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Increased SPHK2 Transcription of Human Colon Cancer Cells in Serum-Depleted Culture: The Involvement of CREB Transcription Factor

Naoki Mizutani,[1] Yukari Omori,[1] Koji Tanaka,[1] Hiromi Ito,[1] Akira Takagi,[1] Tetsuhito Kojima,[1] Masahiro Nakatochi,[2] Hideo Ogiso,[3] Yoshiyuki Kawamoto,[4] Mitsuhiro Nakamura,[5] Motoshi Suzuki,[6] Mamoru Kyogashima,[7] Keiko Tamiya-Koizumi,[1] Yoshinori Nozawa,[8] and Takashi Murate[1]*

[1]*Department of Pathophysiological Laboratory Science, Nagoya University Graduate School of Medicine, Nagoya, Japan*

[2]*Bioinformatics Section, Center for Advanced Medicine and Clinical Research, Nagoya University Hospital, Nagoya, Japan*

[3]*Department of Hematology, Kanazawa Medical University, Kanazawa, Japan*

[4]*College of Life and Health Sciences, Chubu University, Kasugai, Japan*

[5]*Department of Drug Information, Gifu Pharmaceutical University, Gifu, Japan*

[6]*Division of Molecular Carcinogenesis, Nagoya University Graduate School of Medicine, Nagoya, Japan*

[7]*Department of Microbiology and Molecular Biology, Nihon Pharmaceutical University, Saitama, Japan*

[8]*Tokai Gakuin University, Kakamigahara, Japan*

ABSTRACT

Sphingosine kinases (SPHK) are important to determine cells′ fate by producing sphingosine 1-phosphate. Reportedly, exogenous SPHK2 overexpression induces cell cycle arrest or cell death. However, the regulatory mechanism of SPHK2 expression has not been fully elucidated. Here, we analyzed this issue using human colon cancer cell lines under various stress conditions. Serum depletion (FCS(-)) but not hypoxia and glucose depletion increased mRNA, protein and enzyme activity of SPHK2 but not SPHK1. In HCT116 cells mostly used, SPHK2 activity was predominant over SPHK1, and serum depletion increased both nuclear and cytoplasmic SPHK2 activity. Based on previous reports analyzing cellular response after serum depletion, the temporal changes of intracellular signaling molecules and candidate transcription factors for SPHK2 were examined using serum-depleted HCT116 cells, and performed transfection experiments with siRNA or cDNA of candidate transcription factors. Results showed that the rapid and transient JNK activation followed by CREB activation was the major regulator of increased SPHK2 transcription in FCS(−) culture. EMSA and ChIP assay confirmed the direct binding of activated CREB to the CREB binding site of 5′ SPHK2 promoter region. Colon cancer cells examined continued to grow in FCS(−) culture, although mildly, while hypoxia and glucose depletion suppressed cell proliferation or induced cell death, suggesting the different role of SPHK2 in different stress conditions. Because of the unique relationship observed after serum depletion, we examined effects of siRNA for SPHK2, and found the role of SPHK2 as a growth or survival factor but not a cell proliferation inhibitor in FCS(−) culture. J. Cell. Biochem. 116: 2227–2238, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: HUMAN COLON CANCER CELL LINES; SERUM DEPRIVATION; SPHK2; TRANSCRIPTIONAL REGULATION; JNK ACTIVATION; CREB TRANSCRIPTION FACTOR

Abbreviations: SPHK, sphingosine kinase; FCS (−), culture serum depleted culture; QRT-PCR, quantitative reverse transcription-polymerase chain reaction; EMSA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation; S1P, sphingosine 1-phosphate; LC-MS/MS, liquid chromatography followed by tandem mass-spectrometry; GUSB, beta glucuronidase.

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*Correspondence to: Takashi Murate, Department of Pathophysiological Laboratory Science, Nagoya University Graduate School of Medicine, Daiko-minami, 1-1-20 Higashi-ku, Nagoya, Aichi 461-8673, Japan. E-mail: murate@met.nagoya-u.ac.jp

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Sphingolipid has been regarded as an important intra- or intercellular-signaling molecule, which regulates various aspects of cellular function including cell survival, cell proliferation, cell death, differentiation, migration, and inflammation [Hannun and Obeid, 2008; Maceyka et al., 2009]. Among them, ceramide, sphingosine, and sphingosine 1-phosphate (S1P) play a major role, and the sphingolipid rheostat model has been recently hypothesized, proposing that the balance between cellular ceramide, sphingosine, and S1P determines the cell's fate [Pitson, 2011]. Although many enzymes are involved in the sphingolipid metabolism, the sphingosine kinase (SPHK), the only enzyme to produce S1P, is of particular interest. SPHK has two isozymes, SPHK1 and SPHK2. The expression and activity of SPHK1 are regulated by heterogeneous extracellular stimuli and have been extensively studied. The activation process of SPHK1 has recently been reviewed [Alemany et al., 2007], revealing that SPHK1 stimulated cell survival, proliferation, and migration mostly through cell surface S1P receptors [Rosen et al., 2009]. Thus, SPHK1 has been widely regarded as the appropriate target of anti-cancer or anti-inflammatory drugs [Maceyka et al., 2005].

In contrast, investigation of SPHK2 is limited. SPHK2 enzyme activity is stimulated by a number of agonists including EGF, TNFα, IL-1β, and phorbol ester and also crosslinking of the IgE receptor FcεRI [Hait et al., 2005; Mastrandrea et al., 2005; Olivera et al., 2006]. Both SPHKs catalyze the same reaction generating S1P and dihydrosphingosine 1-phosphate, and share regions of high sequence similarity of the catalytic domain and the domains important for ATP and sphingosine binding. However, SPHK2 possesses additional regions at its N-terminus and in the middle of its sequence [Alemany et al., 2007]. Intriguingly, previous reports of SPHK2 have revealed some contradictory results. For example, in some reports, artificial overexpression of SPHK2 induced cell cycle arrest or cell death [Igarashi et al., 2003; Maceyka et al., 2005; Okada et al., 2005], whereas others showed cell proliferative and cancer promoting function of SPHK2 in vivo using mouse tumor models and anti-cancer drug resistance function of SPHK2 in cancer cells [Nemoto et al., 2009; Antoon et al., 2011; Gao and Smith, 2011].

Endogenous SPHK2 expression of cancer cells seems to be cell-type specific. We have recently reported the heterogeneous expression levels of SPHK2 in human colon cancer and mouse cancer cell lines [Nemoto et al., 2009; Mizutani et al., 2013]. SPHK2 is localized in both the nucleus and cytoplasm [Engel et al., 2012]. The cellular localization (cytoplasmic and nuclear) of SPHK2 differs, for example, between HeLa and HEK293 cells [Igarashi et al., 2003]. Moreover, Nakamura's group has reported its shuttling property under different conditions using tagged SPHK2 cDNA overexpression [Igarashi et al., 2003; Okada et al., 2005; Ding et al., 2007]. The apparent discrepancies in the role of SPHK2 might be due to difference of these factors described above. Further elucidation of the regulatory mechanism of SPHK2 expression is helpful for better understanding its physiological roles.

In the present study, we analyzed the changes of SPHK2 expression under different cellular stresses using human colon cancer cell lines exhibiting heterogeneous SPHK2 expression. Interestingly, among three different cellular stresses (serum depletion, hypoxia, and glucose depletion), serum depletion was found to increase SPHK2 expression significantly in all cell lines tested. Our analysis showed that increased SPHK2 after serum depletion was due to transcriptional activation and that activated JNK followed by CREB activation was the major regulatory mechanism of increased SPHK2 expression after serum depletion.

Serum depletion has been reported to induce growth arrest of human glioblastoma cell line, T98G [Canhoto et al., 2000]. It has recently been reported that nuclear SPHK2 inhibits HDAC activity followed by p21 (the cell cycle inhibitor) re-expression and cell growth arrest in MCF-7 breast cancer cells treated with PMA [Hait et al., 2009]. We examined the role of increased SPHK2 by serum depletion on cell survival and proliferation using siRNA for SPHK2, and found that SPHK2 inhibition suppressed cell proliferation of serum-depleted colon cancer cell lines, suggesting cell-type specific role of SPHK2.

MATERIALS AND METHODS

CELL CULTURE

Four human colon cancer cell lines, HCT116, RKO, DLD-1, and HT29 were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) as described before [Nemoto et al., 2009]. The cell lines were analyzed in serum-depleted condition (cultured in DMEM without FCS), hypoxic condition using Personal CO_2/Multigas incubator APM-30D (Astek KK. Fukuoka, Japan) (1% O_2), or glucose-depleted condition (cultured with 10% FCS in glucose-free DMEM), respectively.

REAGENTS

C18 S1P, C17 sphingosine, and C17 S1P were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). A JNK specific inhibitor, SP600125 was purchased from Sigma (St. Louis, MO).

ANTIBODIES AND WESTERN BLOTTING

Anti-SPHK1 (Cat. No. 3297) antibody was purchased from Cell Signaling Technology, (Beverly, MA). Anti-SPHK2 (Cat. No. sc-22704), anti-p-JNK (Cat. No. sc-12882), anti-JNK (Cat. No. sc-474), anti-p-Akt (Cat. No. sc-7985-R), anti-Akt (Cat. No. sc-8312), anti-p-p38 (Cat. No. sc-7975-R), anti-p38 (Cat. No. sc-535), anti-p-ERK (Cat. No. sc-7383), anti-ERK (Cat. No. sc-94), anti-p-ATF2 (Cat. No. sc-8398), anti-ATF2 (Cat. No. sc-6233), anti-p-CREB (Cat. No. sc-101663), anti-CREB-1 (Cat. No. sc-25785), anti-p-c-JUN (Cat. No. sc-16312), anti-c-JUN (Cat. No. sc-44), and anti-Lamin A/C (Cat. No. sc-20681) antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-β-actin antibody (Cat. No. 3598-100) was purchased from Bio Vision (San Francisco, CA), and anti-α-tubulin antibody (Cat. No. T8203) was from Sigma. Anti-COX IV antibody (Cat. No. ab14744) was purchased from Abcam (Cambridge, UK). Western blotting was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA).

QUANTITATIVE RT-PCR (QRT-PCR)

Total RNA was extracted from cells using the RNeasy mini kit (QIAGEN, Hilden, Germany) and the first strand cDNA was prepared from 1 μg of total RNA using the PrimeScript RT Master Mix

(TaKaRa, Shiga, Japan) for the detection of mRNAs. Quantitive RT-PCR was performed with SYBR Premix EXTaq II (TaKaRa). Relative mRNA expression were calculated as the ratio of target mRNA/β glucuronidase (GUSB) mRNA as described before [Mizutani et al., 2013]. To compare the mRNA expressions between cell lines, especially in glucose free condition, respective mRNA expressions were quantified absolutely. Primer sets were shown in Supplementary Table S2.

RNA LIGASE-MEDIATED RAPID AMPLIFICATION OF 5′-CDNA ENDS (5′RACE), PROMOTER CLONING OF HUMAN SPHK2, AND PROMOTER ASSAY

SPHK2 transcription start site was determined by the 5′ RACE method (Invitrogen, Carlsbad, CA) using the following primer sets: forward; 5′-CGACTGGAGCACGAGGACACTGA-3′, reverse; 5′-GGTGAGGGCAAAGCGTGGG-3′, forward for the nested PCR; 5′-GGACACTGACATGGACTGAAGGAGTA-3′, reverse for the nested PCR; 5′-AGAGGCCCCCGACGTGCT-3′.

Based on the 5′ RACE data illustrated in Supplementary Fig. S3a, showing the transcription site located about 360 bp downstream of the online data (NG_029867), 1.2 kb fragment covering the short 5′ region of SPHK2 was cloned into the *Mlu*I and *Hind*III sites of the pGL3 basic vector (Promega, Madison, WI). Deletion mutants and mutation-inserted promoter vectors were constructed with either restriction enzyme digestion or the PCR-based method (described in Supplementary information and Supplementary Table S1).

Promoter analysis was performed using Dual Luciferase reporter system (Promega) according to the manufacturer's instruction. Briefly, cells were seeded in 24 well plates and grown to 60% confluence. Three hundred ng of SPHK2 promoter luciferase reporter plasmids and 50 ng of pGL4.74 (hRluc/TK) vector, as internal control, were co-transfected by Lipofectamine 2000 (Invitrogen). After 24 h, transfected cells were collected and their luciferase activity was measured. Data were normalized by dividing the *Firefly* luciferase activity with the *Renilla* luciferase activity. The mean $\pm$ SD was shown.

TRANSFECTION OF SIRNA

Colon cancer cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's manual. siRNAs for ATF2 and c-JUN, and non-specific siRNA were purchased from Thermo scientific (Waltham, MA). siRNAs for CREB and SPHK2 were purchased from Sigma. Concentrations of siRNAs were optimized in the preliminary experiments by measuring target protein expression.

NUCLEAR RUN-ON ASSAY

SPHK2 transcription rate was detected by the nuclear run-on assay as described previously [Sobue et al., 2008] with minor modification. Cells were suspended in 1 ml of NP-40 lysis buffer (10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 10 mM NaCl, 0.5% NP-40, and 150 mM sucrose) stayed on ice for 10 min. After washing, collected nuclei were resuspended in glycerol buffer containing 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% Glycerol. Nuclear run-on was performed in the presence of Biotin Labelling RNA Mix (Roche, Basel, Switzerland) and 20 μl of transcription buffer (20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM DTT, 200 mM KCl 200 mM sucrose, and 20% Glycerol) at 29°C for 30 min. Newly synthesised and labeled RNA were isolated from nuclear RNA using Dynabeads M-280 streptavidin (Invitrogen). Captured RNA were washed twice with 15% formamide in 2 × SSC (saline-sodium citrate buffer) followed by 2 × SSC. Bound RNA was eluted in RNase-free H_2O. Newly transcribed SPHK2 mRNA was measured by the QRT-PCR method as described above.

VIABLE CELL COUNT

Viable cells were counted by the trypan blue dye exclusion method.

SPHK ENZYME ACTIVITY

SPHK enzyme activities were measured as described before [Nemoto et al., 2009] with some modifications. For SPHK2 assay, 40 μg of protein was used for the enzyme assay. The reaction was initiated by adding 5 nmol of C17 sphingosine (dissolved in 50 μl of 4 mg/ml fatty acid free BSA), 1 mM ATP, 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 250 μM EDTA, 13.5% glycerol, 2 mM DTT, and 1 M KCl in a final volume of 500 μl. For SPHK1 activity, 40 μg of protein was used. Five nanomoles of C17 sphingosine was dissolved in 50 μl of 2.5% TritonX-100 in KCl free condition. Any other composition was same as for SPHK2. After incubating at 37°C for 30 min, the reaction was terminated with 50 μl of 1 N HCl followed by 2 ml of chloroform: methanol: HCl (100:200:1,v/v). Then, 50 pmol C18 S1P (much higher than S1P in cell lysate) was added to confirm extraction efficiency. After samples were vigorously vortexed for 30 sec and left at the room temperature for 5 min, then 600 μl of 1 M KCl and the same volume of chloroform were added for phase separation. The mixture was again vigorously vortexed for 30 s and kept at the room temperature for 5 min, then centrifuged at 12,000 g for 5 min at 4°C. The upper water phase was completely aspirated and the lower chloroform phase was evaporated by N_2 spray at 60°C. The dried residue was reconstituted in 200 μl of mobile phase, [solvent A (methanol/water/formic acid = 58/41/1 with 5 mM ammonium formate): solvent B (methanol/formic acid = 99/1 with 5 mM ammonium formate) = 6:4].

An aliquot of final solution was injected and C17 S1P level was analyzed by 320 LC-MSMS triple quadrupole tandem mass spectrometer (Agilent Technologies, Santa Clara, CA) or Acquity Ultra Performance LC (Waters, Milford, MA) and 4000 QTRAP LC/MS/MS (ABSciex, Framingham, MA) to detect S1P. The enzyme activity was calculated and expressed as pmol S1P/min/mg protein.

ELECTROPHORESIS MOBILITY SHIFT ASSAY (EMSA)

As described above, nuclear extract was prepared from HCT116 cells cultured with FCS for 24 h. EMSA was performed as described previously [Mizutani et al., 2013]. The sequence of biotin-labeled probes is 5′-CGATGCGTCGCGCGGTGACGCTCTGGCCC-GACGCCGA-3′. A cold competitor was used 10, 100, or 300 times as much as the labeled probe. Anti-CREB, and p-CREB antibodies were used at 0.8, 1.6, or 2.4 μg/each sample, respectively.

DNA-PULL DOWN ASSAY

DNA pull-down assays (known as DNA affinity precipitation or DNAP assay) were performed as described previously [Okuyama et al., 2013]. The nuclear extracts from treated cells were incubated with biotin-labeled DNA probes, which were used in EMSA, and 15 μg of poly d(I-C) (Roche) in DNAP buffer (20 mM HEPES-KOH pH 7.9, 80 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol and 0.1% TritonX-100) on ice for 45 min. Then, 500 μg of Dynabeads M-280 streptavidin (Invitrogen) was added and incubated at 4°C for 1 h. The beads were washed using DNAP buffer, and bound proteins were eluted in SDS sample buffer. Subsequently, pulled down proteins were separated using a 10% SDS–PAGE gel and detected by Western blotting.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

ChIP assay was performed as described previously [Mizutani et al., 2013]. HCT116 cells were used for the crosslinking with 1% formaldehyde for 10 min at 37°C. After crosslinking, nuclei was extracted and sonicated at 4°C. Effect of sonication was checked by electrophoresis. For immunoprecipitation, normal rabbit IgG, or anti-CREB antibody was added (1 μg/each sample) to the same amount of nuclear protein and incubated at 4°C for 5 h. After washing, immunocomplexes were extracted and the crosslinking was reversed by heating elutes at 65°C overnight. Elutes were purified by High Pure PCR Cleanup Micro kit (Roche). Final elution volume was 20 μl. SPHK2 promoter region of the eluted sample was amplified by the following primer set: forward; 5′-TACCCAGGCCGTGTTCTCGATAGCTTT-3′, reverse; 5′-AGCCCCGCCTCCTCACCTCCA-3′. PCR was performed using KOD FX according to the user's manual. One microliter of the purified elutes as template were added in the PCR mixture. Total reaction volume was 10 μl. PCR conditions were 2 min of initial denaturation at 94°C followed by 38 cycles of 98°C for 10 s and 68°C for 15 s. The expected PCR product was 169 bp length.

ESTABLISHMENT OF STABLE TRANSFECTANTS

The original pIRES2 AcGFP vector was derived from Professor Ogretmen. (Medical University of South Carolina, Charleston, SC). CREB cDNA was inserted into the vector by the PCR method. To obtain CREB stable transfectant, HCT116 cells were transfected by CREB/pIRES2 AcGFP and selected with G418 (up to 500 μg/ml). The green fluorescent protein-positive cells were isolated using FACS Area2 (BD, San Jose, CA) (named as bulk cells) and one subclone was picked up from bulk cells named sc I.

STATISTICAL ANALYSIS

Statistical significances were analyzed using Student's *t*-test or one-way factorial analysis of variance and multiple comparison test (Fisher's method) using Excel software (Microsoft).

RESULTS

SPHK EXPRESSION OF HUMAN COLON CANCER CELL LINES

SPHK1 and SPHK2 expressions were examined for four human colon cancer cell lines, HCT116, RKO, DLD-1, and HT29. Consistent with our previous report [Nemoto et al., 2009], SPHK2 protein and mRNA levels showed heterogeneity among these four cell lines, however, the respective SPHK2 protein and mRNA levels were well correlated (Fig. 1a and c). SPHK2 protein and mRNA expressions were not correlated with those of SPHK1. All of these cell lines have been reported to possess either *Kras*- or *Braf*-mutations, which might be connected to the enhanced ERK cellular signaling pathway. However, the signaling pathways including ERK, JNK, p38, and AKT were not correlated with SPHK2 expression level in FCS (+) culture (a). For further analysis, we chose HCT116 and HT29 as representatives of low- and high-SPHK2 expressing cells, respectively.

INCREASED SPHK2 EXPRESSION BY SERUM DEPLETION

It has been reported that serum depletion induced SPHK2 expression in HEK293 cells [Okada et al., 2005]. We examined the effect of serum deprivation on endogenous SPHK2 of colon cancer cell lines. As shown in Figure 2 and Supplementary Fig. S1, SPHK2 but not SPHK1 of all cell lines increased after 12 h or later of serum

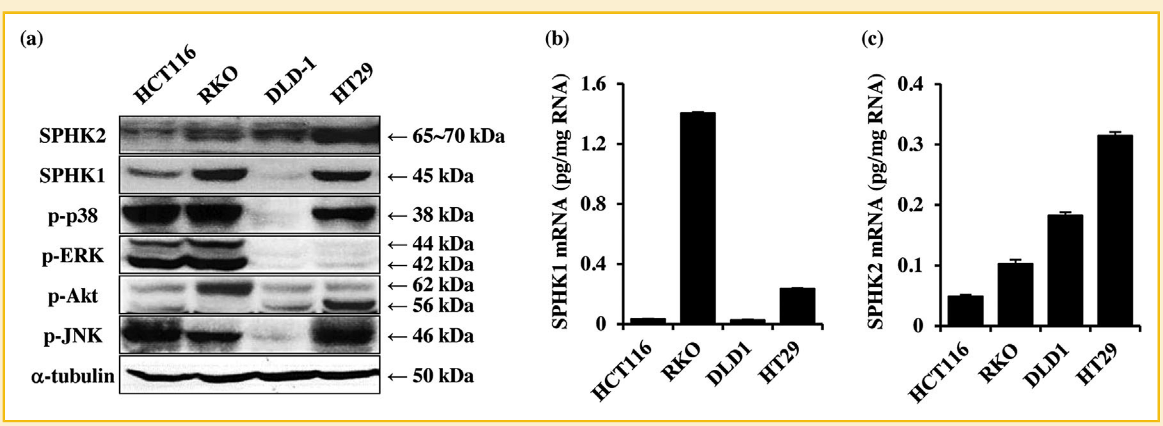

Fig. 1. Expression levels of SPHKs and cellular signaling (a) SPHK1, SPHK2, and activated cellular signaling molecules (p-38, JNK, ERK, and AKT) of four human colon cancer cell lines were examined by Western blotting according to the Materials and methods. Antibodies used were also listed in the Materials and methods. (b), (c) SPHK1 and SPHK2 mRNA of four colon cancer cells were measured by the QRT-PCR method as described in the Materials and methods. The mean ± SD was calculated from three separate experiments.

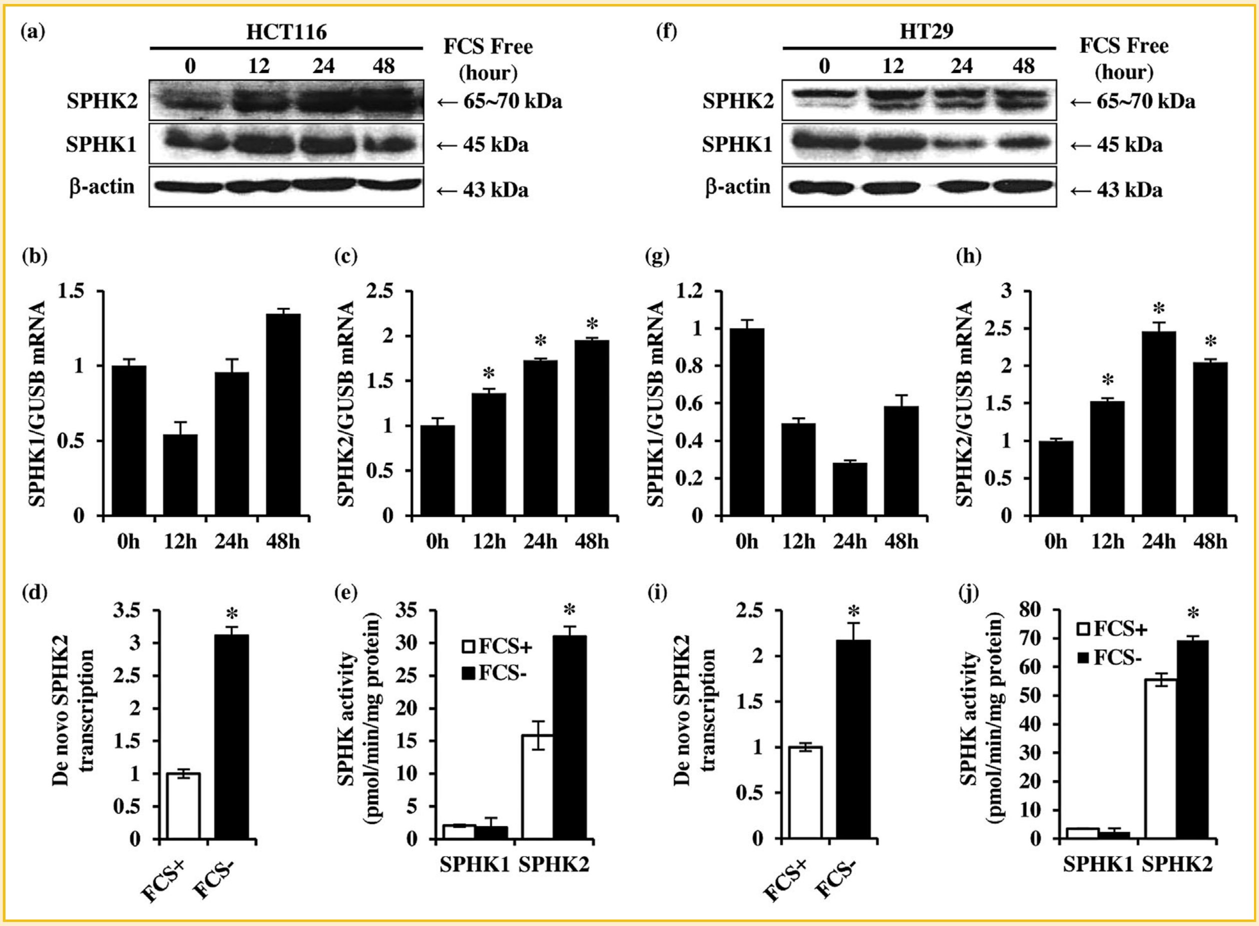

Fig. 2. Serum depletion-induced changes of SPHKs protein and mRNA (a) SPHK2 and SPHK1 protein of HCT116 were examined after serum depletion. β-actin was shown as the internal control. (b), (c) 12 h, 24 h, and 48 h after serum depletion, cellular SPHK1 and SPHK2 mRNA levels were assayed by the QRT-PCR according to the Materials and methods. The mean ± SD was shown from three independent experiments. *denotes $P < 0.001$ between FCS (+) and FCS (−) group. (d) Eighteen hours after serum depletion, de novo SPHK2 mRNA production was measured by the nuclear run-on assay using HCT116 cells described in the Materials and methods. The mean ± SD was shown from three independent experiments. *means $P < 0.001$. (e) Twenty-four hours after serum depletion, SPHK1 and SPHK2 enzyme activities were measured using HCT116 cells described in Material and methods. The mean ± SD was shown from three independent experiments. *means $P < 0.001$.(f–j) Similar experiments were performed using HT29 cells.

depletion, which paralleled its mRNA increase. Nuclear run-on assay of HCT116 and HT29 cells showed that de novo SPHK2 mRNA production increased about threefold (in HCT116) or twofold (in HT29) in FCS (−) culture compared with FCS (+) condition (Fig. 2d and i). SPHK2 but not SPHK1 activity was also increased in FCS (−) culture in both colon cancer cell lines (Fig. 2e and j), and SPHK2 enzyme activities of these cell lines were much higher than that of SPHK1 (Fig. 2e and j). In contrast, other cell stresses, hypoxia and glucose depletion, did not exhibit such an increase as found in SPHK2 of HCT116 and HT29 cells in FCS (−) culture as shown in Figure 3. Contrary, both of these stresses gradually suppressed SPHK2 expression, which was paralleled with the decrease of mRNA level, approximately.

EFFECTS OF SIRNA FOR SPHK2 ON CELL PROLIFERATION

Glucose depletion rapidly induced apoptosis of HCT116 cells, whereas hypoxia suppressed cell proliferation (Fig. 4). Unexpectedly, in FCS (−) culture, colon cancer cells continue to proliferate for at least 4 days, although its proliferation rate was slower than that of FCS (+) culture (Fig. 4a and data not shown). siRNA for SPHK2 but not control siRNA inhibited cell proliferation in FCS (−) culture (Fig. 4b). Similar results were obtained in other colon cancer cell lines examined (data not shown).

SPHK2 enzyme activities of HCT116 and HT29 cells were higher than those of SPHK1, and serum depletion increased SPHK2 but not SPHK1 enzyme activity (Fig. 2e and j). Not only nuclear but also cytoplasmic SPHK2 protein and activity of HCT116 cells increased after serum depletion (Supplementary Fig. S2a and c). Mitochondrial SPHK2 was also increased slightly after serum depletion. However, considering the band intensities of SPHK2 and COX IV (a marker protein of mitochondria) of whole cell and mitochondrial fraction (Supplementary Fig. S2b), the mitochondrial SPHK2 was suggested to be a minor component of SPHK2 in HCT116 cells.

These results suggest that SPHK2 of colon cancer cells is at least pro-survival or proliferative even in the serum-depleted condition. The physiological level of SPHK2 has been reported not to inhibit

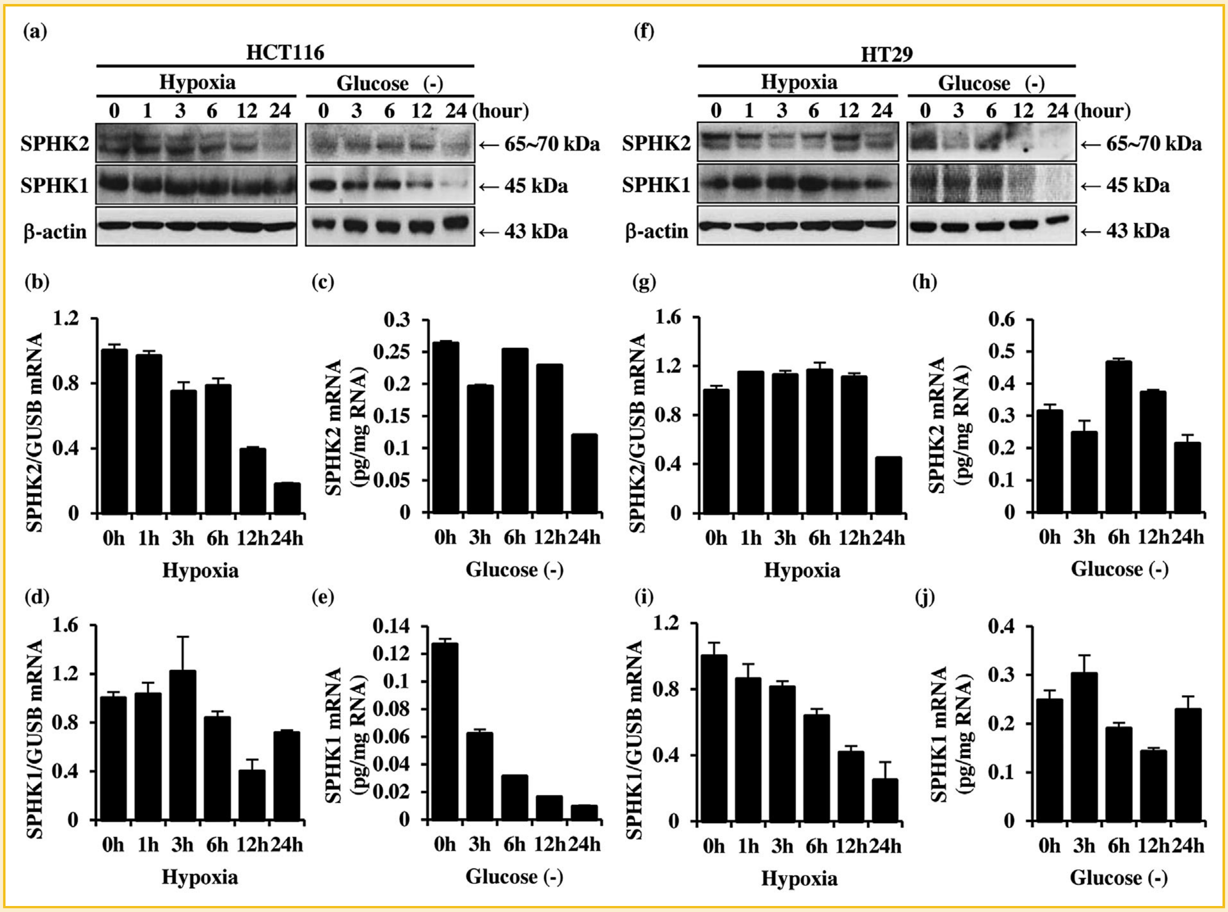

Fig. 3. Effect of hypoxia and glucose depletion on SPHK1 and SPHK2 of HCT116 and HT29 cell lines (a) SPHK1 and SPHK2 protein levels were measured sequentially after hypoxia treatment or glucose depletion of HCT116 cells. β-actin was illustrated as the internal control. (b), (d) SPHK1 and SPHK2 mRNA of hypoxia-treated HCT116 cells were measured by the QRT-PCR method as illustrated in Figures 1 and 2. The mean ± SD was shown from the date of triplicate samples. (c), (e) SPHK1 and SPHK2 mRNA of glucose-depleted condition were measured in a similar way as (b) and (d). In this case, SPHK mRNA were expressed as pg/mg total RNA. (f–j) Similar experiments were performed using HT29 cells.

DNA synthesis, although high SPHK2 expression inhibits DNA synthesis [Okada et al., 2005]. The report by French et al. [2010], using ABC294640, a relatively specific SPHK2 inhibitor, demonstrated the positive role of SPHK2 on various solid tumor cell proliferation. However, further analysis is needed to elucidate how increased SPHK2 and its product, S1P, contribute to cell survival and proliferation in FCS (−) culture.

ANALYSIS OF THE 5′ REGION OF SPHK2

We analyzed the 5′ promoter region in order to search for the main factors to increase SPHK2 transcription in FCS (−) culture. We examined the transcription start point by the 5′ RACE method described in the Materials and methods. We determined 5 transcription start sites located very close to each other and selected the most 3′ side located one as the +1 bp. It was about 360 bp proximal to the transcription site of NG_029867 described in NCBI (Supplementary Fig. S3a). In FCS (+) culture of HCT116, we examined the relationship between the promoter length and its activity. Supplementary Fig. S3b illustrates several potent promoter regions including regions between −126 bp and the first exon, between −181 and −173 bp, between −317 and −225 bp in HCT116 cells. We surveyed the putative transcription factor-binding site through Match gene-regulation (www.gene-regulation.com/pub/programs.html). Because a single putative binding factor was not presumed in these binding sites, several different transcription factors are expected to bind to these different regions and to co-work for the regulation of SPHK2 expression. We unexpectedly observed the similar promoter profiles in other cell line, HT29, which showed high SPHK2 mRNA expression compared with HCT116 cells (Supplementary Fig. S3c), suggesting the contribution of other elements than 5′ SPHK2 promoter region in SPHK2 expression in FCS (+) culture.

We performed similar experiments in FCS (−) culture. However, luciferase activity as well as internal control activity diminished severely in FCS (−) culture, making both reproducibility and data evaluation difficult (data not shown). Thus, we analyzed the temporal change of signaling pathways and transcription factors by serum depletion to examine involvement of the 5′ region in SPHK2 expression.

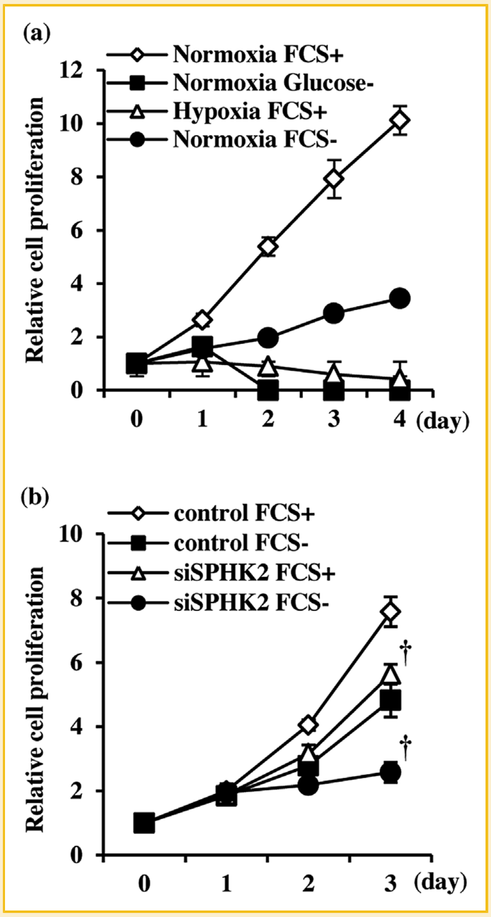

Fig. 4. Effect of several stress condition and SPHK2 inhibition on HCT116 cells proliferation (a) Cell proliferation in normal (FCS (+)) culture, FCS (−), hypoxia, or glucose depleted condition were described. One day after plating, culture medium or concentration of O_2 was changed, respectively. Viable cell number was counted in triplicate using 12-well plates. Relative cell proliferation was illustrated, regarding the cell count of day 0 as 1.0. (b) HCT116 cells were cultured in triplicate in either FCS (+) or FCS (−) culture with or without siRNA for SPHK2 (5 nM). Control means siRNA of negative control transfected cells. Viable cell number was counted sequentially. †$P < 0.005$ between control and siSPHK2. siRNA for SPHK2 was transfected 24 h before day 0.

EFFECT OF SERUM DEPLETION ON SIGNALING PATHWAY AND TRANSCRIPTION FACTORS IN HCT116 CELLS

Activation of some signaling pathways by serum depletion is shown in Figure 5a. AKT and ERK were deactivated gradually in a time-dependent manner. p38 was not or very mildly activated. Interestingly, JNK rapidly and transiently activated with serum depletion (p-JNK, Fig. 5a). Several transcription factors have been reported to increase during serum depletion including CREB, HIF-1, HSF1, NF-κB, FOXO3, KLF4, and SMAD4 [Zhang et al., 2000; Leicht et al., 2001; Piret et al., 2004; Thomas and Kim, 2008; Lee et al., 2011; Wang et al., 2011; Li et al., 2012]. Figure 5b shows that activated (phosphorylated) CREB but not total CREB increased after JNK-activation. c-JUN exhibited a gradual activation 6 h after serum depletion and activated ATF2 was also increased in early term transiently.

CREB AS AN IMPORTANT STIMULATOR OF SERUM DEPLETION-INDUCED SPHK2 UP-REGULATION

Effects of siRNAs for ATF2, c-JUN and CREB were examined in HCT116 cells for both SPHK2 promoter activity and SPHK2 mRNA. Effect of each siRNA is shown in Figure 5c–e, respectively. Only CREB siRNA suppressed both SPHK2 promoter activity and mRNA (Fig. 5f and g). We did not further analyze ATF2 and c-JUN, because SPHK2 mRNA inhibition by respective siRNA was much smaller than that by CREB siRNA. Notably, CREB activation started earlier than SPHK2 increase (Fig. 5a and b). Activated JNK shown in Fig. 5a was supposed to phosphorylate CREB because of the rapid activation after serum depletion. JNK inhibition with a specific inhibitor, SP600125, robustly inhibited CREB activation (phosphorylation) in FCS (−) culture (Fig. 5h). Furthermore, increases of SPHK2 protein and mRNA level in FCS (−) culture were significantly suppressed by JNK inhibitor (Fig. 5h and i). Similar results were observed in HT29 cells (data not shown).

Hypoxia inhibited JNK activation but CREB activation was observed in both HCT116 and HT29 cells. In contrast, glucose depletion did not induce CREB activations in both cell lines (Supplementary Fig. S4). Considering that hypoxia and glucose depletion did not induce SPHK2 expression, these results clearly indicate the major role of the signaling pathway from JNK to CREB in increased SPHK2 transcription only in FCS (−) culture but not in hypoxia and glucose-depleted condition.

CREB transcription factor bind with the CRE binding site. The CRE binding site was located between −181 and −173 bp region of the 5′-SPHK2 promoter and in FCS (+) culture, mutation insertion inhibited the promoter activity of this region (Supplementary Fig. S3b, −181 vs. Mut −181 bp), suggesting some role of this region and probably CREB transcription factor in FCS (+) culture. CREB overexpression increased p-CREB and SPHK2 expression in serum-depleted HCT116 cells (Fig. 6a). siRNA for CREB effectively decreased CREB levels and also inhibited serum depletion-induced increase of SPHK2 protein, mRNA, enzyme activity and de novo SPHK2 transcription measured by the nuclear run-on assay (Fig. 6b–e). Moreover, siRNAs for CREB and SPHK2 did not affect SPHK1 expression (Fig. 6b). On the contrary, SPHK2 overexpression and inhibition using siRNA did not affect CREB activation (data not shown).

EMSA AND CHIP ASSAY

Figure 7a illustrates the primer set for ChIP assay and DNA probe used in EMSA and pull down assay covering important CREB binding sites around −180 bp from the first exon. Two shifted bands were observed in EMSA, and a cold probe erased these shifted bands in a dose-dependent manner (Fig. 7b). Because the super shift assay using anti-CREB and anti-p-CREB antibodies demonstrated only a moderate decrease of the band 2 (Fig. 7c: in short exposure time), we further performed DNA pull-down assay. Increased binding of CREB to DNA probe in FCS (−) culture is demonstrated in Figure 7d. ChIP assay (Fig. 7e) showed the increased PCR product from the immunoprecipitate of FCS (−) cultured cells with anti-CERB antibody, supporting EMSA and DNA pull-down data shown above.

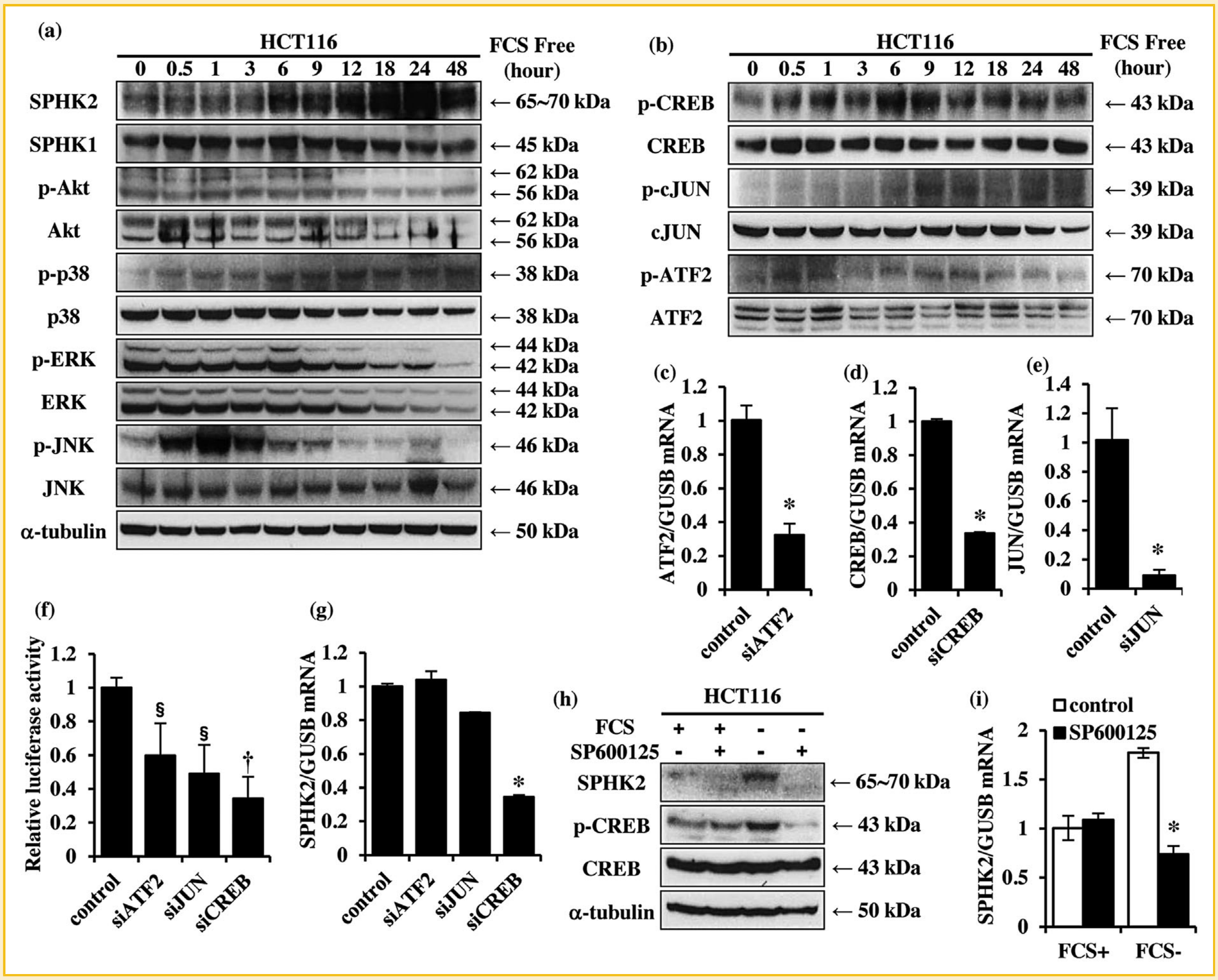

Fig. 5. Temporal changes of cellular signaling pathways and transcription factors by serum depletion in HCT116 cells (a) Effect of serum depletion on various cellular signaling was analyzed by Western blotting. Antibodies used were listed in the Materials and methods. α-tubulin was used as the internal control. (b) Activated (phosphorylated) ATF2, activated (phosphorylated) CREB, and activated (phosphorylated) c-JUN were analyzed by Western blotting. (c–e) Effects of siRNA for ATF2, CREB, and c-JUN were examined. Respective mRNA expression was measured according to the Materials and methods, and was calculated as the ratio of respective mRNA/GUSB mRNA. Assay was performed in triplicate. The mean ± SD was shown, and control siRNA value was regarded as 1.0. *means $P < 0.001$ compared with control siRNA. (f), (g) Effects of siRNAs for ATF2, c-JUN and CREB on SPHK2 promoter activity and SPHK2 mRNA level. The control or respective siRNA was transfected for 24 h, and SPHK2 promoter activity (by the luciferase assay) and relative SPHK2 mRNA level were examined according to the Materials and methods. Cells were plated in triplicate, transfected and collected. The mean ± SD was calculated. In (f) and (g), §indicates $P < 0.05$. †denotes $P < 0.005$. *Means $P < 0.001$ compared with control siRNA. (h) Cells were cultured in FCS (+) condition with or without 10 μM of SP600125 (JNK inhibitor) for 3 h, and culture medium was replaced to FCS (−) with or without 10 μM of SP600125. After additional 12 h culture, cells were collected and analyzed the effect of JNK inhibitor on activated CREB (p-CREB) and SPHK2 protein expression by Western blotting. (i) HCT116 was treated in the same way as Figure 5h. Relative SPHK2 mRNA expression was analyzed according to Materials and methods. The mean ± SD was shown from three independent experiments. *Denotes $P < 0.001$ between control and SP600125 treated group.

DISCUSSION

It has been recognized that the delicate balance between ceramide, sphingosine and S1P determines the cell's fate [Pyne and Pyne, 2010]. SPHK is one of the central regulators of this delicate balance, and extensive studies have mostly focused on SPHK1, which functions as anti-apoptotic and pro-survival or cell proliferation factor. In contrast, reports analyzing another isozyme, SPHK2, have shown its pro-apoptotic and cell cycle arresting function. Thus, SPHK1 and SPHK2 have been regarded to play distinct role, although both produce the same product, S1P. In contrast to the report showing apoptotic function of SPHK2 overexpression [Maceyka et al., 2005], knockdown of SPHK2 sensitizes breast cancer cells to doxorubicin-induced apoptosis [Sankala et al., 2007], and activation of SPHK2 protects cerebral ischemia [Pfeilschifter et al., 2011].

It is of great interest to elucidate the regulatory mechanism of endogenous SPHK2 expression to gain more information regarding the preferential roles of SPHK1 and SPHK2 in the clinical field including immunological disease and cancer. For further analysis, we selected 4 different colon cancer cell lines and found the heterogeneous SPHK2 mRNA and protein expression (Fig. 1), supporting our previous report mainly analyzed enzyme activity

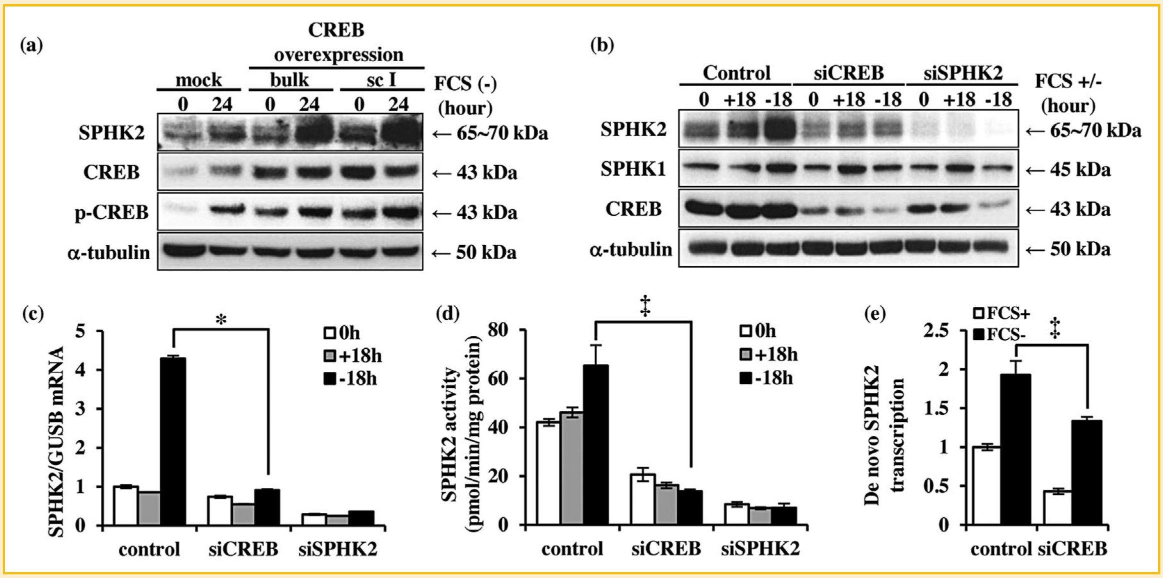

Fig. 6. Effect of CREB overexpression and siRNAs for CREB on SPHK2 expression in HCT116 cells (a) High CREB expressing cells selected by G418 after cDNA transfection followed by cell sorting with their GFP expression (named as the bulk) and one stable subclone (named as sc I) selected from bulk cells (described in the Materials and methods) were analyzed in FCS (−) condition. Twenty-four hours after serum depletion, cells were collected and processed for Western blotting. (b) siRNAs for control, CREB and SPHK2 mRNA were transfected for 24 h and culture medium was replaced with FCS (+) or FCS (−) medium for another 18 h. Cells were collected and processed for Western blotting assay. 0 means collected 24 h after transfection, +18 means 18 h after FCS (+) culture, whereas −18 denotes FCS (−) culture for 18 h. α-tubulin was used as the internal control. (c), (d) Using the same cell samples as Figure 6b, relative SPHK2 mRNA level and SPHK2 enzyme activity were measured. *indicates $P < 0.001$ and ‡ shows $P < 0.01$. (e) Nuclear run-on assay. Effect of siRNA on de novo SPHK2 transcription was analyzed with the nuclear run-on assay described in the Materials and methods. Cells were plated in triplicates. The mean ± SD was illustrated. ‡$P < 0.01$.

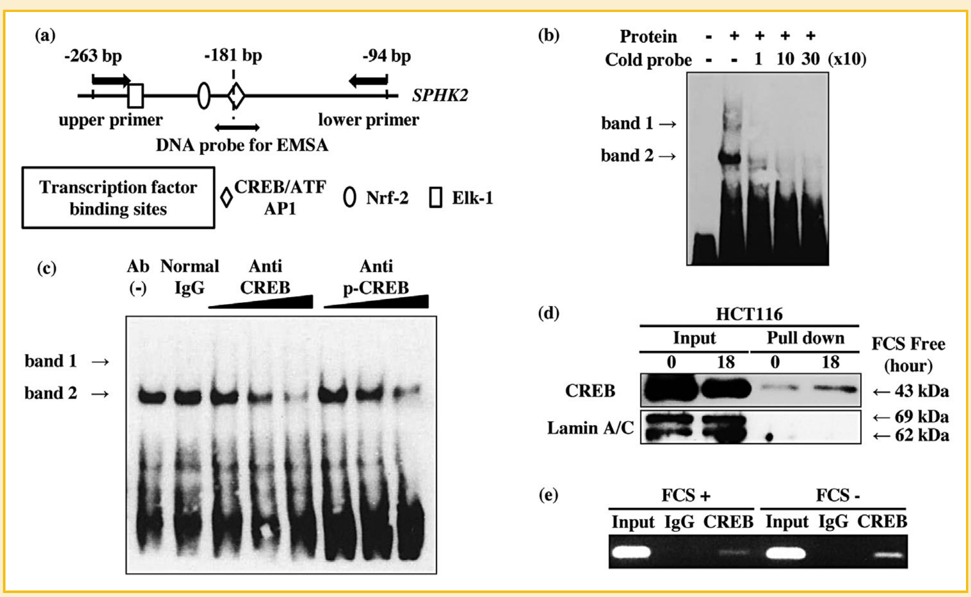

Fig. 7. EMSA, DNA pull-down, and ChIP assay (a) illustrates the 5′ promoter region of SPHK2 analyzed, the site of the PCR primer set for ChIP assay and a DNA probe for EMSA as well as for DNA-pull down assay.(b) EMSA was performed according to the Materials and methods. Cold competitor was added with three different doses (×10, ×100, and ×300 times the labeled probe). (c) EMSA Supershift assay: Ab (−) denotes no antibody addition. Normal IgG, anti-CREB and anti-phosphoryated CREB were added 30 min before EMSA at room temperature. Because short exposure was adopted to exhibit the effect of each antibody clearly, band 1 was not visible in this figure. (d) DNA pull-down assay was performed according to the Materials and methods. Anti-Lamin antibody was used as a marker of nuclear fraction. Nuclear extracts from FCS (−) culture for 0 and 18 h were used for the pull-down assay. (e) ChIP assay was performed as described in the Materials and methods. FCS (+) and (−) denote cells cultured with or without FCS for 24 h, respectively. Input denotes the sample before immunoprecipitation. IgG and CREB means immunoprecipitation with rabbit normal IgG or CREB antibody respectively.

[Nemoto et al., 2009]. The promoter analysis of SPHK2 has not been precisely reported, and our present analysis for the first time revealed that several 5′ promoter regions were involved in SPHK2 transcription of human colon cancer cell lines in FCS (+) culture, also suggesting involvement of several different transcription factors. Unexpectedly, the profile of promoter activity was similar between high- and low-SPHK2 expressing cells, indicating the presence of mechanisms other than transcription factors (Supplementary Fig. S3). This issue is an interesting subject for the future study.

Okada et al. [2005] reported SPHK2 in serum-depleted culture using stably overexpressed SPHK2 in HEK293 cells. We examined effects of several cellular stresses on endogenous SPHK expression in human colon cancer cell lines. Interestingly, serum depletion, but not hypoxia and glucose depletion, induced SPHK2 protein expression (Figs. 2, 3, and Supplementary Fig. S1). Increased SPHK2 was observed in both nuclear and cellular fraction, and the involvement of mitochondrial SPHK2 is less likely (Supplementary Fig. S2). Increased transcription of SPHK2 in serum-depleted culture was confirmed by the nuclear run-on assay (Fig. 2d and i).

Interestingly, in serum-depleted culture but not hypoxia and glucose depletion, HCT116 cells continued to grow, although slowly as compared with serum-containing culture (Fig. 4). SPHK2 activity was higher in HCT116 cells than SPHK1. Therefore, the role of SPHK2 was examined using siRNA for SPHK2, and it was shown that SPHK2 was pro-survival but not cell cycle inhibitor in serum-depleted HCT116 cells. Similar data were also obtained in HT29 cells (data not shown). It has been reported that SPHK2 is pro-survival and proliferative rather than pro-apoptotic or macroautophagy-inducing [Antoon et al., 2011; Beljanski et al., 2011; Gestaut et al., 2014], and Gao and Smith [2011] found that selective inhibition of SPHK2 may provide optimal targeting of this pathway in cancer chemotherapy.

Moreover, the equivalent roles of SPHK1 and SPHK2 have been revealed in mediating insulin′s mitogenic action [Dai et al., 2014], and the pro-survival and anti-cancer drug-resistant functions of endogenous SPHK2 in some cancer cells have recently been shown [Nemoto et al., 2009; Weigert et al., 2009; Antoon et al., 2011]. Intriguingly, glucose depletion induced very rapid cell death or apoptosis and hypoxia induced cell growth arrest. These two stresses affected SPHK2 expression of colon cancer cells differently from that of serum depletion, suggesting different contribution of SPHK2 in different stress conditions.

Recent report indicated the novel function of nuclear SPHK2 as the HDAC inhibitor of MCF7 cells [Hait et al., 2009]. However, our preliminary experiments revealed that the nuclear HDAC activity was not decreased in serum-depleted HCT116 cells, suggesting that the pro-survival effect of SPHK2 observed in serum-depleted culture of human colon cancer cells was not related to the HDAC inhibition by activated SPHK2. We also examined the localization of SPHK2 in mitochondria, and found that mitochondrial SPHK2 expression level was very low and was not increased so much after serum depletion. Thus, mitochondrial SPHK2 might not be involved greatly with the growth or survival after serum depletion.

Based on these results, we attempted to analyze the promoter activity in FCS (−) culture condition. As unexpectedly, the promoter analysis was practically impossible (data not shown), other approaches such as the analysis of signal transduction pathways and the survey for candidate transcription factors were adopted. Figure 5a demonstrates the temporal changes in activation of JNK but not p38, AKT, and ERK pathway after serum depletion.

The on-line survey through Match gene-generation disclosed several putative transcription factor-binding sites of the 5′ promoter region of SPHK2, and we focused on CREB, ATF2, and c-JUN. Figure 5b shows the early activation of ATF2, CREB, and c-JUN. Effect of siRNA for ATF2 and c-JUN on SPHK2 expression was smaller than that of CREB siRNA (Fig. 5f and g). Based on Figures 5 and 6, CREB was proved to be a major transcription factor responsible for SPHK2 transcription after serum depletion, and JNK activation lead to CREB activation followed by increased SPHK2 transcription. The rapid phosphorylation of CREB has been reported after serum depletion [Leicht et al., 2001]. In hypoxia or glucose-depleted condition, activated CREB was not the main regulator of SPHK2 expression (Supplementary Fig. S4), suggesting the different regulatory mechanism of SPHK2 in hypoxia and glucose-depleted condition.

The c-Jun N-terminal protein kinases (JNK) are evolutionary-conserved family of serine/threonine protein kinases and have been shown to be activated with various extracellular signals including growth factors, cytokines, as well as various cellular stresses [Barr and Bogoyevitch, 2001]. The role of JNK is heterogeneous and sometimes paradoxical; cell death enhancing effect and cell proliferation stimulator depending on analyzed cells and agonists/treatments. Although c-JUN is a typical JNK substrate, JNK can also phosphorylate and activate other transcription factors including ELK-1 and ATF2 [Cavigelli et al., 1995; van Dam et al., 1995]. Using JNK specific inhibitor, SP600125 (Fig. 5h), we clearly demonstrated that JNK phosphorylated CERB. Similar to our results, the protective role of JNK activation has been reported in thymosin β4-treated endothelial progenitor cells under serum depletion [Zhao et al., 2011].

CREB is the first transcription factor, whose activity has been shown to be regulated by phosphorylation [Mayr and Montminy, 2001]. CREB is activated by Ca^{2+} and cAMP. These two regulate both ERK1/2 and protein kinase A (PKA) activity, followed by CREB phosphorylation [Barlow et al., 2008]. Phosphorylation of Ser133 is a predominant mechanism for regulating the kinase-inducible domain (KID) activity of CREB protein [Arias et al., 1994]. Phosphorylation of ser133 enhances the KID activity by promoting the recruitment of transcription co-activators, CREB-binding protein (CBP) and p300 to the promoter of target genes [Kwok et al., 1994]. Important target genes of CREB in cell proliferation and apoptosis include c-fos and bcl-2 [Mayr and Montminy, 2001]. Studies of transgenic models expressing dominant-negative CREB have revealed a role of CREB family in controlling cell survival and proliferation [Mayr and Montminy, 2001]. In addition to the role as cAMP-responsive activator, various growth factors phosphorylate CREB [Deak et al., 1998].

The CREB/CRE transcriptional pathway protects against oxidative stress-mediated neuronal cell death [Lee et al., 2009]. SPHK2 is the predominant S1P-synthesizing isoform in the brain parenchyma, and it has recently been reported that exogenous S1P increases p-CERB expression of 1-methyl-4 phenylpyridinium-treated MN9D dopaminergic neuronal cell line (in vitro model of Parkinson disease)

[Sivasubramanian et al., 2015], suggesting the positive feedback between CERB-induced SPHK2 expression and S1P-induced p-CERB induction. Because the role of each SPHK must be considered from its cellular localization, accessibility to the substrate, and interaction with other proteins, it is an interesting future project to elucidate the precise mechanism how SPHK2 and its product, S1P, maintain cell survival or proliferation in FCS (−) culture.

In our analysis, ESMA, DNA-pull down assay and ChIP assay shown in Figure 7 support the direct binding of CREB with the 5′ promoter region of SPHK2 and its positive role in SPHK2 transcription. Because the precise analysis of SPHK2 transcription and involved transcription factors has not been reported, the present study is the first demonstration that SPHK2 is the direct target of CERB transcription factor of serum-depleted colon cancer cells.

Taken together, our present experiments using human colon cancer cells suggest (1) that serum depletion but not hypoxia and glucose depletion-induced SPHK2 expression, (2) that the role of SPHK2 in serum-depleted cells is pro-survival but not pro-apoptotic, and (3) that serum depletion induced SPHK2 expression is dependent on activated JNK/CREB pathway.

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