Received: 11 October 2021 | Revised: 19 April 2022 | Accepted: 25 April 2022

DOI: 10.1111/cas.15386

ORIGINAL ARTICLE

Cancer Science WILEY

Extracellular PKCδ signals to epidermal growth factor receptor for tumor proliferation in liver cancer cells

Kohji Yamada[1] | Ryusuke Kizawa[1] | Ayano Yoshida[1] | Rei Koizumi[1] | Saya Motohashi[1] | Yuya Shimoyama[1] | Yoshito Hannya[1] | Saishu Yoshida[1] | Tsunekazu Oikawa[2] | Masayuki Shimoda[3] | Kiyotsugu Yoshida[1]

[1]Department of Biochemistry, The Jikei University School of Medicine, Tokyo, Japan

[2]Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan

[3]Department of Pathology, The Jikei University School of Medicine, Tokyo, Japan

Correspondence
Kiyotsugu Yoshida, Department of Biochemistry, The Jikei University School of Medicine, 3-25-8 Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan.
Email: kyoshida@jikei.ac.jp

Funding information
Japan Society for the Promotion of Science (KAKENHI), Grant/Award Number: 20K07621, 19H03519, 16K18434, 18K19484, 21K06192, 17H03584, 18K15253, 20H03519; AMED, Grant/Award Number B326TS; The Jikei University Graduate Research Fund; Science Research Promotion Fund

Abstract

Protein kinase C delta (PKCδ) is a multifunctional PKC family member and has been implicated in many types of cancers, including liver cancer. Recently, we have reported that PKCδ is secreted from liver cancer cells, and involved in cell proliferation and tumor growth. However, it remains unclear whether the extracellular PKCδ directly regulates cell surface growth factor receptors. Here, we identify epidermal growth factor receptor (EGFR) as a novel interacting protein of the cell surface PKCδ in liver cancer cells. Imaging studies showed that secreted PKCδ interacted with EGFR-expressing cells in both autocrine and paracrine manners. Biochemical analysis revealed that PKCδ bound to the extracellular domain of EGFR. We further found that a part of the amino acid sequence on the C-terminal region of PKCδ was similar to the putative EGFR binding site of EGF. In this regard, the point mutant of PKCδ in the binding site lacked the ability to bind to the extracellular domain of EGFR. Upon an extracellular PKCδ-EGFR association, ERK1/2 activation, downstream of EGFR signaling, was apparently induced in liver cancer cells. This study indicates that extracellular PKCδ behaves as a growth factor and provides a molecular basis for extracellular PKCδ-targeting therapy for liver cancer.

KEYWORDS
epidermal growth factor receptor, extracellular secretion, liver cancer, PKCδ

1 | INTRODUCTION

Human liver cancer is one of the most common and deadly tumors in the world.[1] Accumulating studies have shown that the major risk factors for liver cancer are well identified, such as nonalcoholic fatty liver disease as well as viral infections.[2] However, the molecular mechanisms of carcinogenesis and tumor progression in liver cancer remain poorly understood.[3]

The PKC family comprise serine/threonine protein kinases, which are generally categorized as conventional (α, βI, βII, and γ), novel (δ, ε, η, and θ), and atypical (ζ, λ/ι) PKC subfamilies.[4,5] These PKC isoforms are commonly comprised of an N-terminal regulatory domain and

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPC3, glypican3; IGF1R, insulin-like growth factor 1 receptor; PKCδ, protein kinase C delta; PMSF, phenylmethylsulphonyl fluoride; rPKCδ, recombinant protein kinase C delta; RTK, receptor tyrosine kinase.

a C-terminal kinase core domain.[5] Although the C-terminal kinase core domain is conserved between PKC isoforms and includes an ATP-binding site, a kinase core along with a substrate-binding site, the N-terminal regulatory domain is much less conserved and contains specific motifs for each isoform that are activated by unique signals.[6,7] Indeed, each isoform differs in structure, function, and biological properties. Especially, PKCδ, one of a novel PKC subfamily, is known to be structurally and functionally affected by a variety of cellular stimuli, including growth factors and DNA damage.[8]

Human PKCδ is ubiquitously expressed in many types of tissues, and has been implicated in the regulation of various cellular processes, including proliferation, migration, and apoptosis as well as tumor promotion and tumor suppression.[9,10] It is well known to be functionally translocated to diverse locations, including the cytosol and nucleus, in response to different stimuli and cell types.[11] After PKCδ is translated by the ribosome at the cytosol, it is generated as an inactive cytosolic form. Similar to other conventional and novel PKC subfamilies, in response to diacylglycerol, PKCδ migrates just below the plasma membrane, which triggers subsequent phosphorylation cascades. For example, PKCδ activation is known to be required for Akt activation by Ras.[12] Protein kinase Cδ is also known to induce ERK1/2 activation.[13,14] These PKCδ pathways are implicated in anchorage-independent growth and resistance of pancreatic ductal cancer cells to apoptotic stimuli.[15] Conversely, cytosolic PKCδ has been reported to induce apoptosis by activating p38 MAPK to inhibit Akt.[11] These findings indicate that PKCδ might also act as a pro-survival or pro-apoptotic factor, depending on its subcellular localization. Furthermore, PKCδ contains a nuclear localization signal in its C-terminal kinase core domain, which is necessary for nuclear proteins to transit across the nuclear pore.[16,17] Nuclear localization of PKCδ is associated with pro-apoptotic functions.[16] However, it has been recently reported that nuclear PKCδ is involved in EGFR-kinase inhibitor resistance in EGFR-activating mutant lung cancer.[18] These results suggest that PKCδ has a diversity in subcellular localization and functions according to tissues and stimuli. Recently, we have revealed that PKCδ is observed at the cell surface and in the extracellular space in liver cancer.[19] Extracellular PKCδ was associated with GPC3, a heparan sulfate proteoglycan highly expressed on the cell surface of liver cancer cells, to activate IGF1R, which consequently contributes to cell proliferation and tumorigenesis.[19] On the contrary, other types of cells, such as normal hepatocytes, did not show extracellular localization of PKCδ, although they express PKCδ in the intracellular area. In addition, treatment with the mAb against PKCδ has shown antitumor effects in liver cancer cells, suggesting that extracellular PKCδ acts in tumor promotion and could be a suitable therapeutic target for liver cancer. However, the impact of extracellular PKCδ on the cell surface is still not well characterized.

In this study, we explored additional receptors activated following extracellular PKCδ treatment in a liver cancer cell line, and found EGFR to be one of the targeting receptors. Extracellular PKCδ was found to bind to the extracellular domain of EGFR, leading to the activation of EGFR signaling. Furthermore, cellular imaging analysis revealed that secreted PKCδ interacted with the surrounding EGFR-expressing cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human liver cancer line HepG2, human embryonic kidney cell line HEK293, human gastric cancer line AGS, and mouse fibroblast NIH3T3 were obtained from the Japanese Collection of Research Bioresources. Tetracycline-inducible HepG2 cells were purchased from Takara in 2018. HepG2, HEK293, and NIH3T3 cells were maintained in DMEM (Sigma) supplemented with 10% FBS (Sigma). AGS cells were maintained in RPMI-1640 (Sigma) supplemented with 10% FBS (Sigma). Tetracycline-inducible HepG2 cells were maintained in α-MEM (Nacalai Tesque) supplemented with 0.1 mM nonessential amino acids, 500 μg/ml G418, and 10% Tet system-approved FBS (Takara).[19] Cell lines were routinely monitored for *Mycoplasma* (4A Biotech Co.). Authentication of the cell lines was confirmed by short tandem repeating profiling every 6 months. The cells used for experiments were passaged within 10 times after thawing.

2.1.1 | Clinical samples

Tumor specimens from liver cancer patients were obtained from Jikei University Hospital. The protocol used in the study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the Jikei University School of Medicine Ethics Review Committee (ethics approval license: 29-038 [8654] and 29-006 [8622]), and written informed consent was obtained from all patients.

2.1.2 | Plasmid constructs and transfection

Protein kinase Cδ, EGFR, GFP, and mCherry cDNA were cloned into the pcDNA3 vector by a HiFi DNA Assembly kit (New England Biolabs). Various deletion and point mutations were introduced by site-directed mutagenesis (Toyobo). Each plasmid construct was transfected using Lipofectamine 3000 (Thermo Fisher Scientific).

2.1.3 | Secreted protein preparation

Cells were maintained in a 0.1% FBS-containing medium for 24 h. After incubation, conditioned media (containing secreted proteins) were collected and subjected to sequential centrifugation: first at 300 *g* for 5 min to remove contaminated cells, then at 1000 *g* for 10 min to remove cell debris. Finally, the media were passed through a Millex 0.22 μm GV filter (Merck Millipore).

2.1.4 | Immunoprecipitation and immunoblotting

Cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1% NP-40) with or without phosphatase inhibitor (10 mM NaF and 1 mM Na_3VO_4). The supernatants were isolated by centrifugation and used as cell lysates. For immunoprecipitation, lysates were resuspended in a washing buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.1% NP-40) with or without phosphatase inhibitor (10 mM NaF and 1 mM Na_3VO_4), and incubated with each Ab or GFP beads for 1 h. The solutions were then incubated with protein A-Sepharose for 2 h. The beads were washed five times in the washing buffer. The lysates and immunoprecipitates were boiled for 10 min, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. The membranes were probed with the following Abs: anti-PKCδ rabbit mAb (1:2000 dilution; Abcam), anti-phospho-EGFR rabbit mAb (1:1000 dilution; Cell Signaling Technology), anti-EGFR rabbit mAb (1:1000 dilution; Cell Signaling Technology), anti-phospho-ERK1/2 rabbit mAb (1:1000 dilution; Cell Signaling Technology), anti-ERK1/2 rabbit mAb (1:1000 dilution; Cell Signaling Technology), anti-actin mouse mAb (1:1000 dilution; Abcam), anti-GFP mouse mAb (1:1000 dilution; Nacalai Tesque), and anti-HA rat mAb (1:1000 dilution; Roche). The signals were detected by enhanced chemiluminescence (Thermo Fisher Scientific).

2.1.5 | Characterization of RTK activity

For analysis of RTK activity in HepG2 cells, the Human Phospho-RTK array kit (ARY001B; R&D Systems) was utilized to profile the RTK activity of the HepG2 cells treated with rPKCδ. Concisely, lysates were extracted using the lysis buffer, incubated overnight over the array, then incubated with HRP detection Ab, and visualized with the chemiluminescent reagent.

2.1.6 | Cell-to-cell PKCδ transmission analysis

Two distinct cells were cocultured and observed using ImageXpress Pico system (Molecular Devices). Images were processed using ImageXpress software to generate analysis mask.

3 | RESULTS

3.1 | Extracellular PKCδ activates EGFR in liver cancer cells

To examine the biological impact of the extracellular PKCδ on liver cancer cells, we performed an antibody array for phosphorylated RTKs using liver cancer cell line HepG2, which secretes PKCδ to the extracellular space (Figure 1A). Lysates of HepG2 cells were

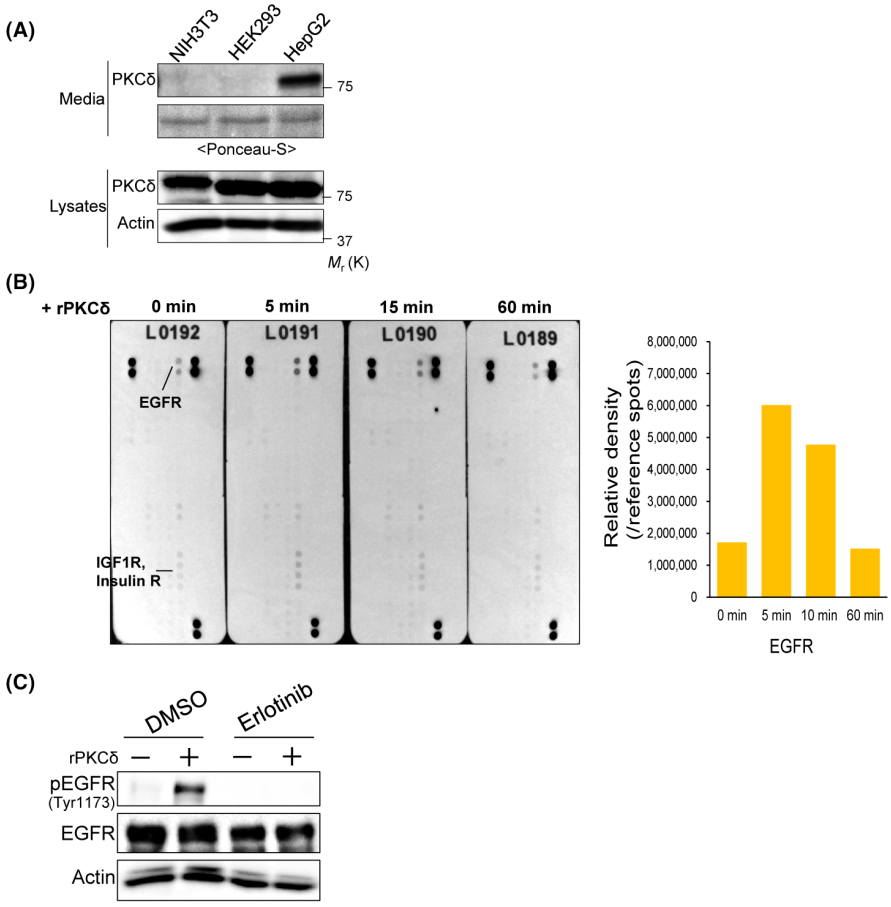

FIGURE 1 Extracellular protein kinase C delta (PKCδ) activates epidermal growth factor receptor (EGFR) in liver cancer cells. (A) Immunoblot analysis of PKCδ in media and lysates of NIH3T3, HEK293, or HepG2 cells. The Abs against PKCδ and actin (positive marker) were used. Ponceau S staining was used as loading control. (B) Human phospho-receptor tyrosine kinase Ab array analysis of starved HepG2 cells, followed by recombinant PKCδ (rPKCδ; 100 ng/ml) treatment for 0, 5, 15, and 60 min. (C) HepG2 cells were pretreated with DMSO or erlotinib (1 μM), followed by rPKCδ treatment for 5 min, and were subjected to immunoblot analysis with phospho-EGFR (pEGFR; Tyr1173), total EGFR, and actin (loading control). IGF1R, insulin-like growth factor 1 receptor

collected after treatment with sufficient rPKCδ (100 ng/ml) for 0, 5, 15, or 60 min, and reacted with each membrane of the Ab array (Figure S1). As shown in our previous results with HEK293 cells,[19] rPKCδ treatment was shown to induce phosphorylation of IGF1R (Figure 1B and Figure S2). Interestingly, we found that EGFR1 (EGFR) was also activated by rPKCδ treatment in HepG2 cells, peaking at 5 min and then decreasing by 1 h to close to the original level (Figure 1B). It has been well known that EGFR is expressed in various tumor tissues, including liver cancer.[18,20] The expression and the rPKCδ-induced activation of EGFR in HepG2 cells was confirmed by western blot analysis (Figure 1C). The activity of other EGFR families was not affected in this phosphorylation array, suggesting that PKCδ might be EGFR-specific (Figure 1B). We further confirmed the rPKCδ-mediated EGFR activation by pretreatment of HepG2 cells with erlotinib, an EGFR inhibitor (Figure 1C), indicating that extracellular PKCδ activates EGFR in liver cancer cells.

3.2 | Protein kinase Cδ binds to the extracellular domain of EGFR

Given that IGF1R activation by rPKCδ treatment has been mediated by the PKCδ-GPC3 interaction,[19] we investigated the involvement of GPC3 expression in activation of EGFR following PKCδ treatment. Knockout of GPC3 had no effect on EGFR activation in response to PKCδ treatment (Figure S3), indicating that GPC3 is not engaged in the activation of EGFR following PKCδ treatment. Next, we further examined the relationship between extracellular PKCδ and EGFR. When we carried out co-immunoprecipitation experiments using HEK293 cells, PKCδ was associated not only with full length of EGFR, but also a mutant lacking the intracellular domain of EGFR (Figure 2A), indicating that PKCδ interacts with the extracellular domain of EGFR. Similar results were obtained by co-immunoprecipitation analysis using HEK293 cells transfected with other Tag-fused constructs (Figure 2B). These data suggested that secreted PKCδ was likely to directly bind to EGFR in the extracellular space.

3.3 | Protein kinase Cδ contains putative EGFR binding sites similar to EGF

To test this hypothesis, we explored the binding region of PKCδ to extracellular EGFR. First, we generated plasmid constructs of several PKCδ deletion mutants, and introduced them into HEK293 cells (Figure 3A). Co-immunoprecipitation analysis showed that the C-terminal kinase core domain of PKCδ (amino acids 330-676; ΔN) interacted with the extracellular domain of EGFR, whereas the association with that of EGFR was abolished when the N-terminal regulatory domain of PKCδ (amino acids 1-329; ΔC) was expressed (Figure 3B), indicating that the C-terminal region of PKCδ was required for the extracellular interaction with EGFR. Next, to determine the amino acid sites in the C-terminal kinase core domain of PKCδ that can

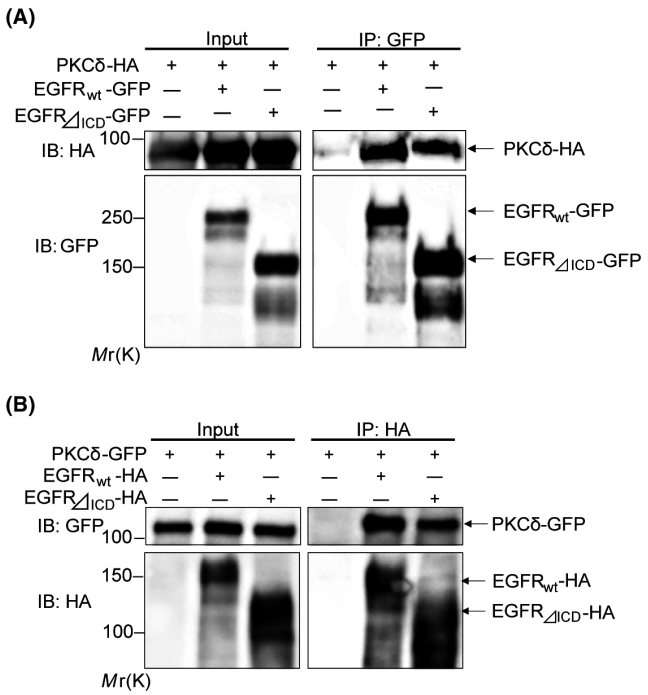

FIGURE 2 Protein kinase C delta (PKCδ) associates with the extracellular domain of epidermal growth factor receptor (EGFR). (A) HEK293 cells were cotransfected with HA-tagged PKCδ and GFP-tagged EGFR containing full-length (wt) or deleted intracellular domain (ΔICD) expression plasmids. GFP beads were used for immunoprecipitation. (B) HepG2 cells were cotransfected with GFP-tagged PKCδ and HA-tagged EGFR containing wt or ΔICD expression plasmids. Anti-HA Ab was used for immunoprecipitation. IB, immunoblot

bind to EGFR, we compared the sequence similarity between PKCδ and EGF, one of the major ligands of EGFR. Among the sequences of EGF, we noticed that PKCδ contained the identical sequences to the putative EGFR binding sites (amino acids 44–48; YRDLK) of EGF, which are common recognition sites for cetuximab, an EGFR-specific inhibitor (Figure 3C).[21] To verify whether this sequence is crucial for the association of PKCδ with EGFR, we generated a plasmid construct of the point mutant of PKCδ, in which three representative amino acids were replaced with alanine (Y471A/L473A/K475A). Co-immunoprecipitation analysis showed that this mutant apparently lacked the ability to bind to EGFR (Figure 3D). These results suggested that PKCδ directly bound to EGFR as a humoral factor.

3.4 | Extracellular PKCδ-dependent EGFR signaling is transmitted to surrounding cells

To investigate whether PKCδ acts as a growth factor after its secretion, we undertook cell-to-cell transport analysis on the secreted PKCδ. We cocultured HepG2 cells expressing GFP-fused PKCδ with HepG2 or NIH3T3 cells expressing mCherry-fused EGFR (1:1), and then detected mCherry-positive and GFP-positive cells (Figure 4A). The results indicated that GFP(+)-mCherry(+) double-positive cells

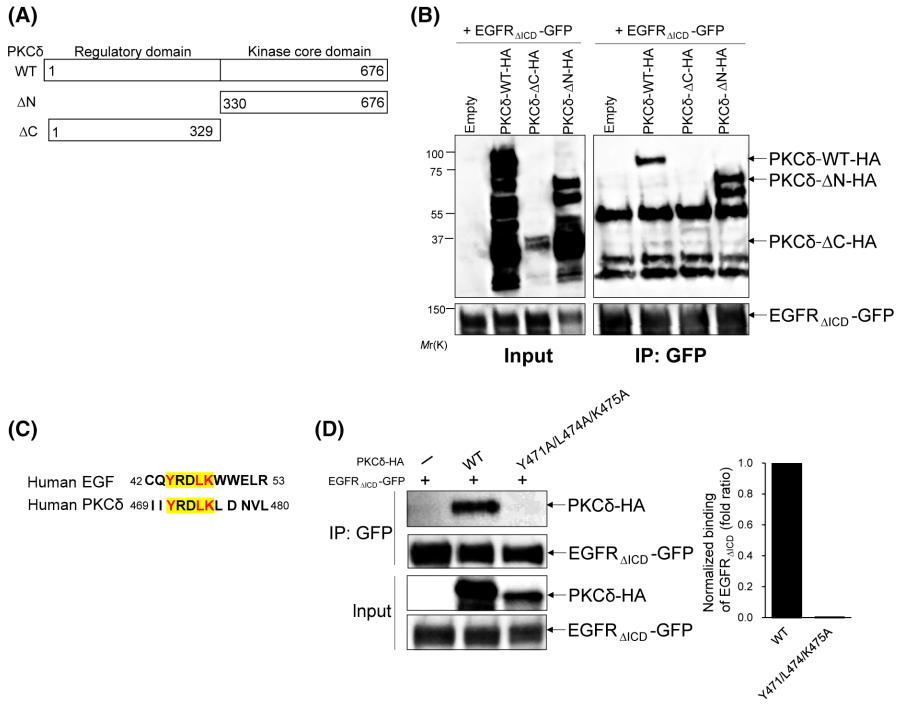

FIGURE 3 Part of the amino acid sequences of protein kinase C delta (PKCδ) is necessary for the association with epidermal growth factor receptor (EGFR). (A) Schematic diagram of the WT, C-terminal cartelistic domain (amino acids 330–676; ΔN), and N-terminal regulatory domain (amino acids 1-329; ΔC) constructs of PKCδ-HA. (B) HEK293 cells were cotransfected with HA-tagged PKCδ and GFP-tagged intracellular domain-deleted EGFR expression plasmids. GFP beads were used for immunoprecipitation. (C) Sequence alignment of human epidermal growth factor (EGF) and human PKCδ. Filled areas indicate 100% identity, and red characters show EGF–EGFR binding sites. (D) HEK293 cells were cotransfected with WT or point mutant (Y471A/L474A/K475A) of HA-tagged PKCδ, and GFP-tagged intracellular domain-deleted EGFR expression plasmids. GFP beads were used for immunoprecipitation (IP). ΔICD, deleted intracellular domain

were observed in both HepG2 (7.5%) and NIH3T3 cells (0.2%) (Figure 4B and Figure S4), suggesting that the secreted PKCδ not only interacted with liver cancer cells in an autocrine manner, but also with surrounding EGFR-positive heterogeneous cells in a paracrine manner. This higher percentage of GFP(+)-mCherry(+) double-positive cells in HepG2 cells might be due to the fact that HepG2 cells highly express additional cell surface receptors, such as GPC3, which can interact with the extracellular PKCδ.[19] We also observed that the GFP(+)-mCherry(+) double-positive cells were located in the vicinity of GFP(+)-only positive HepG2 cells (Figure 4B,C), indicating that PKCδ secretion was likely to affect the perisecretory environment. Furthermore, we confirmed EGFR-positive fibroblasts in tumors of patients with liver cancer (Figure S5).

To further understand the biological significance of PKCδ in communication between cells, we evaluated the activation of downstream signals of EGFR by western blot analysis. According to previous results of intracellular kinase phosphorylation array using HepG2 cells, we found that rPKCδ treatment strongly induced activation of ERK1/2, one of the downstream signaling molecules of EGFR.[19] Therefore, we investigated whether ERK1/2 is activated by rPKCδ treatment in EGFR-expressing cells. Exogenous rPKCδ treatment increased ERK1/2 activation not only in the HepG2 liver cancer cell line, which secretes PKCδ endogenously, but also in other EGFR-expressing cells (AGS and EGFR-transfected NIH3T3 cells), which do not secrete PKCδ (Figures 1A and 4D–F).[19] Taken together, these results indicate that PKCδ acts as a direct agonistic ligand for EGFR after its secretion.

4 | DISCUSSION

On the basis of our current findings, we provide a model demonstrating that PKCδ secretion in the liver cancer microenvironment induces activation of EGFR signaling in an autocrine or paracrine manner. In addition to enhancement of liver cancer cell proliferation and tumorigenesis by extracellular PKCδ,[19] it has been known that EGFR expression is involved in tumorigenesis and tumor progression in various types of cancers, including lung, pancreatic, and liver cancers.[22,23] Taken together, these facts suggest that the PKCδ–EGFR axis could play a critical role in the pathogenesis of liver cancer. Furthermore, our current study observed that secreted PKCδ could interact with a type of stroma cell (fibroblasts), which were increased in EGFR signaling (Figure 4B). Given that tumor-associated endothelial cells also express high levels of EGFR, and the EGFR signaling is required for their proliferation and angiogenesis,[24] we propose a mechanistic insight into the interplay between PKCδ and EGFR in the modulation of the liver cancer microenvironment (Figure 5).

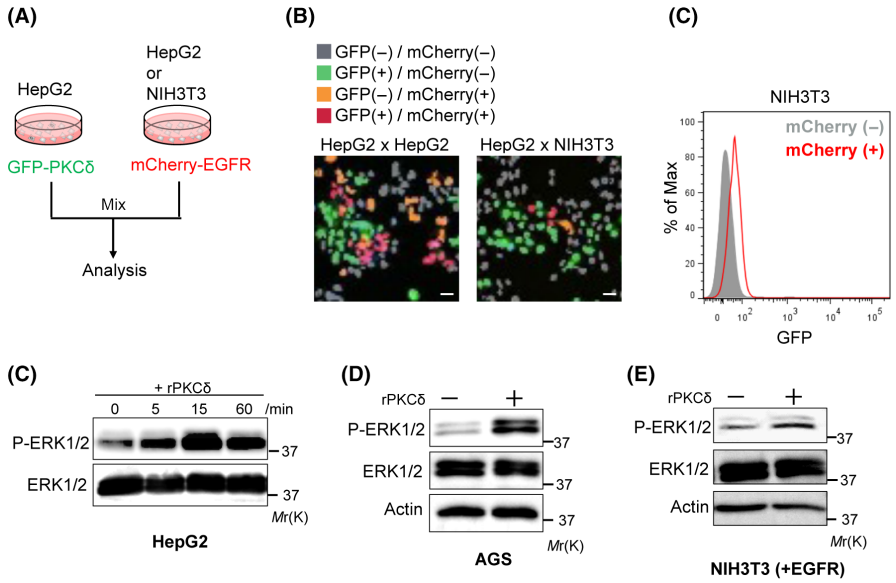

FIGURE 4 Extracellular protein kinase C delta (PKCδ) functions as a growth factor to activate epidermal growth factor receptor (EGFR) signaling. (A) Schematic outline of a mixture experiment of two distinct cell lines. (B) Cell-to-cell PKCδ transmission analysis using the ImageXpress Pico system in co-culture with doxycycline-inducible HepG2 cells expressing PKCδ-GFP and HepG2 (left panel) or NIH3T3 (right panel) cells expressing EGFR-mCherry. Image analysis masks showing GFP(−)-mCherry(−) cells (gray), GFP(+)-mCherry(−) cells (green), GFP(−)-mCherry(+) cells (orange), or GFP(+)-mCherry(+) cells (red). Each cell was analyzed for more than 10,000 cells. Scale bars, 20 μm. (C) Flow cytometric analysis of mCherry-EGFR or mCherry-control-expressing NIH3T3 cells. These cells were treated with conditioned medium of PKCδ-GFP-expressing HepG2 cells for 24 h. (D) Immunoblot analysis of phospho-ERK1/2 (P-ERK1/2) and total ERK1/2 (loading control) in HepG2 cells treated with recombinant PKCδ (rPKCδ) for 0, 5, 15, and 60 min. (E) Immunoblot analysis of P-ERK1/2, total ERK1/2, and actin (loading control) in AGS cells treated with rPKCδ for 15 min. (F) Immunoblot analysis of P-ERK1/2, total ERK1/2, and actin (loading control) in EGFR-overexpressing NIH3T3 cells treated with rPKCδ for 15 min

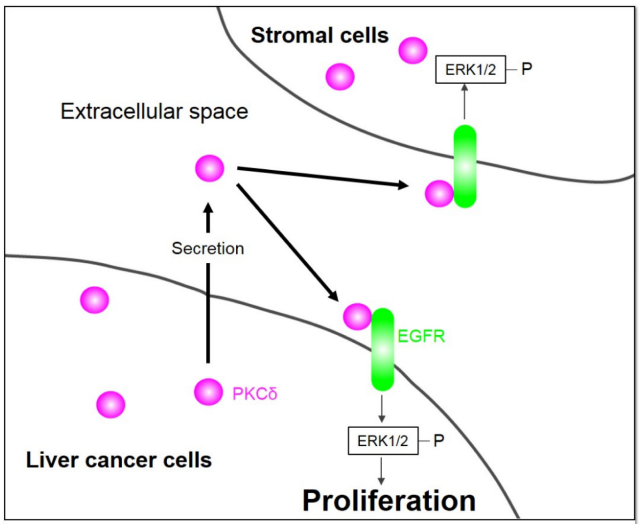

FIGURE 5 Protein kinase C delta (PKCδ)–epidermal growth factor receptor (EGFR) interaction at the cell surface. A model for PKCδ secreted from liver cancer cells to act on surrounding EGFR-positive cells in a paracrine and autocrine manner

We have previously found extracellular distribution of PKCδ.[19] Generally, PKCδ is usually thought of as an intracellular protein because it does not have a signal peptide. Similarly, we and other groups have reported the extracellular presence of importin α1 and nucleolin in several cancer cells.[25,26] An important point is that these extracellular localizations are observed under normal cell culture conditions, which are apparently independent of cell death. In the current study, we also observed that PKCδ, which is expressed in a doxycycline-inducible manner, was delivered to EGFR-positive cells through the extracellular space. Given the fact that these secreted nonsignal peptide molecules are involved in tumor enhancement, such as cell proliferation and angiogenesis,[27] this unconventional secretion system appears to be crucial for tumorigenesis. Indeed, the development of aptamer targeting cell surface nucleolin has been implemented in various diseases such as gastric and renal cancers.[28,29] Together with the evidence that the Ab to PKCδ has antitumor activity in liver cancer,[19] it can be assumed that many drugs targeting unconventional secretory proteins will be developed in the future.

In this study, we found that PKCδ directly bound and activated to one of the RTKs. Our previous work has also found that extracellular PKCδ activates IGF1R, mediated by GPC3.[19] Thus, it is conceivable that extracellular PKCδ is an auxiliary ligand for growth factors that enhances proliferative signaling and contributes to tumorigenesis. Indeed, the extracellular PKCδ enhances proliferative signals such as ERK1/2 and STAT3.[19] Given that PKCδ secretion is specific to liver cancer and is missing in normal hepatocytes and precancerous patients,[19] we speculated that the PKCδ-EGFR-ERK1/2 pathway might play an indispensable role in tumor initiation or progression.

In this current study, we found that PKCδ has a sequence similar to that of EGF, which is important for binding to EGFR (Figure 3).

Although the relationship between EGF and PKCδ, such as the difference in its affinity to associate with EGFR, is not uncovered in the current study, the functional similarity suggests that they mutually complement each other in increasing the proliferation of liver cancer cells. Likewise, there is a possibility that PKCδ binds to EGFR sequences that are quite distinct from EGF, requiring further work to clarify.

Epidermal growth factor receptor is correlated with the promotion of various cancerous tissues, and has been reported to regulate cell proliferation through a variety of biological mechanisms.[30,31] For example, although EGFR typically localizes to the cell surface, it has also been shown that EGFR can be translocated to the nucleus. Nuclear EGFR cooperates with E2F1 to transactivate the *B-MYB* gene in a cell cycle-dependent manner.[32] It has also been found that in pancreatic ductal adenocarcinoma, extracellular human RNase 5 (angiogenin) binds to EGFR, which contributes to tumorigenesis.[33] In view of such functional redundancies, our present study indicated that PKCδ was a novel active ligand for EGFR in liver cancer cells. Taken together with EGFR-activating mutations in other cancer types, such as lung cancer,[34] these findings strongly support that EGFR redundantly regulates cell proliferation. In fact, EGFR tyrosine kinase inhibitors are now considered promising drugs to treat various cancers, such as lung and pancreatic cancers.[35,36] Therefore, in the future, the utilization of EGFR inhibitors could be evaluated for their efficacy in the treatment of liver cancer in accordance with the expression and secretion of PKCδ.

This study showed that EGFR activation is independent of GPC3 expression (Figure S3). The secretion of EGF, a normally secreted protein, appears to be diminished upon nutritional starvation, whereas PKCδ secretion occurs consistently[19] (Figure 1A and Figure S1). Thus, it is likely that EGF is responsible for EGFR activation under a normal nutritional condition, whereas PKCδ activates EGFR during nutrient starvation.

In conclusion, we identified EGFR as a new receptor for extracellular PKCδ, and showed that extracellular PKCδ behaves as its ligand. The fact that PKCδ acts on liver cancer cell membranes to enhance proliferative signaling provides a rationale for the development of drugs targeting extracellular PKCδ for the treatment of liver cancer.

AUTHOR CONTRIBUTIONS

K. Yamada, R. Kizawa, and K. Yoshida contributed to the experimental concept and design and wrote the manuscript. K. Yamada and R. Kizawa contributed to data analysis and interpretation. K. Yamada, R. Kizawa, and K. Yoshida developed the methodology. K. Yamada, R. Kizawa, A.Y., S.M., R. Koizumi, S.Y., Y.S., and Y.H. performed in vitro experiments. T.O. and M.S. obtained and analyzed clinical samples, and K. Yoshida supervised the project.

ACKNOWLEDGMENTS

We thank Ms. N. Tago for secretarial and technical assistance. We also thank Dr. Yamamoto and Dr. Shima for technical assistance in the ImageXpress Pico system. This work was supported by grants from the Japan Society for the Promotion of Science (KAKENHI Grant Numbers 20K07621, 19H03519, 16K18434, and 18K19484 to K. Yamada, 21K06192 to S. Yoshida, 17H03584, 18K15253, and 20H03519 to K. Yoshida), AMED under Grant Number B326TS to K. Yamada., The Jikei University Graduate Research Fund to K. Yamada., and the Science Research Promotion Fund to K. Yoshida.

DISCLOSURE

No potential conflicts of interest were disclosed.

ORCID

Kohji Yamada https://orcid.org/0000-0001-9493-5269
Ryusuke Kizawa https://orcid.org/0000-0001-8558-2190
Saishu Yoshida https://orcid.org/0000-0002-2574-6410
Tsunekazu Oikawa https://orcid.org/0000-0003-1365-5391
Kiyotsugu Yoshida https://orcid.org/0000-0003-3108-7383

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
2. Tateishi R, Okanoue T, Fujiwara N, et al. Clinical characteristics, treatment, and prognosis of non-B, non-C hepatocellular carcinoma: a large retrospective multicenter cohort study. *J Gastroenterol*. 2015;50:350-360.
3. Villanueva A, Newell P, Chiang DY, Friedman SL, Llovet JM. Genomics and signaling pathways in hepatocellular carcinoma. *Semin Liver Dis*. 2007;27:55-76.
4. Ohno S, Nishizuka Y. Protein kinase C isotypes and their specific functions: prologue. *J Biochem*. 2002;132:509-511.
5. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiol Rev*. 2008;88:1341-1378.
6. Gold MG, Barford D, Komander D. Lining the pockets of kinases and phosphatases. *Curr Opin Struct Biol*. 2006;16:693-701.
7. Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem J*. 1998;332(Pt 2):281-292.
8. Parker PJ, Brown SJ, Calleja V, et al. Equivocal, explicit and emergent actions of PKC isoforms in cancer. *Nat Rev Cancer*. 2021;21:51-63.
9. Wetsel WC, Khan WA, Merchenthaler I, et al. Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. *J Cell Biol*. 1992;117:121-133.
10. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer*. 2007;7:281-294.
11. Gomel R, Xiang C, Finniss S, et al. The localization of protein kinase Cdelta in different subcellular sites affects its proapoptotic and antiapoptotic functions and the activation of distinct downstream signaling pathways. *Mol Cancer Res*. 2007;5:627-639.
12. Xia S, Chen Z, Forman LW, Faller DV. PKCdelta survival signaling in cells containing an activated p21Ras protein requires PDK1. *Cell Signal*. 2009;21:502-508.
13. He P, Shen N, Gao G, et al. Periodic mechanical stress activates PKCδ-dependent EGFR mitogenic signals in rat chondrocytes via PI3K-Akt and ERK1/2. *Cell Physiol Biochem*. 2016;39:1281-1294.
14. Yan D, Chen D, Im HJ. Fibroblast growth factor-2 promotes catabolism via FGFR1-Ras-Raf-MEK1/2-ERK1/2 axis that coordinates with the PKCδ pathway in human articular chondrocytes. *J Cell Biochem*. 2012;113:2856-2865.
15. Mauro LV, Grossoni VC, Urtreger AJ, et al. PKC Delta (PKCdelta) promotes tumoral progression of human ductal pancreatic cancer. *Pancreas*. 2010;39:e31-41.

16. Lomonaco SL, Kahana S, Blass M, et al. Phosphorylation of protein kinase Cdelta on distinct tyrosine residues induces sustained activation of Erk1/2 via down-regulation of MKP-1: role in the apoptotic effect of etoposide. *J Biol Chem*. 2008;283:17731-17739.
17. Adwan TS, Ohm AM, Jones DNM, Humphries MJ, Reyland ME. Regulated binding of importin-α to protein kinase Cδ in response to apoptotic signals facilitates nuclear import. *J Biol Chem*. 2011;286:35716-35724.
18. Lee PC, Fang YF, Yamaguchi H, et al. Targeting PKCδ as a therapeutic strategy against heterogeneous mechanisms of EGFR inhibitor resistance in EGFR-mutant lung cancer. *Cancer Cell*. 2018;34:954-969.e954.
19. Yamada K, Oikawa T, Kizawa R, et al. Unconventional secretion of PKCδ exerts tumorigenic function via stimulation of ERK1/2 signaling in liver cancer. *Cancer Res*. 2021;81:414-425.
20. Dong Q, Du Y, Li H, et al. EGFR and c-MET cooperate to enhance resistance to PARP inhibitors in hepatocellular carcinoma. *Cancer Res*. 2019;79:819-829.
21. Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell*. 2005;7:301-311.
22. Avraham R, Yarden Y. Feedback regulation of EGFR signalling: decision making by early and delayed loops. *Nat Rev Mol Cell Biol*. 2011;12:104-117.
23. Komposch K, Sibilia M. EGFR signaling in liver diseases. *Int J Mol Sci*. 2015;17.
24. Amin DN, Hida K, Bielenberg DR, Klagsbrun M. Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. *Cancer Res*. 2006;66:2173-2180.
25. Yamada K, Miyamoto Y, Tsujii A, et al. Cell surface localization of importin α1/KPNA2 affects cancer cell proliferation by regulating FGF1 signalling. *Sci Rep*. 2016;6:21410.
26. Hovanessian AG, Puvion-Dutilleul F, Nisole S, et al. The cell-surface-expressed nucleolin is associated with the actin cytoskeleton. *Exp Cell Res*. 2000;261:312-328.
27. Destouches D, El Khoury D, Hamma-Kourbali Y, et al. Suppression of tumor growth and angiogenesis by a specific antagonist of the cell-surface expressed nucleolin. *PLoS One*. 2008;3:e2518.
28. Soundararajan S, Wang L, Sridharan V, et al. Plasma membrane nucleolin is a receptor for the anticancer aptamer AS1411 in MV4-11 leukemia cells. *Mol Pharmacol*. 2009;76:984-991.
29. Mosafer J, Mokhtarzadeh A. Cell surface nucleolin as a promising receptor for effective AS1411 Aptamer-mediated targeted drug delivery into cancer cells. *Curr Drug Deliv*. 2018;15:1323-1329.
30. Wang YN, Lee HH, Lee HJ, Du Y, Yamaguchi H, Hung MC. Membrane-bound trafficking regulates nuclear transport of integral epidermal growth factor receptor (EGFR) and ErbB-2. *J Biol Chem*. 2012;287:16869-16879.
31. Wang SC, Hung MC. Nuclear translocation of the epidermal growth factor receptor family membrane tyrosine kinase receptors. *Clin Cancer Res*. 2009;15:6484-6489.
32. Hanada N, Lo HW, Day CP, Pan Y, Nakajima Y, Hung MC. Co-regulation of B-Myb expression by E2F1 and EGF receptor. *Mol Carcinog*. 2006;45:10-17.
33. Wang YN, Lee HH, Chou CK, et al. Angiogenin/ribonuclease 5 Is an EGFR ligand and a serum biomarker for erlotinib sensitivity in pancreatic cancer. *Cancer Cell*. 2018;33:752-769.e758.
34. Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol*. 2012;13:239-246.
35. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007;25:1960-1966.
36. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med*. 2005;353:123-132.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Yamada K, Kizawa R, Yoshida A, et al. Extracellular PKCδ signals to epidermal growth factor receptor for tumor proliferation in liver cancer cells. *Cancer Sci*. 2022;113:2378–2385. doi:10.1111/cas.15386