCCAGGTCCACCACCAGACAGGTGA (annealing at 65°C); CD23b, sense CAAGCAGAATTTAGCATA and antisense CTTGCCGAAGTAGTAGCA (annealing at 52°C); β-actin, sense TGTGCTATCCCTGTACGCCTCT and antisense ATCTCCTTCTGCATCCTGTCGG (annealing at 60°C). The amplified products were verified to increase in proportion to the amount of template.

Electrophoretic mobility shift assay (EMSA). Binding reactions with 5 μg of nuclear extracts were carried out according to the protocol described previously (29). For competition and supershift experiments, extracts were preincubated with a 100-fold excess of nonlabeled oligonucleotide or 1 μg antibody for 30 min on ice. Protein-DNA complexes were resolved on a native 4% polyacrylamide gel in 0.5× TBE at 150 V for 2 h. Dried gels were analyzed by BAS system (Fujifilm, Tokyo, Japan). The following oligonucleotides were used: STAT6, 5′-GTCAACTTCCCAAGAACAGAA; C/EBP, 5′-AGCTGTTGCTCAATCGACC; PU.1/NF-κB1, 5′-GAGAGAAAAGGGAACTTCCAGGGCGGCCC; NF-κB2, TCCGGGGGTTCCCACCC.

RESULTS

PKCδ and ζ Are Involved in Germline ϵ Transcription in DND39 Cells

Activation of PKC was described as being associated with a translocation of the enzyme to the plasma mem-

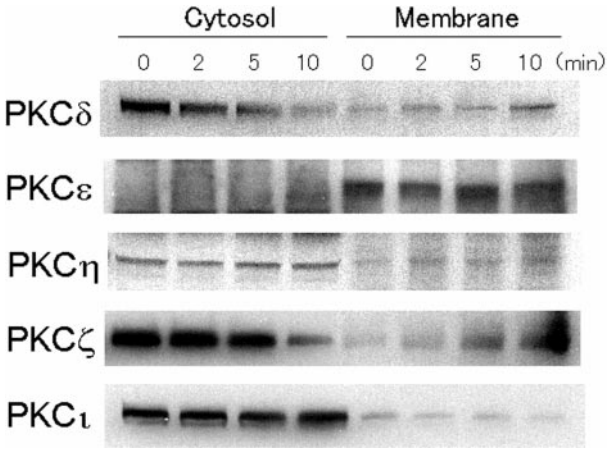

FIG. 1. Translocation of PKC δ and ζ in IL-4-stimulated DND39 cells. The cells were stimulated with IL-4 (5 ng/ml) for the indicated times, followed by the separation into the cytosol and the membrane fractions. Both fractions were resolved by SDS–PAGE, transferred to membrane, and blotted with monoclonal antibodies specific for each PKC isozyme.

brane. We have previously reported that IL-4 induces the translocation of PKCζ in a human B cell line DND39 (21). To examine whether other PKC isozymes are activated by IL-4 stimulation, subcellular distribution of each isozyme were analyzed by Western blot analysis. Since no significant change in intracellular Ca^{2+} levels was observed in IL-4-stimulated DND39 cells (30), we focused on the Ca^{2+}-insensitive isozymes. Expression of δ, ϵ, η, ζ and ι forms of PKC were detected in DND39 cells (Fig. 1), whereas PKCθ was not detected (data not shown). In addition to PKCζ, PKCδ also translocated from the cytosol to the membrane fraction after IL-4 stimulation. Although PKCϵ was detected in the membrane fraction, it was not dependent on IL-4 stimulation.

To determine whether PKCδ and ζ are functionally involved in IL-4 signaling, we introduced dominant negative mutants of these isoforms together with a reporter construct of germline ϵ promoter into DND39 cells. These mutants harbor amino acid substitution at lysine residue in the catalytic domain, which is crucial for ATP binding and is conserved among several protein kinases (31). As shown in Fig. 2A, IL-4 enhanced luciferase activity driven by the germline ϵ promoter about fourfold. Cotransfection of dominant-negative form of either PKCδ or ζ clearly reduced the IL-4-dependent promoter activation. Although the complete inhibition was not observed, transfection with higher amounts of plasmids generated toxic effects in our methods. To further confirm these results, we used a PKCδ-selective inhibitor, rottlerin, and a cell-permeable peptide inhibitor of PKCζ. Rottlerin inhibits PKCδ activity at low concentrations (3–5 μM), while it needs high concentrations (30-100 μM) to inhibit the other PKC isoforms (32). Myristoylated peptide identical to the pseudosubstrate region of PKCζ (amino acids 113–125) inhibits the enzyme activity at 10–30 μM (33). As shown in Fig. 2B, both inhibitors strongly inhibited IL-4-induced germline ϵ promoter activation at the concentration exerting an isozyme-selective inhibition.

We next examined whether PKCδ and ζ are also involved in IL-13 signaling. This was tested using

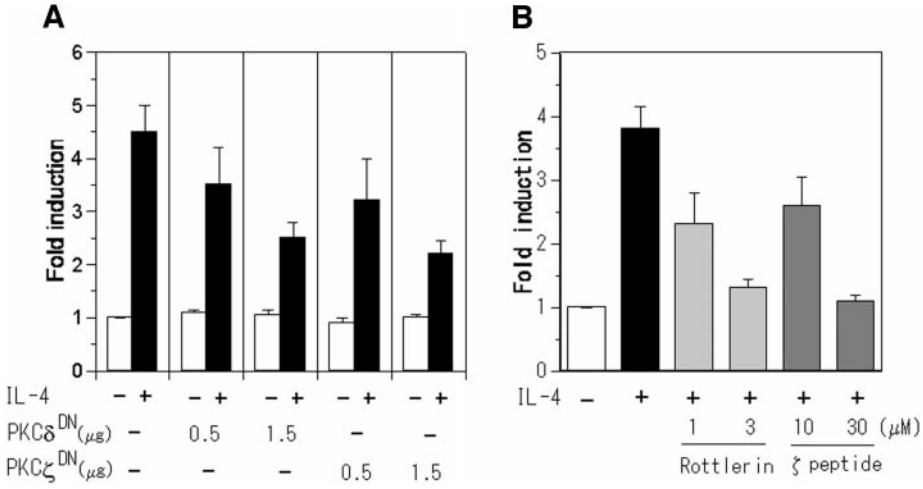

FIG. 2. PKCδ and ζ are required for IL-4-induced germline ϵ transcription. (A) Effects of dominant-negative PKCδ and ζ. DND39 cells were transiently transfected with germline ϵ promoter plasmid (0.5 μg), pRL-SV40 second reporter (40 ng), and indicated amounts of dominant-negative PKC expression plasmid or empty plasmid to keep total amount of DNA constant. After 24 h, the cells were stimulated with IL-4 (5 ng/ml) for an additional 24 h. (B) Effects of isozyme-specific PKC inhibitors. DND39 cells, transiently transfected with germline ϵ promoter plasmid (0.5 μg), were pretreated with rottlerin or myristoylated PKCζ peptide inhibitor for 30 min and then stimulated with IL-4 for 24 h. Luciferase activities are expressed as fold induction over the basal activity of unstimulated cells cotransfected with an empty expression plasmid. Results are mean values and SDs of three independent experiments.

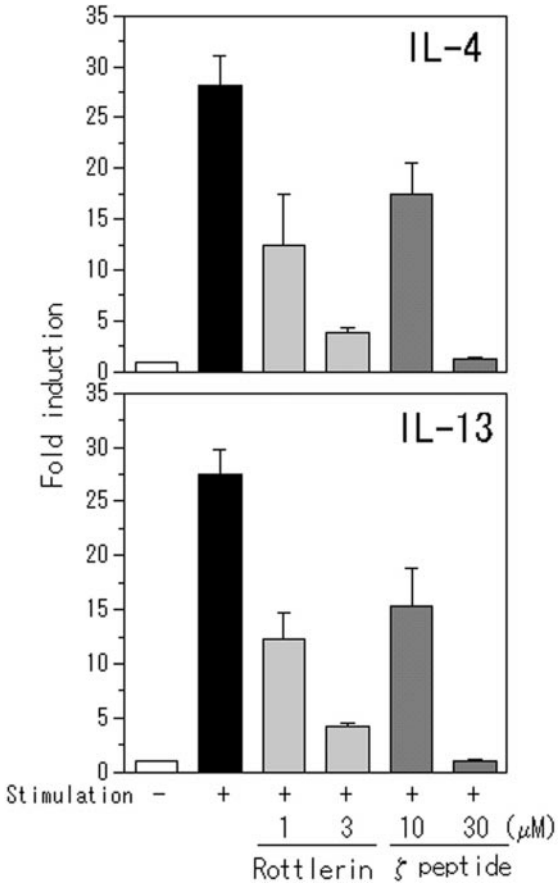

FIG. 3. IL-13-induced germline ϵ transcription is dependent on PKCδ and ζ. DND39/Gϵ/13Rα1 transfectant was pretreated with rottlerin or PKCζ peptide inhibitor for 30 min and stimulated with IL-4 (5 ng/ml) or IL-13 (10 ng/ml) for 24 h. Luciferase activities are expressed as fold induction over the basal activity of unstimulated cells. Results are mean values and SDs of three independent experiments.

DND39 cells stably transfected with the IL-13Rα1 (13R/ϵ/DND39) because this receptor chain is not expressed on parental DND39 cells, as a feature of Burkitt's lymphoma. Optimal concentrations of IL-4 and IL-13 enhanced the germline ϵ promoter activity to a similar extent and these responses were suppressed by rottlerin and PKCζ peptide inhibitor (Fig. 3). The potency of each inhibitor against the IL-4 induction was equal to that against the IL-13 induction.

Germline ϵ Transcription, but Not CD23b mRNA Expression, Is Dependent on PKCδ and ζ in Peripheral Blood B Cells

Next, we determined whether the PKC-dependent IL-4/IL-13 signaling pathway identified in DND39 cells are similarly activated in peripheral blood B cells. Purified B cells were stimulated with IL-4 in the absence or presence of the PKC inhibitors and the expression of germline ϵ mRNA was analyzed by RT-PCR. As shown in Fig. 4, rottlerin at 3 μM and PKCζ peptide inhibitor at 10 μM suppressed the expression of germline ϵ mRNA without affecting the expression of β-actin mRNA. In this experiment, we also examined the expression of CD23b mRNA, another IL-4-inducible gene in human B cells, and found that its expression was unaffected by rottlerin and PKCζ peptide inhibitor. Similar results were obtained with purified B cells stimulated with IL-13 (data not shown). These results suggest that PKCδ and ζ are involved in the induction of germline ϵ mRNA, but not in that of CD23b mRNA, in IL-4- and IL-13-stimulated human B cells.

DNA Binding of Transcription Factors to the Germline ϵ Promoter Are Independent of PKC

Previous studies have revealed that the binding sites for STAT6, C/EBP, NF-κB, and PU.1 are important elements in the human germline ϵ promoter. To examine whether rottlerin and PKCζ peptide inhibitor affect the DNA binding activity of these transcription factors, EMSAs were performed using oligonucleotides containing the binding site for each factor (Fig. 5A). IL-4-induced STAT6 activation, which was confirmed by anti-STAT6, was unaffected by the PKC inhibitors (Fig. 5B). In the experiments with other probes, no IL-4-inducible complex was detected, while there were several constitutive complexes that were unsusceptible to the PKC inhibitors. The complexes with the C/EBP and the PU.1/NF-κB1 motifs contained C/EBPβ and PU.1, respectively, as evidenced by supershifted bands with the specific antibodies. The combination of anti-NF-κB p50 and p65 diminished the major band with the NF-κB2 motif but scarcely affected the band with NF-κB1 motif. Antibodies against c-Rel, RelB and NF-κB p52 had no influence (data not shown). Taken

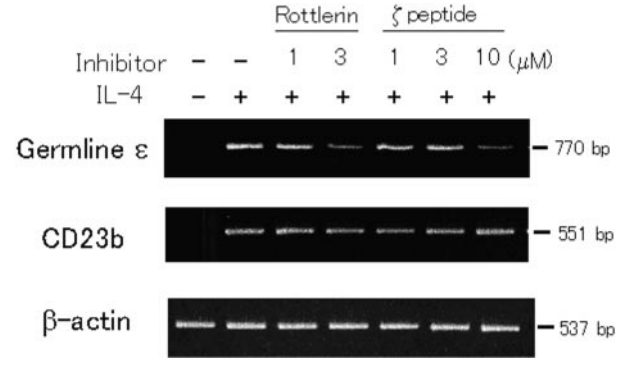

FIG. 4. PKCδ and ζ mediate the expression of germline ϵ mRNA, but not CD23b mRNA, in IL-4-stimulated peripheral blood B cells. Purified B cells were pretreated with rottlerin or PKCζ peptide inhibitor for 30 min and then stimulated with IL-4 (5 ng/ml) for 24 h. Total RNA were extracted and the expression of mRNA for germline ϵ, CD23b and β-actin were analyzed by RT-PCR. Amplified products were subjected to electrophoresis and visualized by ethidium bromide. Similar results were obtained in two other experiments.

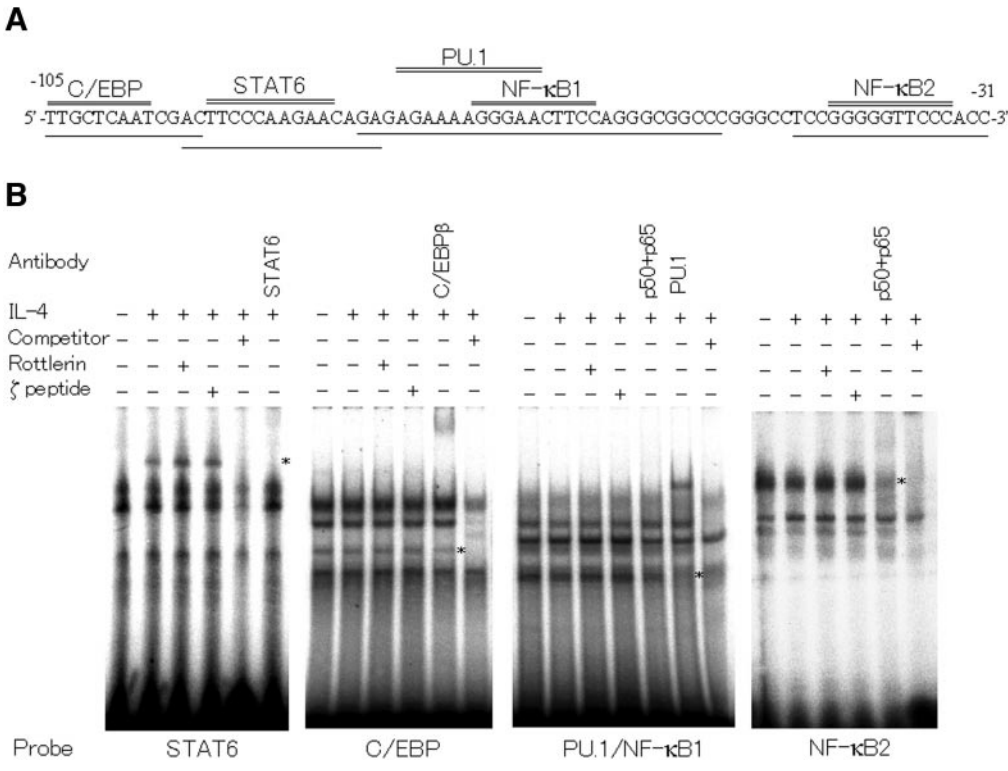

FIG. 5. Effects of rottlerin and PKCζ peptide inhibitor on the binding of transcription factors to regulatory elements in the human germline ϵ promoter. (A) The nucleotide sequence of the human germline ϵ promoter from position -105 to -31. Consensus sequences of *cis*-acting elements are indicated. Locations of oligonucleotides used as probes are underlined. (B) EMSAs of nuclear extracts from DND39 cells stimulated with IL-4 for 1 h in the absence or presence of rottlerin (3 μM) or myristoylated PKCζ peptide inhibitor (30 μM). Antibodies for supershift analysis and unlabeled probes were added as indicated. Asterisks mark the positions of complexes affected by addition of the antibodies.

together, DNA binding activity of STAT6, NF-κB, and PU.1 in IL-4-stimulated DND39 cells were independent of PKCδ and ζ.

Threonine Phosphorylation of PU.1 Induced by IL-4

It has been shown that the transactivation function of PU.1 and C/EBPβ are regulated by the phosphorylation state of serine/threonine residues, in addition to the DNA binding activity (34–37). Thus, immunoprecipitates of PU.1 and C/EBPβ were prepared from the nuclear extracts of DND39 cells and analyzed using anti-phosphoserine and anti-phosphothreonine antibodies. As reported previously (38), the apparent mobility of PU.1 on SDS–PAGE was 40–42-kDa despite the predicted molecular size of 29-kDa. IL-4 stimulation was found to phosphorylate threonine residues of PU.1, which was abrogated by rottlerin and PKCζ peptide inhibitor (Fig. 6). On the other hand, anti-phosphoserine showed no signal (data not shown), although the same monoclonal antibody was reported to be able to detect serine phosphorylation of PU.1 (38). Reprobing with anti-PU.1 indicated that equivalent levels of PU.1, regardless of IL-4 stimulation and of treatment with the inhibitors, were present in the nuclear extracts. The presence of C/EBPβ was also confirmed by immunoblotting, but the phosphorylation of neither threonine nor serine residues could be detected (data not shown).

DISCUSSION

The present study demonstrates that both PKCδ and ζ are involved in IL-4 and IL-13 signaling in human B cells. Based on the previous reports showing that IL-4 hardly affects Ca^{2+} mobilization in human B cells (26, 27, 30), we have focused on Ca^{2+}-insensitive isozymes and found the translocation of these two isozymes in response to IL-4. Their functional involvement was demonstrated by two different approaches; dominant-negative mutants of PKC and isozyme-selective inhibitors, rottlerin, and myristoylated PKCζ pseudosubstrate peptide. These inhibitors have been recently developed and shown to exert an isozyme-selective inhibition (32, 33).

Because both IL-4 and IL-13 signaling are dependent on PKCδ and ζ, it appears that the IL-4Rα chain, a common component of the IL-4R and the IL-13R, is responsible for the activation of these PKC isozymes.

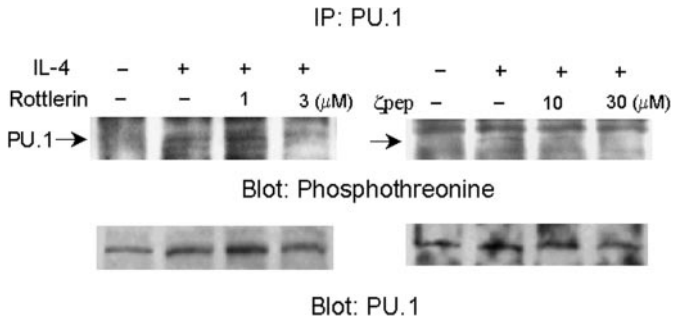

FIG. 6. IL-4 induces threonine phosphorylation of PU.1 in a PKC-dependent manner. DND39 cells were pretreated with rottlerin (3 μM) or PKCζ peptide inhibitor (30 μM) and stimulated with IL-4 for 1 h. Anti-PU.1 immunoprecipitates from nuclear extracts of the cells were probed with biotinylated monoclonal anti-phosphothreonine plus peroxidase-conjugated avidin (upper left panel) or with polyclonal anti-phosphothreonine plus peroxidase-conjugated anti-rabbit IgG (upper right panel). After stripping, the membranes were reprobed with anti-PU.1 antibody (lower panels). Similar results were obtained by both anti-phosphothreonine antibodies and representative data are shown.

Separate sites on the cytoplasmic region of the IL-4Rα chain interact with IRS-1/2 and Fes kinase, and these molecules in turn recruit PI3K. As we have previously reported (21, 29), PI3K inhibitors, wortmannin and LY294002, suppress both the translocation of PKCζ and germline ϵ transcription in IL-4-stimulated DND39 cells, suggesting that PI3K mediates the activation of PKCζ. Similar results have been reported using a murine T cell line (23). On the other hand, the generation of DAG, which is prerequisite for the activation of PKCδ, was shown to be induced by IL-4 not only in DND39 cells but also in normal human B cells and monocytes (18, 26, 39). Whether the roles of these two isozymes in IL-4/IL-13 signaling are distinct or mutually substitutive is not clarified in our study. However, almost complete inhibition of germline ϵ promoter activity by the inhibitor of either isozyme appears to argue against the latter role.

Our data also demonstrated that PKC activity is required for germline ϵ transcription but not for CD23b expression. This result was obtained in peripheral B cells, whereas DND39 cells did not express CD23 even after IL-4 stimulation as is often the case with most transformed B cell lines (data not shown). Considering the fact that both germline ϵ and CD23b gene induction are dependent on STAT6 (40, 41), the failure to inhibit CD23b mRNA expression by rottlerin and PKCζ peptide inhibitor indicates that STAT6 activation is independent of PKCδ and ζ. This is consistent with a recent report by Pesu *et al.* (42) that H7, a broad inhibitor of serine/threonine kinases, blocks IL-4-induced germline ϵ transcription without affecting phosphorylation and DNA binding activity of STAT6.

The germline ϵ promoter contains the binding sites for NF-κB, PU.1 and C/EBP, which cooperate with STAT6 (41, 43–46). In our EMSA analysis, neither rottlerin nor PKCζ peptide inhibitor affected the DNA binding activities of these transcription factors in DND39 cells. But we found the IL-4-induced threonine phosphorylation of PU.1, which was susceptible to these inhibitors. Because it is known that phosphorylation of PU.1 at serine 148, located within a casein kinase II consensus motif, leads to the interaction with another transcription factor (34), phosphorylation at threonine residues might play an analogous role in the transactivation function of PU.1 through the germline ϵ promoter.

In summary, our results show that the induction of germline ϵ transcription depends on the activation of PKCδ and ζ, possibly through the regulation of PU.1. By contrast, CD23b expression does not require PKC activation. Consistent with this, conditional activation of STAT6 is sufficient for CD23 induction in murine B cells (47). Thus, differential regulation of germline ϵ transcription and CD23b expression might be based on a common mechanism in humans and mice.

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