Diacylglycerol Kinase η Augments C-Raf Activity and B-Raf/C-Raf Heterodimerization

Satoshi Yasuda 1,†, Masahiro Kaito, Shin-ichi Imai, Kazuki Takeishi, Akinobu Taketomi, Minoru Toyota, Hideo Kanoh,† and Fumio Sakane‡

From the Department of Biochemistry, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, and the Department of Chemistry, Graduate School of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

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The Ras/B-Raf/C-Raf/MEK/ERK signaling cascade is critical for the control of many fundamental cellular processes, including proliferation, survival, and differentiation. This study demonstrated that small interfering RNA-dependent knockdown of diacylglycerol kinase η (DGKη) impaired the Ras/B-Raf/C-Raf/MEK/ERK pathway activated by epidermal growth factor (EGF) in HeLa cells. Conversely, the overexpression of DGKη could activate the Ras/B-Raf/C-Raf/MEK/ERK pathway in a DGK activity-independent manner, suggesting that DGKη serves as a scaffold/adaptor protein. By determining the activity of all the components of the pathway in DGKη-silenced HeLa cells, this study revealed that DGKη activated C-Raf but not B-Raf. Moreover, this study demonstrated that DGKη enhanced EGF-induced heterodimerization of C-Raf with B-Raf, which transmits the signal to C-Raf. DGKη physically interacted with B-Raf and C-Raf, regulating EGF-induced recruitment of B-Raf and C-Raf from the cytosol to membranes. The DGKη-dependent activation of C-Raf occurred downstream or independently of the already known C-Raf modifications, such as dephosphorylation at Ser-259, phosphorylation at Ser-338, and interaction with 14-3-3 protein. Taken together, the results obtained strongly support that DGKη acts as a novel critical regulatory component of the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade via a previously unidentified mechanism.

The Ras/Raf/MEK/ERK signaling pathway is critical for the transduction of the extracellular signals to the nucleus, regulating diverse physiological processes such as cell proliferation, differentiation, and survival (1, 2). The binding of extracellular ligands, such as growth factors and cytokines, to cell surface receptors activates Ras. The Raf serine/threonine kinase transmits signals from activated Ras to the downstream protein kinases, MEK1 and MEK2, subsequently leading to activation of ERK1 and ERK2.

In mammals, the Raf kinase consists of three isoforms, A-Raf, B-Raf, and C-Raf (Raf-1). It is clinically known that both B-Raf and C-Raf mutations are associated with human cancers (3–5). Knock-out mouse studies demonstrated that each individual Raf isoform has distinct functions, although the three Raf isoforms have high homology in the amino acid sequence (6). The mechanisms underlying C-Raf activation are complicated and thus are not completely understood (3). In response to extracellular signals, C-Raf is initially recruited from cytosol to the plasma membrane and undergo conformational changes by binding directly to the active Ras (7). In addition, other modifications and factors are required for the sufficient activation of C-Raf. For example, dephosphorylation of Ser-259 and phosphorylation of Ser-338, Tyr-341, Thr-491, and Ser-494 are critical for the activation of C-Raf (8–11). Feedback phosphorylation of C-Raf by ERK was also reported to be important for the modulation of C-Raf activity (12, 13). C-Raf activity is regulated by the interaction with 14–3–3 protein (14). Moreover, the heterodimerization of C-Raf with B-Raf, which transmits the signal to C-Raf, has been reported to play an essential role in the activation of the MEK-ERK signaling pathway (15–17). Although B-Raf and C-Raf are the central regulatory components in the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade involved in a variety of pathophysiological events, the activation mechanisms of C-Raf by B-Raf are still unclear.

Diacylglycerol kinase (DGK) catalyzes the phosphorylation of diacylglycerol to generate phosphatidic acid. DGK has been recently recognized as an emerging key regulator in a wide range of cell signaling systems (18–20). To date, 10 mammalian DGK isozymes have been identified. They characteristically contain two or three protein kinase C-like C1 domains and a catalytic region and are subdivided into five subtypes according to their structural features (18–20). Their structural variety and distinct expression patterns in tissues allow us to presume that each DGK isozone has its own biological functions. Indeed, recent studies have revealed that individual DGK isozymes play distinct roles in cell functions through interactions with unique partner proteins such as protein kinase C (21, 22), Ras guanyl nucleotide-releasing protein (23, 24), phosphatidylinositol-4-
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phosphate 5-kinase (25), chimerins (26, 27), AP-2 (28), and PSD-95 (29).

DGK\(\alpha\) belongs to the type II DGKs containing a pleckstrin homology domain at the N terminus and the separated catalytic region (19, 30). Two alternative splicing products of DGK\(\alpha\) have been identified as DGK\(\eta\) and -\(\eta2\) (31). DGK\(\eta2\) possesses a sterile \(\alpha\)-motif (SAM) domain at the C terminus, whereas DGK\(\eta1\) does not. This study demonstrated that the expression levels of DGK\(\eta1\) and -\(\eta2\) were regulated differently by glucocorticoid, and that they were translocated from the cytoplasm to endosomes in response to stress stimuli as osmotic shock and oxidative stress (31). However, the physiological roles of DGK\(\eta\) remain unknown.

This study showed that siRNA-dependent knockdown of DGK\(\eta\) inhibits cell proliferation of the HeLa cells. In addition, DGK\(\eta\) is required for the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade activated by epidermal growth factor (EGF). Intriguingly, DGK\(\eta\) regulates recruitment of B-Raf and C-Raf from cytosol to membranes and their heterodimerization. Moreover, this study demonstrated that DGK\(\eta\) activates C-Raf but not B-Raf in an EGF-dependent manner. The data show DGK\(\eta\) as a novel key regulator of the Ras/B-Raf/C-Raf/MEK/ERK signaling pathway.

EXPERIMENTAL PROCEDURES

Antibodies—To raise anti-DGK\(\eta\) polyclonal antibody, rabbits were immunized by intramuscular multiple injections of 200 \(\mu\)g of glutathione S-transferase (GST) fusion protein containing amino acids 580–736 of human DGK\(\eta\) mixed with adjuvant. The serum obtained after the fourth injection was used. This antibody did not react with human DGK\(\delta\) (data not shown). Anti-DGK\(\delta\) polyclonal antibody was generated as described previously (32). Other antibodies were obtained from commercial sources as follows: anti-FLAG M2 and anti-\(\beta\)-actin (AC-15) antibodies (Sigma); anti-actin and anti-B-Raf antibodies (Santa Cruz Biotechnology); anti-C-Raf (clone 53) antibody (BD Biosciences); anti-Ras (RAS10) antibody (Upstate Biotechnology, Inc.); anti-ERK1/2, anti-phospho-ERK1/2 (Thr-202/ Tyr-204), anti-MEK1/2, anti-phospho-MEK1/2 (Ser-217/221), anti-phospho-C-Raf (Ser-259), and anti-phospho-C-Raf (Ser-338) (56A6) antibodies (Cell Signaling Technology); and anti-14-3-3 antibody (Zymed Laboratories Inc.).

Cell Culture and Transfections—HeLa, COS7, and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 \(\mu\)g/ml) (Invitrogen) at 37 °C in an atmosphere containing 5% CO2. HeLa and HEK293 cells were seeded in 60-mm dishes at a density of 2 \(\times\) \(10^5\) and 4 \(\times\) \(10^5\), respectively. The next day, the cells were transfected with 10 \(\mu\)M of siRNA using LipofectamineTM RNAiMAX (Invitrogen), according to the instructions from the manufacturer. After 72 h of siRNA transfection, the cells were serum-starved for 5 h in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin and stimulated with 100 ng/ml human recombinant EGF (Wako Pure Chemical Industries) for the indicated times. HeLa and COS7 cells (~8 \(\times\) \(10^4\) cells per 60-mm dish) were transiently transfected with 1 \(\mu\)g of plasmid using Effectene (Qiagen), according to instructions from the manufacturer.

siRNAs—To silence the expression of human DGK\(\eta\) (31), DGK\(\delta\) (33), B-Raf, and C-Raf, the following siRNAs were used: DGK\(\eta\) siRNA 1, 5’-CAAGGGAAAUCAUGUUUGGCGGAAA-3’ and 5’-UUUGCCCGCAACAUGAUUCCCUUG-3’ (Invitrogen); DGK\(\delta\) siRNA 2, 5’-CCAAGGCGCUAUGUGGAAAUCUGUAUUAUUCC-3’ and 5’-UUACAGUUCUAACAUGCGCUUCUGG-3’ (Invitrogen); DGK\(\eta\) siRNA 3, 5’-GGACUAGUAGUACCGUGUGAGAAGTCGTCGAAUUAUUCC-3’ (Invitrogen); B-Raf, 5’-GGACAAAGAAAAUGGACUUGGAUAUAAU-3’ and 5’-AUGAUCAGAGAAUCUAUUUGAACCC-3’ (Invitrogen); and C-Raf, 5’-GGUCAAGGUGCGAAGUAGAGC-3’ and 5’-GUCUCAUCAUUCAGCUGACUCACC-3’ (Invitrogen). These siRNA target sequences of B-Raf and C-Raf have been reported previously (34). Stealth RNA interference negative control duplexes (Invitrogen) or negative control siRNA (Qiagen) was used as control.

Plasmid Constructs—p3xFLAG-DGK\(\eta1\) and -DGK\(\eta2\) were generated as described previously (31). Mutations within p3xFLAG-DGK\(\eta1\) were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). An siRNA-resistant DGK\(\eta1\) mutant was constructed by substituting the target sequence of DGK\(\eta\) siRNA 1 to 5’-CAAGGGAGATTATGTTGAGGGCACA-3’ (mutated nucleotides are underlined) without changing the coding amino acids using a PCR-based site-directed mutagenesis method. p3xFLAG-DGK\(\eta\)(-1–328) and p3xFLAG-DGK\(\eta\)(-329–1164) were constructed by inserting PCR fragments encoding amino acids 1–328 and 329–1164, respectively, of human DGK\(\eta1\) into the Sall-BamHI site of p3xFLAG-CMV-7.1 (Sigma). The full-length cDNAs of human DGK\(\eta1\) and DGK\(\eta2\) were amplified by PCR using p3xFLAG-DGK\(\eta1\) and -DGK\(\eta2\), respectively, and subcloned into pCDNA3.1 (Invitrogen) at the site of BamHI-Xhol. pcAcGFP1-DGK\(\eta1\) was constructed by inserting a PCR fragment of DGK\(\eta1\) cDNA into the Sall-BamHI site of pcAcGFP1-C1 (Clontech). pcMV-C-Raf was obtained from Clontech. pcMV-C-Raf-S338D/Y341D/T491E/S494D was generated by PCR-based site-directed mutagenesis. pDs-Red-monomer-C-Raf was constructed by inserting a PCR fragment of C-Raf cDNA into the EcoRI-BamHI site of pDs-Red-monomer-C-Raf (Clontech). pcMV-H-RasV12 and pcMV-H-RasN17 were obtained from Clontech. The full-length cDNAs of H-RasV12 and H-RasN17 were amplified by PCR and inserted into the EcoRI-Sall sites of \(\psi\)ECPF-C1 (Clontech) to generate \(\psi\)ECPF-H-RasV12 and \(\psi\)ECPF-H-RasN17, respectively. Authenticity of the constructs was confirmed by DNA sequencing.

Cell Proliferation Assay—HeLa cells were seeded in 12-well plates at a density of 2 \(\times\) \(10^4\). The next day, the cells were transfected with 10 \(\mu\)M of siRNA. After 0, 48, and 96 h of transfections, the cells were trypsinized. Cells excluding trypan blue were counted using a hemocytometer.

Immunoprecipitation and Western Blot Analysis—The cells were lysed in 500 \(\mu\)l of ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor
mixture (Roche Applied Science)) containing 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, and 1 mM sodium vanadate. The mixture was centrifuged at 12,000 × g for 10 min at 4 °C to obtain the cell lysates. The lysates were precleared by mixing 10 μl of protein G-Sepharose 4 Fast Flow beads (GE Healthcare) for 30 min at 4 °C. The precleared lysates were incubated for 1 h at 4 °C with 1.5 μg of anti-B-Raf antibody, anti-C-Raf antibody, or normal IgG (Santa Cruz Biotechnology), followed by incubation with 10 μl of protein G-Sepharose 4 Fast Flow beads for 1 h at 4 °C. For immunoprecipitation of FLAG-tagged proteins, the cell lysates were incubated for 1 h at 4 °C with 10 μl of anti-FLAG M2 affinity gel (Sigma). For immunoprecipitation of endogenous DGK η, precleared lysates prepared from HEK293 cells were incubated for 2 h at 4 °C with 5 μl of anti-DGK η antibody or preimmune serum, followed by incubation with 10 μl of protein G-Sepharose 4 Fast Flow beads for 1 h at 4 °C. The beads were washed three times with 500 μl of buffer B (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Nonidet P-40) and then boiled in SDS sample buffer. The cell lysates and immunoprecipitants were separated using SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membranes were blocked with 5% low fat milk in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 0.1% Tween 20 for 1 h and incubated with primary antibodies. The immunoreactive bands were visualized with horseradish peroxidase-conjugated anti-mouse or -rabbit IgG antibody (Jackson ImmunoResearch) and ECL Western blotting detection system (GE Healthcare). The detected bands were quantified by densitometric analysis using ImageJ 1.34s.

Affinity Precipitation of Activated Ras—The cells were rinsed with TBS and lysed in 500 μl of ice-cold buffer A containing 5 mM MgCl₂, 1 mM dithiothreitol, and 5% glycerol. After centrifugation at 12,000 × g for 10 min at 4 °C, the supernatant was incubated with 10 μl of Raf-RBD (Ras-binding domain)-GST beads (Cytoskeleton), which selectively interacted with active GTP-bound Ras, for 1 h at 4 °C. The beads were washed three times with 500 μl of buffer B containing 5 mM MgCl₂ and then boiled in SDS sample buffer. The beads were associated with Raf-RBD-GST and total Ras in cell lysates were detected with anti-Ras antibody using Western blot analysis.

In Vitro Raf Kinase Assay—The cells were lysed in 500 μl of ice-cold buffer A containing 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, and 1 mM sodium vanadate, and centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was incubated for 1 h at 4 °C with 1.5 μg of anti-C-Raf or anti-B-Raf antibodies, followed by incubation with 5 μl of protein G-Sepharose 4 Fast Flow beads for an additional hour. The beads were washed three times with 500 μl of buffer B and once with 500 μl of TBS. The immunoprecipitated Raf kinases were incubated in 20 μl of buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 50 μM ATP, and 500 ng of His₆-tagged MEK1-K97R (Upstate Biotechnology) at 30 °C for 20 min. Phosphorylated MEK1-K97R was detected with anti-phospho-MEK1/2 (Ser-217/221) antibody by Western blot.

Cell Fractionation—The cytosol and membrane fractions were isolated using ProteoExtract™ subcellular proteome extraction kit (Calbiochem), according to the manufacturer’s instructions.

Fluorescence Microscopy—HeLa cells grown on type I collagen-coated glass coverslips were transiently transfected with pAcGFP-DGK η, pDsRed-monomer-C-Raf, and either pECFP, pECFP-H-RasV12, or pECFP-H-RasN17. After 24 h of transfection, the cells were serum-starved for 5 h. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min. The coverslips were mounted using Vectashield HardSet (Vector Laboratories). The cells were examined using a confocal laser scanning microscope (LSM510META; Carl Zeiss).

Assay of DGK Activity—The octyl glucoside-mixed micellar assay of DGK activity was performed as described previously (35).

RESULTS

Knockdown of DGK η Inhibits HeLa Cell Proliferation—To determine the roles of DGK η in cell function, initially an attempt was made to knock down the expression of DGK η by transfecting HeLa cells, which are derived from cervical cancer, with siRNA. DGK η-specific siRNA successfully silenced the expression of DGK η protein 48 and 96 h after transfection (Fig. 1A). Of the two DGK η isoforms, DGK η1 was predominantly expressed in HeLa cells, consistent with the mRNA expression patterns of most tissues and tumor-derived cells (31). Intriguingly, knockdown of DGK η in HeLa cells showed a significant
DGK activation of the Ras/B-Raf/C-Raf/MEK/ERK signaling pathway induced by EGF—To elucidate the mechanism by which DGK enhances cell proliferation, EGF-induced ERK1/2 activation in DGK-silenced HeLa cells was subsequently examined. EGF-induced ERK1/2 activation is known to be critical for cell growth. The ERK1/2 activation was assessed by its phosphorylation; EGF stimulation maximally induced phosphorylation of ERK1/2 within 5 min (Fig. 2A). As expected, DGK depletion significantly inhibited the increase in ERK1/2 phosphorylation by ~60% and ~40% after 2 and 5 min of EGF stimulation, respectively. To confirm specific silencing of DGK, another set of DGK siRNA was used. As siRNA 2, which recognizes a DGK sequence different from that of siRNA 1, efficiently inhibited the expression of DGK, EGF-dependent phosphorylation of ERK1/2 was impaired (supplemental Fig. S1A). In addition, DGK depletion by siRNA 3, which also recognizes a DGK sequence different from those of siRNAs 1 and 2, also inhibited ERK1/2 phosphorylation induced by EGF (supplemental Fig. S1B). These results confirm that the down-regulation of ERK1/2 activation is because of the specific silencing of DGK expression. In addition to HeLa cells, DGK knockdown by siRNA also inhibited EGF-induced ERK1/2 activation in HEK293 cells derived from human embryonic kidney cells (supplemental Fig. S1C), indicating that the ERK activation by DGK is not an event restricted to one cell line.

To verify the positive effect of DGK on ERK activity, complementation experiments were performed. DGK-silenced HeLa cells were transiently transfected with a plasmid encoding wild-type DGK1 (DGK siRNA-resistant cDNA), followed by stimulation with EGF. As shown in Fig. 2B, wild-type DGK1 successfully compensated for a defect in ERK1/2 activation induced by DGK depletion, further supporting a critical role of DGK in the regulation of the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade.

To examine whether the catalytic activity of DGK plays a key role in promoting cell growth, DGK1 activates the Ras/B-Raf/C-Raf/MEK/ERK signaling pathway induced by EGF. To elucidate the mechanism by which DGK enhances cell proliferation, EGF-induced ERK1/2 activation in DGK-silenced HeLa cells was subsequently examined. EGF-induced ERK1/2 activation is known to be critical for cell growth. The ERK1/2 activation was assessed by its phosphorylation; EGF stimulation maximally induced phosphorylation of ERK1/2 within 5 min (Fig. 2A). As expected, DGK depletion significantly inhibited the increase in ERK1/2 phosphorylation by ~60% and ~40% after 2 and 5 min of EGF stimulation, respectively. To confirm specific silencing of DGK, another set of DGK siRNA was used. As siRNA 2, which recognizes a DGK sequence different from that of siRNA 1, efficiently inhibited the expression of DGK, EGF-dependent phosphorylation of ERK1/2 was impaired (supplemental Fig. S1A). In addition, DGK depletion by siRNA 3, which also recognizes a DGK sequence different from those of siRNAs 1 and 2, also inhibited ERK1/2 phosphorylation induced by EGF (supplemental Fig. S1B). These results confirm that the down-regulation of ERK1/2 activation is because of the specific silencing of DGK expression. In addition to HeLa cells, DGK knockdown by siRNA also inhibited EGF-induced ERK1/2 activation in HEK293 cells derived from human embryonic kidney cells (supplemental Fig. S1C), indicating that the ERK activation by DGK is not an event restricted to one cell line.

To verify the positive effect of DGK on ERK activity, complementation experiments were performed. DGK-silenced HeLa cells were transiently transfected with a plasmid encoding wild-type DGK1 (DGK siRNA-resistant cDNA), a kinase-dead mutant where Gly-392 is replaced with Asp. It was confirmed that DGK activity of the G392D mutant plasmid encoding wild-type DGK1 successfully compensated for a defect in ERK1/2 phosphorylation induced by DGK depletion, further supporting a critical role of DGK in the regulation of the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade.

To examine whether the catalytic activity of DGK is required for EGF-induced activation of ERK1/2, DGK-knockdown HeLa cells were transiently transfected with a plasmid encoding wild-type DGK1 (DGK siRNA-resistant cDNA), a kinase-dead mutant where Gly-392 is replaced with Asp. It was confirmed that DGK activity of the G392D mutant was less than 1% of the wild-type control, measured by in vitro assay using their immunoprecipitants (data not shown). Unexpectedly, the G392D mutant exhibited almost the same effect as the wild-type enzyme (Fig. 2B). Although the possibility that subtle phosphatidic acid produced by DGK is involved in ERK1/2 activation by EGF cannot be excluded, this study supports the model that DGK activates the ERK1/2 signaling pathway by acting as a scaffold/adaptor protein.

To identify a target molecule(s) of DGK in the Ras/B-Raf/C-Raf/MEK/ERK pathway, the effects of DGK silencing on EGF-induced phosphorylation of upstream kinases of ERK1/2, MEK1/2 was subsequently examined. Phosphorylation of MEK1/2 reached a maximum by 2 min after EGF stimulation and returned to almost a basal level within 10 min (Fig. 3). Depletion of DGK also inhibited MEK1/2 phosphorylation by EGF.
With DGK \eta, the constitutively active form of Ras, is expressed in the cells. Transfection increased 2 min after EGF stimulation (Fig. 4A). However, depletion of DGK\eta did not affect MEK1/2 activation by RasV12 (supplemental Fig. S2). Collectively, these results strongly suggest that DGK\eta specifically regulates the MEK/ERK pathway downstream of Ras.

\textbf{DGK\eta Is Required for EGF-dependent Activation of C-Raf but Not B-Raf—}B-Raf and C-Raf fulfill the criteria that a molecule, the target component of DGK\eta obtained earlier, acts downstream of Ras as well as upstream of MEK in the Ras/B-Raf/C-Raf/MEK/ERK cascade. To observe the effects of DGK\eta on Raf kinase activities, endogenous C-Raf and B-Raf in the lysates from DGK\eta-silenced HeLa cells were immunoprecipitated with anti-C-Raf and B-Raf antibodies, respectively, and used for \textit{in vitro} Raf kinase assay. EGF clearly induced the activation of C-Raf within 2 min after stimulation (Fig. 5A). Interestingly, DGK\eta silencing remarkably inhibited C-Raf kinase activities prepared from EGF-stimulated cells. In contrast, the knockdown of DGK\eta did not affect B-Raf activities (Fig. 5B). MEK phosphorylation by the Raf kinases \textit{in vitro}, but not the endogenous MEK phosphorylation that was carried out, was verified by abolishing the phosphorylation caused by withdrawal of ATP from the assay mixtures. These experiments demonstrate that DGK\eta is indispensable for the activation of C-Raf, but not for B-Raf, in response to EGF.

\textbf{DGK\eta Activates C-Raf Downstream or Independently of Ras—}C-Raf Interaction, C-Raf Phosphorylation, Feedback C-Raf Phosphorylation by ERK, and C-Raf Interaction with 14-3-3 Protein—C-Raf activity is known to be regulated by five pathways as follows: (a) interaction of C-Raf with activated Ras (7, 37); (b) phosphorylation at Ser-338, Tyr-341, Thr-491, and Ser-494, and dephosphorylation at Ser-259 in C-Raf (8–11); (c) interaction of C-Raf with 14-3-3 protein (14, 38); (d) feedback phosphorylation of C-Raf by ERK (12, 13); and (e) heterodimerization of C-Raf with B-Raf (15–17). Thus, the next step was to determine the pathways that are involved in the DGK\eta-dependent C-Raf activation.

First, this study examined if the DGK\eta-dependent C-Raf activation is regulated by Ras–C-Raf interaction (7, 37). Immunoprecipitation experiments showed that DGK\eta depletion did not affect the binding of RasV12 to C-Raf (supplemental Fig. S3), indicating that DGK\eta regulates the C-Raf activity downstream of the active Ras-dependent activation.

Activity of C-Raf is known to be regulated by phosphorylation. Dephosphorylation at Ser-259 and phosphorylation at Ser-338, Tyr-341, Thr-491, and Ser-494 play critical roles in C-Raf activation in response to growth factors (3). A test was conducted to determine whether DGK\eta influences the C-Raf phosphorylation by EGF. However, DGK\eta depletion by siRNA did not significantly affect the C-Raf phosphorylation of Ser-259 (data not shown) or Ser-338 (supplemental Fig. S4A). C-Raf phosphorylation at Tyr-341, Thr-491, or Ser-494 could not be detected with available phosphoantibodies even in cells stimulated with EGF. This could probably be due to their limited levels of phosphorylation. As mutations of Ser-338 to Asp/Tyr-341 to Glu/Ser-494 to Asp (DDED) in C-Raf were reported to mimic their phosphorylation and confer constitutive activity (10), we alternatively examined the effects of DGK\eta depletion on MEK1/2 phosphorylation induced by tran-
sient expression of the C-Raf-DDED mutant in HeLa cells. The C-Raf-DDED mutant remarkably elevated MEK1/2 phosphorylation without any stimulation (supplemental Fig. S4B). However, the effects of DGK\textsubscript{\eta} depletion on MEK1/2 activation were clearly detected even when the C-Raf-DDED mutant was expressed. These results strongly suggest that DGK\textsubscript{\eta} activates C-Raf downstream or independently of the phosphorylation of these residues.

The 14-3-3 proteins were repeatedly reported to interact with and regulate C-Raf (14, 38). Although the interaction of C-Raf with 14-3-3 proteins in DGK/H9257-depleted cells was examined, no significant difference in the interaction was observed with or without EGF stimulation (supplemental Fig. S5). Thus, it has been suggested that DGK\textsubscript{\eta} modulates C-Raf activity downstream or independently of the interaction between C-Raf and the 14-3-3 protein.

Recent studies have shown that feedback of C-Raf phosphorylation by ERK modulates C-Raf activity and that treatment with U0126, a MEK inhibitor, inhibited hyperphosphorylation of C-Raf (12, 13). The effect of U0126 on the in vitro activity of C-Raf isolated from EGF-stimulated HeLa cells was tested. U0126 did not affect the in vitro C-Raf activity 2 min after EGF stimulation despite a strong inhibition of ERK1/2 phosphorylation (supplemental Fig. S6), indicating that feedback C-Raf phosphorylation by ERK is not involved in DGK/H9257-dependent activation of C-Raf under the conditions employed.

DGK\textsubscript{\eta} Enhances EGF-induced Heterodimerization of B-Raf and C-Raf—Recent studies have reported that active Ras and growth factor stimulation induce heterodimerization of B-Raf and C-Raf (15, 17). Heterodimerization of B-Raf and C-Raf exhibits a potent Raf kinase activity in vitro (17). B-Raf, which is biochemically purified as an activator of C-Raf (39), behaves as an upstream activator of C-Raf (16, 40). Finally, it was examined whether DGK\textsubscript{\eta} influences a complex formation between B-Raf and C-Raf. To this end, endogenous C-Raf in cell lysates was immunoprecipitated with anti-C-Raf antibody, and co-immunoprecipitated B-Raf was detected using Western blot. Upon stimulation with EGF, endogenous B-Raf was clearly co-precipitated (Fig. 6A). Interestingly, the depletion of DGK\textsubscript{\eta} by siRNA significantly inhibited the complex formation between C-Raf and B-Raf. Consistent with these findings, DGK\textsubscript{\eta} knockdown was also observed to inhibit heterodimerization of B-Raf and C-Raf in cells that transiently expressed RasV12 (supplemental Fig. S7).

The next attempt was to confirm whether B-Raf, in addition to C-Raf, plays an essential role in MEK1/2 phosphorylation in HeLa cells by silencing its expression using siRNA. A B-Raf-specific siRNA successfully knocked down the expression of B-Raf in HeLa cells (Fig. 6B). Depletion of B-Raf remarkably
inhibited MEK1/2 phosphorylation induced by EGF, as observed in DGK\eta-knocked down cells, supporting that B-Raf is required for DGK\eta-dependent activation of C-Raf. It was verified that the silencing of C-Raf also significantly inhibited EGF-dependent MEK1/2 phosphorylation. Taken together, these results strongly suggest that DGK\eta regulates C-Raf activity, at least partly, through controlling the heterodimerization between C-Raf and B-Raf.

**DGK\eta Interacts with B-Raf and C-Raf**—Earlier experiments showed that DGK\eta regulates heterodimerization between B-Raf and C-Raf (Fig. 6 and supplemental Fig. S7). Moreover, it was previously suggested that DGK\eta activates the ERK1/2 signaling pathway by acting as a scaffold/adaptor protein (Fig. 2B). Therefore, we determined whether DGK\eta physically interacts with B-Raf and C-Raf. To this end, 3xFLAG-tagged DGK\eta1 was transiently expressed in HeLa cells and immunoprecipitated with anti-FLAG antibody. When DGK\eta1 was immunoprecipitated, endogenous B-Raf and C-Raf were apparently co-precipitated (Fig. 7A). As their interaction did not show any dependence on EGF stimulation or RasV12 expression (data not shown), DGK\eta1 may stably interact with B-Raf and C-Raf in cells. To examine the region of DGK\eta1 that is responsible for the interaction with C-Raf, HeLa cells were transfected with the plasmids encoding the 3xFLAG-tagged N- or C-terminal half of DGK\eta1 (Fig. 7B). After immunoprecipitation, the C-terminal half of DGK\eta1 (DGK\eta1-(329–1164)) interacted with C-Raf as strongly as the full-length of DGK\eta1, whereas the N-terminal half of DGK\eta (DGK\eta1-(1–328)) almost failed to interact with C-Raf (Fig. 7C). These immunoprecipitation experiments revealed that the C-terminal half of the DGK\eta1 containing catalytic domains are responsible for the interaction between DGK\eta1 and C-Raf. Further deletion analysis showed that DGK\eta1 interacts with C-Raf via multiple sites in the C-terminal half (data not shown). The regions of DGK\eta1 responsible for their weak interaction with B-Raf could not be accurately determined. To confirm that DGK\eta1 is physiologically associated with C-Raf, interaction of endogenous DGK\eta1 with C-Raf in HEK293 cells was examined. Consistent with the results obtained from transfected cells, endogenous C-Raf was slightly but clearly co-immunoprecipitated with DGK\eta1 (Fig. 7D).

**DGK\eta Enhances Recruitment of B-Raf and C-Raf to Membranes Induced by EGF**—As the translocation of B-Raf and C-Raf to membranes where their upstream factor, Ras, is activated is thought to be an important step to associate with each other and activate C-Raf (16), it was necessary to determine whether DGK\eta knockdown affects the subcellular localizations of B-Raf and C-Raf. A cell fractionation assay showed that EGF stimulation apparently recruited both B-Raf and C-Raf to membrane fractions (Fig. 8A). DGK\eta silencing by siRNA sig-

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**FIGURE 5.** **DGK\eta is required for kinase activation of C-Raf in response to EGF but not B-Raf.** HeLa cells were transfected with control siRNA or DGK\eta siRNA 1. After 72 h, the cells were serum-starved for 5 h and stimulated with 100 ng/ml EGF for 2 min. Endogenous C-Raf and B-Raf in the cell lysates were immunoprecipitated with anti-C-Raf antibody (A) and anti-B-Raf antibody (B), respectively. A, C-Raf kinase activities of the immunoprecipitants were measured in vitro using His\alpha-tagged MEK1-K97R as a substrate in the presence or absence of ATP. Phosphorylation of His\alpha-tagged MEK1-K97R was analyzed by Western blot (WB) using anti-phospho-MEK1/2 antibody. Upper panels, representative results of Western blot analysis are shown. Bottom panel, C-Raf activities were measured in the presence of ATP, and visualized bands of phospho-MEK1 were quantified by densitometry. C-Raf activities of the cells that were transfected with control siRNA and stimulated with EGF for 2 min were set to 100. The data are shown as means ± S.D. of three independent experiments. B, B-Raf kinase activities of immunoprecipitants were measured and quantified using the same methods as the C-Raf activity assay described in A. Upper panels, representative results of Western blot analysis are shown. Bottom panel, B-Raf activities of the cells that were transfected with control siRNA and stimulated with EGF for 2 min were set to 100. The data are shown as means ± S.D. of three independent experiments.
significantly inhibited translocation of both B-Raf and C-Raf from cytosol to membranes after EGF stimulation. The recruitment of both B-Raf and C-Raf was also seen in cells transiently expressing RasV12 (Fig. 8B). Consistent with EGF stimulation, DGK\(\eta\) depletion by siRNA significantly inhibited recruitment of both B-Raf and C-Raf to membranes induced by RasV12 expression. Activated Ras is known to recruit B-Raf and C-Raf to the plasma membrane, leading to activation of the Raf kinases (41). However, as shown in supplemental Fig. S3, immunoprecipitation experiments showed that DGK\(\eta\) depletion did not affect the binding of RasV12 to C-Raf. The binding of B-Raf to RasV12 could not be detected, because the levels of RasV12 interacting with endogenous B-Raf were too low to detect in Western blot. These results suggest that DGK\(\eta\) enhances, at least, the recruitment of C-Raf to membranes in a Ras-C-Raf interaction-independent manner.

Subcellular localization of DGK\(\eta\) was analyzed. In the absence of dominant-active Ras (RasV12), DGK\(\eta\) was located in the cytoplasm (Fig. 8C). On the other hand, RasV12 enhanced translocation of DGK\(\eta\) from the cytoplasm to the plasma membrane and concomitant co-localization of DGK\(\eta\) with the active Ras and C-Raf. However, dominant-negative Ras (RasN17) did not show such effects. Taken together, these results imply that, in addition to an interaction between C-Raf and active Ras, the translocation of DGK\(\eta\) carries at least DGK\(\eta\)-associated C-Raf to the plasma membrane where active Ras and B-Raf are located. Reciprocally, DGK\(\eta\) can also convey B-Raf to the membrane in which active Ras and C-Raf exist.

DISCUSSION

The Ras/B-Raf/C-Raf/MEK/ERK signaling cascade is essential for the transduction of extracellular signals to the nucleus, regulating a wide variety of pathophysiological processes such as cell proliferation, differentiation, and survival and carcinogenesis (1, 2). This study demonstrated for the first time that DGK\(\eta\) is a critical component in the Ras/B-Raf/C-Raf/MEK/ERK signaling pathway activated by EGF in HeLa cells (Fig. 9). In addition to HeLa cells derived from cervical cancer, DGK\(\eta\) critically controlled the EGF-induced ERK1/2 activation in HEK293 cells derived from human embryonic kidney cells also (supplemental Fig. S1C), indicating that the ERK pathway regulation by DGK\(\eta\) is not an event restricted to one cell line. DGK\(\eta\) transcripts were clearly detectable in most of the normal tissues (31). Therefore, these results may lend support to the view that DGK\(\eta\) regulates a wide variety of physiological events including cell growth (e.g. see Fig. 1) through controlling the
expression was detected in 8 of 10 stomach cancers compared with the adjacent noncancerous stomach. Thus, it can be speculated that, in addition to proliferation of normal cells, DGK η may play an important role in pathological processes, development, and/or progression of human cancers, including stomach cancer, via regulating the Ras/B-Raf/C-Raf/MEK/ERK signaling pathway.

B-Raf and C-Raf are the pivotal regulatory components in the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade (3). Recent studies have reported that heterodimerization of B-Raf and C-Raf is required to exhibit a potent Raf kinase activity (17). Interaction of C-Raf with wild-type B-Raf occurs at the plasma membranes, which is dependent on active Ras (16). In addition, B-Raf activates C-Raf, but C-Raf does not activate B-Raf. However, the molecular mechanism of their heterodimerization is poorly understood. In this study, it was demonstrated that DGK η is a novel critical activator of C-Raf but not B-Raf (Fig. 5). Moreover, DGK η enhanced B-Raf-C-Raf heterodimer formation (Fig. 6A), which is essential for C-Raf activation. Moreover, DGK η physically interacted with B-Raf and C-Raf (Fig. 7). Thus, the data reveal DGK η to be a novel key regulator of B-Raf-C-Raf heterodimer formation and subsequent C-Raf activation. Moreover, because B-Raf is essential for the DGK η-dependent C-Raf activation (Fig. 6B), it is strongly suggested that DGK η at least in part regulates C-Raf activity through controlling the heterodimerization between C-Raf and B-Raf. The B-Raf-C-Raf heterodimer formation was reported to be regulated by interaction of C-Raf with 14-3-3 protein (17). However, knockdown of DGK η failed to affect the interaction of C-Raf with 14-3-3 (supplemental Fig. S5), indicating that the DGK η-dependent augmentation of B-Raf-C-Raf heterodimerization occurs independently or downstream of the 14-3-3 protein binding to C-Raf. Rheb, a member of the Ras/Rap/Ral subfamily of Ras proteins, was recently reported to inhibit C-Raf activity and heterodimerization of B-Raf and C-Raf (42). In contrast, DGK η is required for the activation of C-Raf and heterodimerization. Thus, it is possible that DGK η and Rheb reversely regulate each other. However, it is unlikely that DGK η is involved in the Rheb-dependent C-Raf activation mechanism, for the following reasons. (a) Rheb interacts with only B-Raf, but not with C-Raf to which DGK η is bound (Fig. 7). (b) Rheb regulates C-Raf phosphorylation at Ser-338, which is not affected by DGK η knockdown (supplemental Fig. S4). (c) Rheb inhibits kinase activity of B-Raf but not C-Raf, which is regulated by DGK η (Fig. 5).

Expression of DGK η could compensate for ERK phosphorylation impaired by DGK η-siRNA treatment, independent of the catalytic activity of DGK η (Fig. 2B). It is difficult to rule out the possibility that subtle phosphatidic acid produced by DGK η influences the activation of the ERK pathway, because the catalytic activity that remained slightly in the DGK η-G392D mutant may introduce effective levels of phosphatidic acid by its overexpression. It is likely that DGK η could serve as a scaffold/adaptor protein. Indeed, DGK η is physically bound to B-Raf and C-Raf (Fig. 7). These observations support a para-

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digem that regulation of C-Raf activation is controlled within the assembled signaling complex composed of B-Raf, C-Raf, and DGK\(\eta\). Hence, it appears that DGK\(\eta\) does not simply perform the mandated task of converting diacylglycerol to phosphatidic acid, both signaling lipids, but unexpectedly function as a scaffold/adaptor protein bridging the integral members of the MEK/ERK pathway, B-Raf, and C-Raf. As DGK\(\eta\) obviously exhibits a catalytic activity (31), we speculate that, in addition to the function of scaffold/adaptor protein, DGK\(\eta\) has an unknown primary function via its catalytic action. If the catalytic activity-dependent function is explored, then DGK\(\eta\) may become a new nexus/crossroad linking the Ras/B-Raf/C-Raf/MEK/ERK cascade and an unidentified signaling lipid (diacylglycerol and/or phosphatidic acid)-regulated pathway.

DGK\(\eta\) positively regulates the EGF-induced translocation of B-Raf and C-Raf from cytosol to membranes (Fig. 8). The DGK\(\eta\)-dependent translocation is likely to contribute to the heterodimer formation processes, because heterodimerization has been reported to occur at the plasma membrane (16), where their upstream regulator/interaction partner, Ras, is activated. DGK\(\eta\) was associated with B-Raf and C-Raf, and the interactions were not affected by EGF stimulation. Thus, translocation of DGK\(\eta\) to the plasma membrane and its co-localization with active Ras (Fig. 8C) are likely to enhance translocation of at least DGK\(\eta\)-associated C-Raf. Because active Ras and B-Raf are located on the plasma membrane, the concomitant translocation of DGK\(\eta\)-associated C-Raf to the plasma membrane may facilitate heterodimerization of C-Raf with B-Raf. However, the possibility that DGK\(\eta\) has other effects on B-Raf and C-Raf, such as induction of their conformational changes and enhancements of interactions with their regulatory proteins, cannot be denied.

FIGURE 8. DGK\(\eta\) is necessary for recruitment of B-Raf and C-Raf to membranes induced by EGF and active Ras. HeLa cells were transfected with control siRNA or DGK\(\eta\) siRNA 1. A, after 72 h, the cells were serum-starved for 5 h and stimulated with 100 ng/ml EGF for 2 min. B, after 24 h of transfection with siRNAs, the cells were transiently transfected with either pcDNA3.1 or pCMV-H-RasV12. After 48 h of transfection with the expression plasmids, the cells were serum-starved for 5 h. The cytosol and membrane fractions were isolated using ProteoExtract subcellular proteome extraction kit. Each fraction (10 \(\mu\)l) was analyzed by Western blot (WB) using anti-B-Raf and anti-C-Raf antibodies. Upper panels, representative results of Western blot analysis are shown. Bottom panel, intensity of the each band was quantified by densitometry, and the percentages of B-Raf and C-Raf in the membrane fractions are shown as the means ± S.D. of three independent experiments. Statistical significance was determined using Student’s t test (*, \(p < 0.05\); **, \(p < 0.01\)). C, co-localization of DGK\(\eta\) with RasV12 and C-Raf. HeLa cells grown on type I collagen-coated glass coverslips were co-transfected with plasmids encoding AcGFP1-tagged DGK\(\eta\)1, DsRed-monomer-tagged C-Raf, and either ECFP, ECFP-tagged H-RasV12, or ECFP-tagged H-RasN17. After 24 h of transfection, the cells were serum-starved for 5 h. The cells were fixed with 4% paraformaldehyde and observed by confocal laser scanning microscopy. ECFP-, AcGFP1-, and DsRed-monomer fusion proteins are shown in blue, green, and red, respectively. Scale bar, 10 \(\mu\)m.
DGK\(\eta\) is known to consist of two splice variants, DGK\(\eta_1\) and \(\eta_2\). DGK\(\eta_2\) possesses a SAM domain at its C terminus but not DGK\(\eta_1\). The SAM domain is responsible for homo- and hetero-oligomerization of DGK\(\eta_1\) and DGK\(\eta_2\) (31, 43). Unfortunately, as the expression levels of DGK\(\eta_2\) were considerably low in complementation experiments, it was not possible to evaluate whether the SAM domain is essential for DGK\(\eta_1\)-dependent activation of the ERK pathway. However, the SAM domain does not seem to contribute to the interaction of DGK\(\eta_1\) with C-Raf because both the DGKs, \(\eta_1\) and \(\eta_2\), interacted with C-Raf to almost the same extent in the immunoprecipitation assays (data not shown). DGK\(\eta_1\) was detected in most of the normal tissues and all the examined tumor-derived cells, including HeLa cells (Fig. 1A) (31). On the other hand, DGK\(\eta_2\) was expressed in limited tissues. As the Ras/B-Raf/C-Raf/MEK/ERK pathway is a central signaling cascade in most of the tissues and cell lines (1-3), the results imply that DGK\(\eta_1\) is mainly responsible for the regulation of the Ras/B-Raf/C-Raf/MEK/ERK signaling pathway.

DGK\(\delta\) possessing pleckstrin homology and SAM domains also belongs to type II DGKs containing DGK\(\eta\). Analysis using DGK\(\delta\) knock-out mice revealed that knock-out of DGK\(\delta\) attenuates EGF receptor (EGFR) phosphorylation by protein kinase C, resulting in degradation and impaired signaling of EGFR (22). In this study, DGK\(\eta_1\) is also observed to regulate EGFR signaling by activating C-Raf but not EGFR itself (Fig. 6A). Thus, \(\delta\)- and \(\eta\)-isoforms belonging to the same type of DGK (type II) control the same signaling pathway, the EGFR/Ras/B-Raf/C-Raf/MEK/ERK cascade. However, they modulate different sites, EGFR and C-Raf, in the pathway. DGKs, \(\delta\) and \(\eta\), utilize different control mechanisms by consuming a protein kinase C activator, diacylglycerol, in a catalytic activity-dependent manner and by acting as a scaffold/adaptor protein in a catalytic activity-independent manner, respectively. It is therefore evident that DGK isoforms, even in the same subgroup, regulate important cell signal transductions via a variety of mechanisms.

The results of this study reveal a previously unappreciated role for DGK\(\eta\) and expanded the repertoire of DGK function to the scaffold/adaptor protein. The identification of DGK\(\eta_1\) as a novel key regulator of heterodimerization of C-Raf and B-Raf (Fig. 9) may help to elucidate the activation mechanism of C-Raf by B-Raf, which is poorly understood at present, despite its importance in a wide variety of pathophysiological processes, such as cell proliferation and tumorigenesis. Further study on the mechanism underlying the Raf signaling regulated by DGK\(\eta_1\) is needed to better understand the complex regulation of an important signaling pathway, the Ras/B-Raf/C-Raf/MEK/ERK cascade.

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