Phospholipase Cε (PLCε), an effector of Ras and Rap small GTPases, plays a crucial role in inflammation by augmenting proinflammatory cytokine expression. This proinflammatory function of PLCε is implicated in its facilitative role in tumor promotion and progression during skin and colorectal carcinogenesis, although their direct link remains to be established. Moreover, the molecular mechanism underlying these functions of PLCε remains unknown except that PKD works downstream of PLCε. Here we show by employing the colitis-induced colorectal carcinogenesis model, where ApcMin mice are administered with dextran sulfate sodium, that PLCε knock-out alleviates the colitis and suppresses the following tumorigenesis concomitant with marked attenuation of proinflammatory cytokine expression. In human colon epithelial Caco2 cells, TNF-α induces sustained expression of proinflammatory molecules and sustained activation of nuclear factor-κB (NF-κB) and PKD, the late phases of which are suppressed by not only siRNA-mediated PLCε knockdown but also treatment with a lysophosphatidic acid (LPA) receptor antagonist. Also, LPA stimulation induces these events in an early time course, suggesting that LPA mediates TNF-α signaling in an autocrine manner. Moreover, PLCε knockdown results in inhibition of phosphorylation of 1κB by ribosomal S6 kinase (RSK) but not by 1κB kinases. Subcellular fractionation suggests that enhanced phosphorylation of a scaffolding protein, PEA15 (phosphoprotein enriched in astrocytes 15), downstream of the PLCε-PKD axis causes sustained cytoplasmic localization of phosphorylated RSK, thereby facilitating 1κB phosphorylation in the cytoplasm. These results suggest the crucial role of the TNF-α-LPA-LPA receptor-PLCε-PKD-PEA15-RSK-1κB-NF-κB pathway in facilitating inflammation and inflammation-associated carcinogenesis in the colon.

Phosphatidylinositol-specific PLCs catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate into two vital second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, which induce release of Ca2+ from the intracellular stores and activation of diacylglycerol target proteins, including PKC isoforms, respectively. The thirteen PLC isoforms were identified in mammals and divided into six classes, β, δ, γ, ε, η, and ζ, based on the structural similarity (1). PLCε was first identified as an effector of Ras small GTPases (2, 3), and further studies revealed that it is also activated by other small GTPases, such as Rap1, Rap2, and Rho, as well as by heterotrimeric G protein Gα12, Gα13, and Gβ1 γ2 subunits (4–7). Stimulation of G protein-coupled receptors with their ligands, such as LPA, SIP, and thrombin, induces PLCε activation (6, 7). PLCε is expressed in non-immune cells, such as epithelial cells and fibroblasts of various tissues, but not in immune cells, such as lymphocytes, granulocytes, macrophages, and dendritic cells (8).

By employing genetically modified mice for PLCε, we demonstrated that PLCε plays a crucial role in inflammation in various tissues; mice homozygous for an allele devoid of the lipase activity (PLCε<sup>Δκ/Δκ</sup> mice) exhibited markedly attenuated inflammatory responses in various animal models, including the phorbol ester-induced dermatitis, contact dermatitis, and bronchial asthma models (8–11), and, moreover, transgenic mice overexpressing PLCε in the skin keratinocytes spontaneously developed chronic skin inflammation resembling human psoriasis (12). Concurrently, PLCε<sup>Δκ/Δκ</sup> mice exhibited marked resistance to tumor formation in the two-stage skin chemical carcinogenesis using a phorbol ester as a promoter as well as to the<sup>Δκ/Δκ</sup> inverse of intestinal carcinogenesis on the Apc<sup>Min/+</sup> (adenomatous polyposis coli) background, which were associated with attenuation of tumor-associated inflammation exemplified by reduced expression of proinflammatory cytokines (13, 14). These findings suggested that PLCε plays a role in tumor promotion through augmentation of inflammatory responses. Recently, strong support for the crucial role of PLCε in human carcinogenesis came from genome-wide association studies,

**Phospholipase Cε Activates Nuclear Factor-κB Signaling by Causing Cytoplasmic Localization of Ribosomal S6 Kinase and Facilitating Its Phosphorylation of Inhibitor κB in Colon Epithelial Cells**

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3 The abbreviations used are: PLC, phospholipase C; LPA, lysophosphatidic acid; SIP, sphingosine-1-phosphate; NF-κB, nuclear factor-κB; IKK, IκB kinase; IκB kinase; RSK, ribosomal S6 kinase; CK2, casein kinase 2; CREB, cyclic AMP-response element-binding protein; TBP, TATA-binding protein; qRT-PCR, quantitative RT-PCR; DSS, dextran sodium sulfate; MPO, myeloperoxidase; Ab, antibody.
Mechanism for Cytokine Induction by Phospholipase Cε

Experimental Procedures

Materials—The following antibodies were obtained from Cell Signaling Technologies: rabbit anti-p65 RelA mAb (catalog no. 8242), rabbit anti-PKD Ab (catalog no. 2052), rabbit anti-phospho-PKD (Ser-916) Ab (catalog no. 2051), rabbit anti-RSK mAb (catalog no. 5528), rabbit anti-phospho-RSK (Ser-380) mAb (catalog no. 11989), rabbit anti-IκB Ab (catalog no. 9242), rabbit anti-phospho-IκB (Ser-32) mAb (catalog no. 2859), rabbit anti-ERK Ab (catalog no. 9102), rabbit anti-phospho-ERK (Thr-202/Tyr-204) Ab (catalog no. 9101), rabbit anti-phospho-IKKα/β (Ser-176/180) mAb (catalog no. 2697), rabbit anti-PEA15 Ab (catalog no. 2780), rabbit anti-phospho-PEA15 (Ser-104) Ab (catalog no. 2776), mouse anti-HA tag mAb (catalog no. 2367), and anti-α-tubulin (catalog no. 3873) Ab. The following antibodies were commercially obtained: rabbit anti-PLCε Ab (HPA015597, Sigma), mouse anti-actin mAb (MAB1501R, Chemicon), rabbit anti-IκB Ab (sc-7607, Santa Cruz Biotechnology, Inc.), rabbit anti-TBP Ab (sc-273, Santa Cruz Biotechnology), rabbit anti-FLAG-tag Ab (PM020, MBL), goat anti-CXCL2 (chemokine C-X-C motif ligand 2) Ab (AF-452-NA, R&D Systems). An anti-PLCe antibody raised against the C-terminal peptide of mouse PLCε was generated in house (34). Recombinant TNF-α (300-01A) was obtained from Peprotech. The following chemicals were commercially obtained: 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate (LPA, Avanti), tetracycl phosphate (LPA receptor antagonist, Cayman Chemical Co.), JTE0013 (SIP receptor antagonist, Cayman Chemical Co.), CID755673 (PKD inhibitor, Millipore), CID2011756 (PKD inhibitor, Tocris Bioscience), U0126 (MEK inhibitor, Calbiochem), Trametinib (MEK inhibitor, Selleckchem), BI-D1870 (RSK inhibitor, Enzo Life Technologies), MG-132 (proteasome inhibitor, Calbiochem), and TBB (CK2 inhibitor, Sigma). Protein G-Sepharose was obtained from GE Healthcare. Chemicals were used according to the manufacturers’ recommendations.

Plasmids—The full-length cDNA of human PLCε was pur chased as Flex ORF clones (Promega) and cloned into pFLAG-CMV2 (Sigma) for expression as FLAG fusions. The lipase-dead human PLCε mutant, PLCεΔX, was generated by PCR-mediated mutagenesis to delete amino acids 1391–1541. pCMV-HA-PEA15 was constructed by inserting the human PEA15 cDNA, obtained by RT-PCR using Caco2 cell mRNA as a template, into pCMV-HA (Clontech). pCMV-HA-PEA15S104A and pCMV-HA-PEA15S104D were derived from it by site-directed mutagenesis. The primers used are listed in Table 1.

Cell Culture—A human colon cancer epithelial cell line Caco2 was purchased from the RIKEN cell bank (RBRC-RCB0988) and maintained in 5% CO2 at 37 °C in modified Eagle’s minimum essential medium supplemented with 20% fetal bovine serum, non-essential amino acids, and 100 μg/ml penicillin-streptomycin. Cells were washed with PBS and serum-starved for 3 h before subjecting to various experiments. Cells were pretreated with inhibitors and antagonists before stimulation by ligands, such as TNF-α and LPA.
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**Transfection of siRNAs and Plasmids**—Caco2 cells (8 × 10^6 cells) were transfected with Stealth siRNA (Life Technologies, Inc.) targeting PLCε (HSS181915, siRNA 1) or its control (12935-400) by electroporation using GenePulser (Bio-Rad) as described before (8). Another siRNA (5′-gcaatactacgacctgcttgt-3′) was also used to target PLCε (13) and PLCγ (see the Jackson Laboratory Web site) were determined by PCR of the tail DNAs. All of the animals were maintained in the animal facility of Kobe University Graduate School of Medicine, and the use and care of the animals were reviewed and approved by the Institutional Animal Care and Use Committee of Kobe University.

**Induction of Colitis and Colitis-induced Colorectal Cancer**—To induce colitis, drinking water containing 2.5% (w/v) DSS (molecular weight = 36,000–50,000; Wako Pure Chemical, Osaka, Japan), dissolved in tap water, was orally administered to 8-week-old mice for 5 days (38, 39). After that, the drinking water was substituted by tap water, and the mice were maintained for 16 days to develop colon tumors.

**Histopathological Classification of Tumors**—Tumors in H&E-stained sections were observed under a microscope and classified into early and late adenomas and adenocarcinomas according to the histopathological criteria recommended for the study of mouse models of intestinal cancer as described (41). The classification was carried out by a pathologist blinded in regard to the mouse genotypes.

**Immunohistochemistry**—The whole colon was rolled up in a “Swiss roll” configuration and embedded in OCT compound for sectioning. After fixation with 4% paraformaldehyde, the sections (10 μm thick) were subjected to treatment with primary antibody and visualized with the secondary antibody conjugated to the fluorophore. The images were taken using a confocal microscopy and ImageJ software.
Statistical Analysis—Data are expressed as the mean ± S.D. An unpaired Student’s t test was performed for determination of p values. In cases where the p values were <0.05, differences were considered to be statistically significant. All of the data were obtained from at least three independent experiments.

Results

Role of PLCε in Inflammation-induced Carcinogenesis—We employed the ApcMin+/+ mouse colitis-induced colorectal carcinogenesis model, where tumor development is dependent on the DSS-induced colon inflammation (38, 39). At first,
Mechanism for Cytokine Induction by Phospholipase Cε

PLCe+/- and PLCeΔX/ΔX mice were orally administered with 2.5% DSS for 5 days to induce acute colitis, the severity of which was evaluated by the body weight loss and histological examination. Compared with PLCe+/- mice, the reduction in the body weight was attenuated in PLCeΔX/ΔX mice (Fig. 1A). Likewise, mucosal erosion and leukocyte infiltration were alleviated in PLCeΔX/ΔX mice (Fig. 1B). In this model, infiltration of neutrophils expressing CXCR2 (CXC chemokine receptor 2) and macrophages expressing CCR2 (CC chemokine receptor 2) is known to play critical roles in the pathogenesis of colitis (42, 43). Indeed, we observed marked increases in the numbers of leukocytes and macrophages in the lesional area as detected by MPO staining and immunostaining for CD68, respectively (Fig. 1C). Marked elevation of CXCR2 and CCR2 mRNAs was observed in the DSS-administered colons of PLCe+/- mice (Fig. 1D). In contrast, the colons of PLCeΔX/ΔX mice exhibited markedly attenuated responses to DSS administration. Moreover, the expression of other inflammation-associated genes, such as Tnfα, Cox-2, Cxcl1, Cxcl2, and Ccl2, showed a substantial increase after DSS administration in PLCe+/- mice, which was also markedly suppressed in PLCeΔX/ΔX mice (Fig. 1D). Intriguingly, CXCL2 and CCL2, the ligands for CXCR2 and CCR2, respectively, were abundantly expressed after DSS administration in the colon epithelial cells expressing PLCe, and this expression was markedly suppressed in PLCeΔX/ΔX mice (Fig. 1, E and F). These results suggested that PLCe might augment colon inflammation by facilitating the secretion from the colon epithelial cells of proinflammatory factors, such as CXCL2 and CCL2, which recruit neutrophils and macrophages, respectively.

We next analyzed the effects of the PLCe genotypes on the colitis-induced colorectal carcinogenesis of ApcMin/+ mice (38, 39). As a result, PLCeΔX/ΔX mice exhibited substantial reduction in the total numbers of colon tumors observed on the 16th day after DSS administration, compared with PLCe+/- mice (Fig. 1G). Histopathological classification of the tumors revealed that the ratios of low grade and high grade adenomas and adenocarcinomas were ~40, 30, and 30%, respectively, in PLCe+/- mice. In contrast, >80% of the tumors were low grade adenomas and essentially no adenocarcinoma was formed in PLCeΔX/ΔX mice. These results taken together gave further support for our notion that PLCe facilitates tumor promotion and malignant progression by augmenting inflammation.

Role of PLCe in NF-κB-dependent Proinflammatory Gene Expression—The molecular mechanism underlying the action of PLCe on the expression of proinflammatory factors was analyzed by using a human colon epithelial cell line, Caco2. Because PLCe had been implicated in the induction of the inflammation-associated genes in response to TNF-α stimulation (8, 11, 17), we first examined the effects of the siRNA-mediated knockdown of PLCe on TNF-α-induced expression of these genes (Fig. 2). In Caco2 cells transfected with an siRNA control, the expression of CXCL8 and CCL2 was elevated in 1–2 h after TNF-α stimulation (Fig. 2A). The transfecion of two distinct siRNAs against PLCe, siRNA 1 and siRNA 2, which efficiently knocked down PLCe expression (Fig. 2B), inhibited the expression of both CXCL8 and CCL2 significantly at the 2-h time point but not effectively at the 1-h time point (Fig. 2A). In addition, the expression of COX-2 showed sustained elevation in 1–2 h, as was the case with CXCL8 (Fig. 2C). The expression of CCL20 was mainly elevated in 2 h only, whereas expression of CXCL1 was mainly elevated in 1 h only. In these cases, too, the transfection of siRNA 1 inhibited the expression at the 2-h time point of COX-2 and CCL20 (Fig. 2C).

TNF-α-induced expression of the inflammation-associated genes, shown here to be regulated by PLCe, was known to be mediated by the NF-κB pathway (44). This led us to examine the effects of PLCe knockdown on the nuclear translocation of p65 (Fig. 3). Nuclear accumulation of p65 was detected by immunofluorescence staining (A) and by semiquantitative RT-PCR (B). The β-actin mRNA was used as a control. Three experiments performed independently yielded equivalent results.
ulation in cells transfected with the control siRNA (Fig. 3B). Transfection of the two PLCε siRNAs lowered the peak at 90 min but not at 10 min. The p65 nuclear localization observed at 30 min seemed to result from the IκB phosphorylation at 10 min, taking account of the time lag between the two events. These results suggested that PLCε might be involved in the late phase of the TNF-α-induced NF-κB activation.

Roles of PKD and LPA Receptor in PLCε-dependent NF-κB Activation—We next examined the role of PKD downstream of PLCε. Treatment of Caco2 cells with the two distinct PKD inhibitors, CID755673 and CID2011756, inhibited the IκB phosphorylation at 90 min but not at 10 min as well as the p65 nuclear translocation at 90 min but not at 30 min after TNF-α stimulation (Fig. 4, A and B). These results were very similar to those obtained with the siRNA-mediated knockdown of PLCε (Fig. 3). Moreover, the Ser-916 phosphorylation of PKD induced by TNF-α stimulation was suppressed by transfection of the two PLCε siRNAs (Fig. 4C), suggesting that PKD might function downstream of PLCε for regulation of the NF-κB activation.

The delayed action of PLCε upon the TNF-α-induced NF-κB activation led us to think of the possibility that another humoral factor might mediate the activation of PLCε induced by TNF-α stimulation. It was reported that TNF-α stimulation of cells induced the production of LPA and S1P (45, 46), which were known to activate PLCε (6, 7). This prompted us to examine the effects of the antagonists of the receptors for LPA and S1P on the TNF-α-induced cytokine expression and NF-κB activation (Fig. 5). The expression of CXCL8 was abrogated by treatment of cells with the LPA receptor antagonist tetradecl phosphonate (Fig. 5A). In contrast, treatment with the S1P receptor antagonist JTE013 seemed to have little effect on the CXCL8 expression at the 2-h time point, although it unexpectedly
exhibited a stimulatory effect even in the absence of TNF-α (Fig. 5A). Presently, we do not have any idea for the mechanism underlying this stimulatory effect. Tetradecyl phosphonate inhibited the IκB phosphorylation at 90 min but not at 10 min as well as the Ser-916 phosphorylation of PKD at 90 min after TNF-α stimulation (Fig. 5B). Furthermore, LPA stimulation of Caco2 cells induced both the IκB phosphorylation and PKD phosphorylation in an early time course of 30 min, which was abrogated by the siRNA-mediated knockdown of PLCε (Fig. 5C). These results strongly suggested that TNF-α stimulation
induces LPA production and subsequent activation of LPA receptors in an autocrine manner, thereby leading to activation of the PLC/H9280-PKD axis.

Role of RSK in PLC/H9280-dependent NF-κB Activation—We next analyzed the molecular mechanism by which PLC/H9280 induced the IκB phosphorylation, depending on the stimulation by TNF-α or LPA. To this end, we tested the involvement of three kinases, IKK, RSK, and CK2, which had been known to phosphorylate IκB at Ser-32 (25–28). The phosphorylation of IKKα/β at Ser-176 and Ser-180, which is responsible for IKK activation (47, 48), was induced in 10 min but not later after TNF-α stimulation (Fig. 6A). This early IKK phosphorylation was unaffected

FIGURE 5. **Role of LPA receptor activation in PLCe-dependent IκB phosphorylation.** A, Caco2 cells were serum-starved for 3 h, treated with 15 μg/ml tetradecyl phosphonate or 10 μg/ml JTE013 for 30 min, and subsequently stimulated with 20 ng/ml TNF-α for the indicated times. The CXCL8 mRNA levels were measured by qRT-PCR with the GAPDH mRNA as an internal control. B, Caco2 cells were treated as described in A except that 10 μM MG-132 treatment for 30 min was included. IκB phosphorylation and PKD phosphorylation were measured as described in the legends to Figs. 3B and 4C, respectively. Actin was detected by the anti-actin Ab. The averages of the intensities of the immunoreactive signals of the indicated phosphoproteins at 90 min of three independent experiments are expressed as the mean ± S.D. (error bars) in arbitrary units (AU) with p values (right). C, Caco2 cells transfected with the indicated siRNAs were cultured for 38–42 h, serum-starved for 3 h, and stimulated with 50 μM LPA for the indicated times. IκB phosphorylation and PKD phosphorylation were measured as described in the legends to Fig. 3B and 4C, respectively. PLCe and actin were detected by immunoblotting with the anti-PLCe and anti-actin Abs, respectively. The averages of the intensities of the immunoreactive signals of the indicated phosphoproteins at 30 min of three independent experiments are expressed as the mean ± S.D. in arbitrary units with p values (right). Three experiments performed independently yielded equivalent results.
by the siRNA-mediated PLCε knockdown and likely to account for the early phase of the IκB phosphorylation directly caused by the TNF-α receptor signaling (Fig. 3B). Because no increase in phosphorylation was observed after 10 min, it is unlikely that IKK is involved in the PLCε-dependent IκB phosphorylation. This led us to test RSK and CK2. The TNF-α-induced CCL8 expression was suppressed by treatment with the RSK inhibitor BI-D1870 but not the CK2 inhibitor TBB (Fig. 6B). In Caco2 cells transfected with the control siRNA, TNF-α stimulation induced elevation of RSK phosphorylation at Ser-380 in 90 min, which was diminished in cells transfected with the PLCε siRNA (Fig. 6C) or pretreated with CID755673 and CID2011756 (Fig. 6D). Moreover, the RSK Ser-380 phosphorylation induced in 10–30 min after LPA stimulation was abrogated by the siRNA-mediated PLCε knockdown (Fig. 6E). In another case, phosphorylation of ERK1/2 at Thr-202 and Tyr-204 was elevated in a similar time course with the RSK phosphorylation and unaffected by the PLCε knockdown. Furthermore, pretreatment with the MEK inhibitors, U0126 (49) and trametinib (50), or the RSK inhibitor BI-D1870 abrogated the IκB phosphorylation at 90 min after TNF-α stimulation (Fig. 6, F and G). The two MEK inhibitors almost completely abolished the RSK phosphorylation, confirming that ERK activation is necessary for the Ser-380 phosphorylation and activation of RSK (29). These results taken together indicated that the PLCε-PDK axis facilitates the phosphorylation and activation of RSK by ERK, leading to the phosphorylation and activation of IκB by RSK. The ERK activation did not seem to be regulated by it.

**Molecular Mechanism for RSK Regulation by PLCε—RSK,** activated through phosphorylation by ERK and PDK1 in the cytoplasm, was located in the cytoplasm and the nuclei, where it phosphorylates IκB and CREB, respectively (26–31), suggesting that blocking the nuclear translocation of RSK might enhance IκB phosphorylation. Thus, we examined whether PLCε affects the subcellular localization of RSK. In control Caco2 cells, the amount of the phosphorylated RSK located in the cytoplasm was increased in 90 min after TNF-α stimulation (Fig. 7A). Notably, transfection of the two distinct PLCε siRNAs markedly reduced the amount of the phosphorylated RSK in the cytoplasm at 90 min, which was accompanied by its increase in the nuclei. Likewise, treatment with CID755673 or CID2011756 also resulted in disappearance from the cytoplasm and nuclear accumulation of the phosphorylated RSK at 90 min (Fig. 7B). Furthermore, overexpression of PLCε, but not the lipase-dead mutant, PLCεΔX, increased the amount of the phosphorylated RSK located in the cytoplasm, decreased the amount of IκB in the cytoplasm, and increased the nuclear translocation of p65 upon stimulation by a low dose of LPA (Fig. 7C), suggesting that the lipase activity of PLCε was involved. These phenomena were suppressed by treatment with CID755673. These results taken together indicated that activation of the PLCε-PKD axis causes preferential localization of the phosphorylated RSK in the cytoplasm, leading to enhancement of the IκB phosphorylation and nuclear translocation of p65.

We next analyzed the molecular mechanism by which PLCε causes preferential cytoplasmic localization of RSK. We focused on PEA15, a scaffold protein for RSK in the cytoplasm, which had been reported to regulate subcellular localization of RSK, depending on the phosphorylation state (32, 33). RSK could be co-immunoprecipitated with PEA15 in Caco2 cells stimulated with LPA, which was markedly enhanced by PLCε overexpression (Fig. 7D). The association of RSK with PEA15 seemed independent of its Ser-380 phosphorylation. Moreover, LPA stimulation elevated the phosphorylation of PEA15 at Ser-104, a putative PKC target site implicated in enhancing the cytoplasmic localization of RSK, which was also enhanced by PLCε overexpression (33) (Fig. 7E). In either case, the phenomenon was effectively inhibited by treatment with CID755673. These results suggested that the Ser-104 phosphorylation of PEA15, induced by the activated PLCε-PKD axis, might enhance its association with the phosphorylated RSK and facilitate its cytoplasmic localization. To test this hypothesis, we analyzed the effects of the mutations, S104A and S104D, of

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**FIGURE 6. Role of RSK in PLCε-dependent IκB phosphorylation.** A, Caco2 cells transfected with the indicated siRNAs were cultured for 38–42 h, serum-starved for 3 h, and stimulated with 20 ng/mL TNF-α for the indicated times. IKKα/β phosphorylated at Ser-176 and Ser-180, total IKKα/β, and actin in the cell lysates were detected by immunoblotting with the anti-phospho-IKKα/β, anti-IKKα/β, and anti-actin Abs, respectively. The numbers below the immunoblots indicate -fold changes of the phospho-IKKα/β signals divided by the total IKKα/β signals over that at 0 min after TNF-α stimulation of the control cells. The averages of the intensities of the immunoreactive signals of phospho-IKKα/β at 90 min of three independent experiments are expressed as the mean ± S.D. (error bars) in arbitrary units (AU) with p values (right). B, Caco2 cells were serum-starved for 3 h, treated with 10 μg/mL BI-D1870 or 28 μg/mL TBB for 30 min, and stimulated with 20 ng/mL TNF-α for the indicated times. The CCL8 mRNA levels were measured as described in the legend to Fig. 5A. C, Caco2 cells were treated with the indicated siRNAs and stimulated with TNF-α as described in A. RSK phosphorylated at Ser-916 and total RSK in the cell lysates were detected by immunoblotting with the anti-phospho-RSK and anti-RSK Abs, respectively. ERK1/2 phosphorylated at Thr-202 and Tyr-204 were detected by immunoblotting with the anti-phospho-ERK and anti-ERK Abs, respectively. PLCε and actin were detected by immunoblotting with the anti-PLCε and anti-actin Abs, respectively. The numbers below the immunoblots indicate -fold changes of the signals of the phosphorylated proteins divided by the signals of the total proteins over those at 0 min after TNF-α stimulation of the control cells. The averages of the intensities of the immunoreactive signals of the indicated phosphoproteins at 90 min of three independent experiments are expressed as the mean ± S.D. in arbitrary units with p values (bottom). D, Caco2 cells were serum-starved for 3 h, treated with 10 μg/mL CID755673 or 50 μM CID2011756 for 30 min, and stimulated with 20 ng/mL TNF-α for the indicated times. The phosphorylations of RSK and ERK1/2 were measured as described in C. The averages of the intensities of the immunoreactive signals of the indicated phosphoproteins at 90 min of three independent experiments are expressed as the mean ± S.D. in arbitrary units with p values (bottom). E, Caco2 cells were serum-starved for 3 h, treated with the indicated siRNAs, and stimulated with 50 μM LPA for the indicated times. The phosphorylations of RSK and ERK1/2 were measured as described in C. The averages of the intensities of the immunoreactive signals of the indicated phosphoproteins at 90 min of three independent experiments are expressed as the mean ± S.D. in arbitrary units with p values (bottom). F, Caco2 cells were serum-starved for 3 h, treated with 10 μg/mL U0126 or 10 μM trametinib in combination with 10 μM MG-132 for 30 min, and stimulated with 20 ng/mL TNF-α for the indicated times. IκB phosphorylation was measured as described in the legend to Fig. 3B, and the phosphorylations of RSK and ERK1/2 were measured as described in C. The averages of the intensities of the immunoreactive signals of the indicated phosphoproteins at 90 min of three independent experiments are expressed as the mean ± S.D. in arbitrary units with p values (bottom).
PEA15. The phosphorylation-mimicking mutant S104D efficiently associated with RSK, including the phosphorylated one, even in the absence of LPA stimulation, whereas the S104A mutant failed to associate with RSK (Fig. 7F). Moreover, over-expression of the S104D mutant enhanced the cytoplasmic localization of the phosphorylated RSK in the absence of LPA stimulation, whereas that of the S104A mutant showed inhibition of the LPA-induced cytoplasmic localization (Fig. 7G). These results further supported the crucial role of the Ser-104 phosphorylation of PEA15, mediated by the PLCe-PKD axis, in holding the phosphorylated RSK in the cytoplasm.

Discussion

In this study, we have shown that PLCe augments inflammatory reactions by facilitating the proinflammatory cytokine expression in the colon administered with DSS. By employing the colitis-induced colorectal carcinogenesis model, we obtained further support for our hypothesis that this proinflammatory function of PLCe is responsible for its crucial role in facilitating tumor promotion and malignant progression. PLCeΔ/Δ mice showed markedly attenuated responses to DSS administration, represented by reduced expression of the proinflammatory molecules, such as TNF-α, COX-2, CXCL1, CXCL2, and CCL2 (Fig. 1D), all of which had been implicated in the pathogenesis of DSS-induced colitis and human inflammatory bowel diseases (42, 43, 51–58). Although this result indicated the involvement of PLCe in augmenting the expression of the proinflammatory molecules, it was difficult to distinguish whether the observed increase of the proinflammatory molecules was the direct consequence of the PLCe activation or the secondary phenomenon accompanying the infiltration of immune inflammatory cells. In this regard, it is of particular interest that CXCR2 ligands, CXCL1 and CXCL2, and a CCR2 ligand, CCL2, were overexpressed in the colon epithelial cells of DSS-administered PLCe+/+ mice (Fig. 1, E and F). Because PLCe is not expressed in immune cells (8), it was likely that the epithelial expression of these chemokines represents one of the direct consequences of the PLCe activation. Because mice carrying knock-out of CXCR2 or CCR2 had been shown to become less susceptible to DSS-induced colitis (42, 43, 51), these results suggested a mechanism whereby PLCe might augment colon inflammation by facilitating the production from the colon epithelial cells of proinflammatory chemokines, such as CXCL2 and CCL2, which recruit neutrophils and macrophages, respectively, to the inflamed sites.

By taking our results obtained with the human colon epithelial cell line Caco2 together, we propose the following model for the molecular mechanism by which PLCe augments the expression of proinflammatory molecules (Fig. 8). TNF-α stimulation induces the early phase of NF-κB activation through phosphorylation and activation of IκB by IκK, leading to the expression of the inflammation-associated genes, such as CXCL1, CXCL8, and COX-2, observed in 1 h (Fig. 2, A and C). Although this phase of the inflammation-associated gene expression seems to be partially abrogated by PLCe knockdown, we presently have no mechanistic explanation except that the PKD-RSK-IκB-NF-κB pathway is not involved. Alternatively, the gene expression observed at the 1-h time point might be significantly contributed by that in the late phase, which will be explained below. In parallel with the early phase of the NF-κB activation, TNF-α stimulation enhances the production and secretion of LPA, which induces activation of PLCe through engagement of its receptors in an autocrine manner. PLCe produces diacylglycerol and thereby induces PKC activation, leading to the activation of PKD through direct binding of diacylglycerol to its C1 domain and phosphorylation by PKC (59). Subsequently, the activated PKD induces phosphorylation of PEA15 at Ser-104. The phosphorylated PEA15 binds to RSK activated through phosphorylation by ERK, which is also activated by the LPA receptor engagement (Fig. 6, C–E). The activated RSK, held in the cytoplasm by association with the phosphorylated PEA15,
phosphorylates IκB and thereby causes the late phase of the TNF-α-induced NF-κB activation, leading to the expression of CXCL8, COX-2, CCL2, and CCL20. CXCL8, whose counter-part is nonexistent in mice, is a ligand of CXCR2, causes neutrophil recruitment, and hence is regarded as a functional homologue of mouse CXCL1 and CXCL2 (60–62). Thus, the PLCε-dependent induction of CXCL8 observed in Caco2 cells may functionally substitute for CXCL2, which is induced in mouse colon epithelial cells in a PLCε-dependent manner after DSS administration. The delayed kinetics of the PLCε-dependent cytokine expression in response to TNF-α stimulation was observed before in dermal fibroblasts and epidermal keratinocytes, which led us to propose that unknown humoral factors might mediate activation of PLCε in an autocrine manner (8). This is consistent with our present observation. The results taken together indicate that the differential actions of IKK and RSK on IκB phosphorylation account for the sustained NF-κB activation and the sustained expression of proinflammatory molecules, which seem to be involved in augmentation of inflammatory reactions and promotion of inflammation-associated carcinogenesis. Several questions, including the following, remain to be addressed experimentally: whether TNF-α stimulation of cells really induces the increased production of LPA as reported (45), by what mechanism PLCε is activated downstream of the LPA receptors, and what kinase is directly responsible for the Ser-104 phosphorylation of PEA15.

The results of our present study may give a new mechanistic insight into not only the pathogenesis of human inflammatory bowel diseases and colorectal carcinogenesis but also of other inflammatory diseases and inflammation-associated carcinogenesis. Moreover, our study suggests that PLCε may become a good candidate target for the development of anti-inflammatory and cancer-preventing drugs.

FIGURE 8. A model for TNF-α-induced expression of the inflammation-associated genes mediated by PLCε. In Caco2 cells, TNF-α stimulation induces the early phase of NF-κB activation via the canonical NF-κB pathway involving IKK, leading to the expression of the inflammation-associated genes (left). Concomitantly, it induces the late phase of NF-κB activation via enhancing the production and secretion of LPA, which activates the PLCε-PKD axis in an autocrine manner and leads to phosphorylation of PEA15. The phosphorylated PEA15 binds to the activated RSK and holds it in the cytoplasm, thereby inducing IκB phosphorylation.

Author Contributions—M. W., H. E., and T. K. designed the study and analyzed the data. M. W. conducted most of the experiments. M. L. and A. E. conducted a part of the experiments. S. K. conducted the histopathological classification. T. K. conceived the idea for the project and wrote the paper with M. W. All of the authors reviewed the results and approved the final version of the manuscript.

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