Molecular Biology of the Cell
Vol. 21, 1088–1096, March 15, 2010

Ras and Calcium Signaling Pathways Converge at Raf1 via the Shoc2 Scaffold Protein

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Submitted June 4, 2009; Revised December 18, 2009; Accepted January 7, 2010
Monitoring Editor: J. Silvio Gutkind

Situated downstream of Ras is a key signaling molecule, Raf1. Increase in Ca^{2+} concentration has been shown to modulate the Ras-dependent activation of Raf1; however, the mechanism underlying this effect remains elusive. Here, to characterize the role of Ca^{2+} in Ras signaling to Raf1, we used a synthetic guanine nucleotide exchange factor (GEF) for Ras, eGRF. In HeLa cells expressing eGRF, Ras was activated by the cAMP analogue 007 as efficiently as by epidermal growth factor (EGF), whereas the activation of Raf1, MEK, and ERK by 007 was about half of that by EGF. Using a biosensor based on fluorescence resonance energy transfer, it was found that activation of Raf1 at the plasma membrane required not only Ras activation but also an increase in Ca^{2+} concentration or inhibition of calmodulin. Furthermore, the Ca^{2+}-dependent activation of Raf1 was found to be abrogated by knockdown of Shoc2, a scaffold protein that binds both Ras and Raf1. These observations indicated that the Shoc2 scaffold protein modulates Ras-dependent Raf1 activation in a Ca^{2+}- and calmodulin-dependent manner.

INTRODUCTION

On growth factor stimulation, many receptor-type or non-receptor-type tyrosine kinases are activated, followed by the activation of the Ras/Raf/MEK/ERK signaling cascades, which regulate various aspects of cellular function, including the proliferation, differentiation, and oncogenic transformation of high eukaryotes (Murphy and Blenis, 2006; Katz *et al.*, 2007). It is now widely accepted that, in addition to the canonical pathway mediated by adaptor proteins and guanine nucleotide exchange factors (GEFs) for Ras-family GTPases, other signaling cascades stimulated simultaneously by growth factors also play roles in the regulation of Ras/Raf/MEK/ERK signaling cascades. For example, various ligands activate or suppress the Ras/Raf/MEK/ERK signaling cascades in a Ca^{2+}- and calmodulin-dependent manner (reviewed in Agell *et al.*, 2002). The activities of many tyrosine kinases are regulated by Ca^{2+}-regulated kinases, such as conventional protein kinase C (PKC) isozymes, the proline-rich tyrosine kinase Pyk2 or calmodulin-dependent kinases (Kolch *et al.*, 1993; Blaukat *et al.*, 1999; Agell *et al.*, 2002). Furthermore, Ras is also regulated either positively or negatively by Ca^{2+}-responsive GEFs or GTPase-activating proteins (GAPs; reviewed in Cullen, 2006). Therefore, the effect of Ca^{2+} on the Ras/Raf/MEK/ERK cascade is always the summation of the activity changes of these upstream regulators.

Under these circumstances, it is not surprising that there are only a few reports that refer to the specific role of Ca^{2+} in the signaling from Ras to Raf (Egea *et al.*, 1999; Villalonga *et al.*, 2001; Ren *et al.*, 2008), because the role of calcium has been studied mostly in ligand-stimulated cells treated with calcium ionophores, calcium chelators, or calmodulin inhibitors. In PC12 cells, which respond to an increase in Ca^{2+} concentration by activating extracellular signal–regulated kinase (ERK), it has been suggested that calmodulin directly or indirectly activates MEK (mitogen-activated protein [MAP]/ERK kinase) in a manner dependent on Ras, but independent of Raf, suggesting the presence of an unidentified MEK kinase (Egea *et al.*, 1999). In NIH3T3 cells, calmodulin binds to KRas, thereby decreasing activities of Raf and ERK (Villalonga *et al.*, 2001). In agreement with this observation, inhibition of calmodulin increases KRas-mediated Raf activation (Moreto *et al.*, 2009). Intriguingly, calmodulin was shown to increase the signaling from HRas to Raf1 (Moreto *et al.*, 2008). Therefore, the overall role of calmodulin in ERK signaling may be determined by the ratio of HRas and KRas and the cell-specific contribution of each isoform in Raf1 activation.

The Shoc2 protein, also known as Sur-8, which was originally identified as a pivotal regulator in the Ras/ERK MAP kinase cascade, has been found in all multicellular organisms (Sieburth *et al.*, 1998; Selfors *et al.*, 1998). By two-hybrid analysis, Shoc2 was found to interact with Ras but not with other related small GTPases (Li *et al.*, 2000). Shoc2 has also been found to form a complex with Raf1 in mammalian cells and to enhance the signaling strength of Ras by promoting the Ras–Raf1 interaction (Li *et al.*, 2000). It has been reported that Erbin, a member of the leucine-rich repeat and PDZ domain family, inhibits the interaction of Shoc2 with Ras and Raf and thereby inhibits the Ras/ERK MAP kinase

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–06–0455) on January 13, 2010.

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Abbreviations used: EGF, epidermal growth factor; GEF, guanine nucleotide exchange factor; PKC, protein kinase C; PLC, phospholipase C.

cascade, suggesting that the binding of Ras and Raf1 to the Shoc2 scaffold protein is also subject to regulation (Dai *et al.*, 2006). More recently, Shoc2 has been shown to associate with a phosphatase holoenzyme complex and to bind to MRas, thereby activating Raf1 by means of the dephosphorylation of 14-3-3 binding sites (Rodriguez-Viciana *et al.*, 2006).

Here, by the use of a synthetic guanine nucleotide exchange factor named eGRF (Ohba *et al.*, 2003), we have developed an experimental system wherein Ras can be rapidly activated without perturbing other signaling molecules. With this system in hand, we show that Shoc2 enhances Ras-dependent activation and plasma membrane translocation of Raf1 in a Ca^{2+}-dependent manner.

MATERIALS AND METHODS

Reagents and Antibodies

Epidermal growth factor (EGF), puromycin, ionomycin, BAPTA-AM, U0126, LY294002, and Gö6983 were obtained from Sigma (St. Louis, MO). 007 was purchased from Tocris Cookson (Ballwin, MO), and U73133 and W-13 were purchased from Calbiochem (La Jolla, CA). To obtain the anti-Shoc2 rabbit serum, a polypeptide corresponding to amino acids 70-83 of human Shoc2 was synthesized, bound to keyhole limpet hemocyanin, and inoculated into rabbits with complete Freund's adjuvant (Covalab, Villeurbanne, France). The following antibodies were also used in this study: anti-Raf1, anti-MEK1, and anti-pan ERK antibody were purchased from BD Transduction Laboratories (San Jose, CA); anti-Pan Ras (AB-3) antibody was from Calbiochem; anti-FLAG M5 antibody was purchased from Sigma; anti-Myc (9E10) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against phospho-Raf1 (Ser^{338}), phospho-MEK1/2 ($Ser^{217/221}$), and anti-phospho ERK (Thr^{202}/Tyr^{204}) were obtained from Cell Signaling Technology (Beverly, MA); Alexa 488 anti-rabbit IgG and Alexa 594 anti-rat IgG were from Molecular Probes (Eugene, OR); and anti-mouse IRDye 800 and anti-rabbit IRDye 680 were from Li-Cor Biosciences (Lincoln, NE).

Plasmids

The construction and characterization of eGRF, which consists of the regulatory domain of Epac (amino acids 1–619) and the Ras GEF domain of RasGRF (amino acids 1005-1244), was reported previously (Ohba *et al.*, 2003). This eGRF cDNA was subcloned into MSCVpac to generate MSCVpac-eGRF. cDNA of Shoc2 was obtained from Michael A. White (Department of Cell Biology, University of Texas Southwestern Medical Center). The cDNA of wild-type HRas was subcloned into pERedNES-FLAG, which contained the FLAG tag and an internal ribosomal entry site followed by the cDNA of Express Red (BD Biosciences, San Jose, CA) fused to the nuclear export signal at the 3′ side of the cloning site. A small interfering RNA (siRNA)-resistant Shoc2 cDNA was created by introducing two point mutations in the target sequences. The cDNA was inserted into a retroviral vector to generate pCX4bleo-5myc-Sur8-resi. A fluorescent resonance energy transfer (FRET) biosensor for Raf1, Prin-Raf1, was reported previously (Terai and Matsuda, 2005). For the stable expression, we reassembled Prin-Raf1 and developed a new biosensor named Prin-Raf1-1931, which consists of monomeric TFP (teal fluorescent protein; Ai *et al.*, 2006), a linker; Raf1, a linker; and monomeric Venus. The essential properties of Prin-Raf1-1931 were the same as those of the original Prin-Raf1. For the sake of brevity, Prin-Raf1-1931 is referred to simply as Prin-Raf1 in this study. The reasons we chose mTFP instead of cyan fluorescent protein (CFP) were, first, recombination of CFP and yellow fluorescent protein (YFP) was inevitable when the prototype Prin-Raf1 was introduced into the target cells, and, second, mTFP is brighter than CFP and is probably a better donor, as reported previously (Day *et al.*, 2008). The cDNA of Prin-Raf1 was inserted into pCX4bsr (Akagi *et al.*, 2003) to generate pCX4bsr-Prin-Raf1.

RNA Interference Experiments

RNA interference (RNAi) oligomers containing 19 nucleotides were synthesized in the sense and antisense directions with dTdT overhangs at each 3′ terminus. Human Shoc2-targeting siRNAs, Shoc2-1 (5′-UACCUUCGCUUUAAUCGUAUAdTdT-3′) and Shoc2-2 (5′-AAGCUGCCGAUGCUUGAUUUAdTdT-3′), and a scramble control siRNA (5′-CACCUAAUCCGUGGUUCAA-3′) were synthesized by Gene Design (Mino-shi, Osaka, Japan). siRNAs were transfected with RNAiMAX (Invitrogen, Carlsbad, CA) in HeLa cells according to the manufacturer's instructions. After transfection, cells were incubated for at least 42 h before analysis. For the rescue experiments, the cells were cotransfected with an siRNA and a plasmid carrying an RNAi-resistant Shoc2 gene.

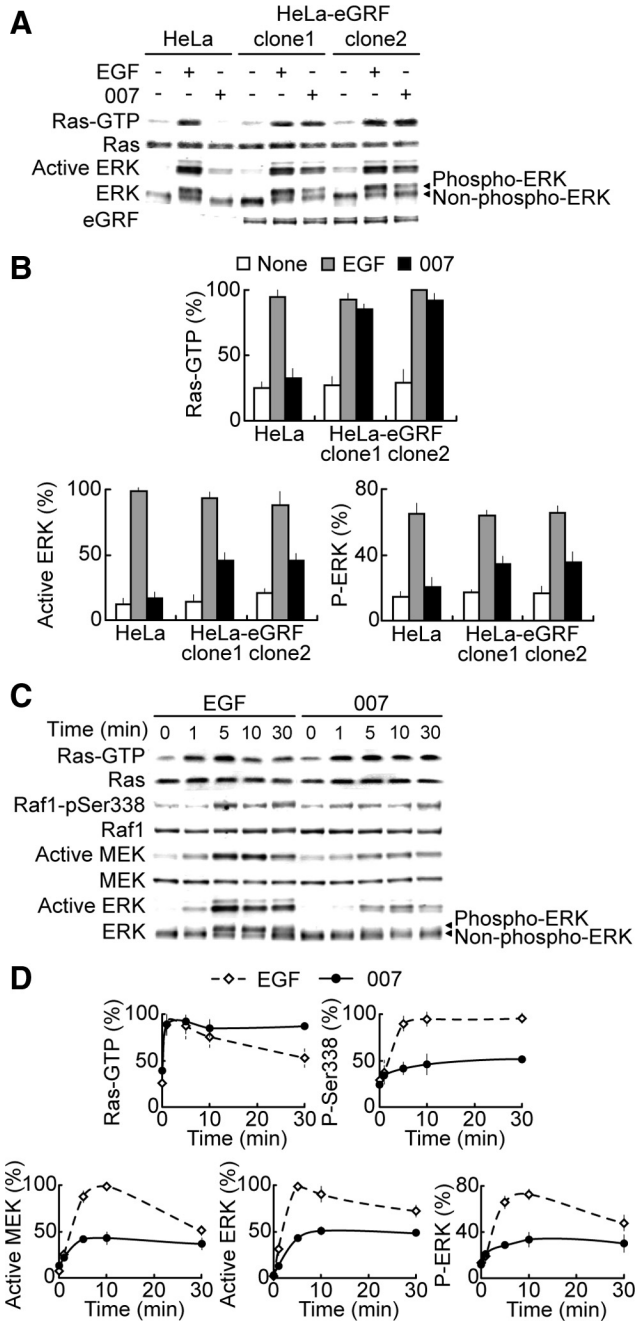

Figure 1. Establishment of HeLa-eGRF cell lines expressing eGRF. (A) Two independent HeLa cell clones expressing eGRF were established and named HeLa-eGRF clone 1 and clone 2. Cells were serum-starved for 6 h and stimulated with either 100 μM 007 or 5 ng/ml EGF for 5 min, followed by Ras-GTP pulldown analysis or immunoblotting analysis. (B) Ras-GTP was normalized for the nonstimulated HeLa cells. Active ERK detected with anti-phospho-ERK antibody in B was normalized for the EGF-stimulated parent HeLa cells. The fraction of phosphorylated ERK (P-ERK) was calculated from the ratio of phospho-ERK and nonphospho-ERK in A. (C) HeLa-eGRF-clone 1 (HeLa-eGRF1) cells were stimulated with 100 μM 007 or 5 ng/ml EGF for the indicated periods and subjected to immunoblotting analysis with the antibodies shown on the left. (D) The activity of Ras as measured by Bos' pulldown analysis and the activities of MEK, Raf1, and ERK as measured by phospho-specific antibodies in C were normalized to the maximum values during the time course and are shown by line plots. The proportion of phosphorylated ERK (P-ERK) was calculated as in B. Results of three independent experiments were averaged; error bars, ±SD (n = 3).

Cells

HeLa cells were purchased from the Human Science Research Resources Bank (Sennan-shi, Japan). The murine ecotropic retrovirus receptor (EcoVR) was first introduced by using the virus produced from BOSC23 cells by transfecting with pCX4hyg-EcoVR, the packaging plasmid pGP, and the envelope plasmid pVSV-G (Akagi *et al.*, 2003; Terai and Matsuda, 2006). Then, two HeLa-eGRF lines, clones 1 and 2 (or simply HeLa-eGRF1 and HeLa-eGRF2), were generated by infecting HeLa-EcoVR cells with a retrovirus obtained from MSCVpac-eGRF–transfected BOSC23 cells and selection with puromycin. The HeLa-eGRF1 cells were further infected with retroviruses obtained from the BOSC23 cells transfected with pCX4bsr-Prin-Raf1. Cells were maintained in DME (Sigma) supplemented with 10% FBS.

HeLa and HeLa-eGRF1 cells were serum-starved for 6 h and stimulated with either 5–50 ng/ml EGF, 100 μM 007, 1 μM ionomycin, or a combination of these reagents. The final concentrations of the inhibitors were as follows: 10 μM LY294002, 1 μM Gö6983, 10 μM U73133, 10 μM U73343, 10 μM U0126, and 100 μM BAPTA. The cells were pretreated with these inhibitors for 20 min before stimulation.

Fluorescence Live Cell Imaging

The cells were plated on 35-mm glass-base dishes (Asahi Techno Glass, Tokyo, Japan), which were coated with collagen type I (Nitta Gelatin, Osaka, Japan), and maintained in phenol red–free MEM (Nissui, Tokyo, Japan). Cos-1 cells and HeLa cells expressing the Prin probes were imaged every 1 min on an Olympus IX81 inverted microscope (Tokyo, Japan) that was equipped with a cooled CCD camera, CoolSNAP HQ (Roper Scientific, Tucson, AZ), and controlled by MetaMorph software (Universal Imaging, West Chester, PA). For the dual-emission ratio imaging of the probes, we used a 440AF21 excitation filter, a 455DRLP dichroic mirror, and two emission filters, 480AF30 for CFP and 535AF26 for FRET (Omega Optical, Brattleboro, VT). The cells were illuminated with a 75-W Xenon lamp through a 25% neutral density filter (Olympus) and a 60× oil immersion objective lens. The exposure time was 0.5 s when the binning of the CCD camera was set to 8 × 8. After background subtraction, the ratio image of FRET to CFP was created with MetaMorph software, and the results were used to represent FRET efficiency. In some experiments, coexpression of monomeric red fluorescent protein (mRFP) was confirmed with a 580AF20 excitation filter and a 635DF55 emission filter. The translocation index was calculated as described previously (Aoki *et al.*, 2007).

Intermolecular FRET imaging was conducted as described previously (Terai and Matsuda, 2005). Briefly, HeLa cells expressing CFP-KRas and YFP or CFP-KRas and Shoc2-YFP were time-lapse imaged by the use of the following filter sets: CFP (a 440AF21 excitation filter, a glass dichroic mirror, and a 480AF30 emission filter), FRET (a 440AF21 excitation filter, a glass dichroic mirror, and a 535AF26 emission filter), and YFP (a 510DF23 excitation filter, a glass dichroic mirror, and a 560DF15 emission filter). From the FRET images, spectral bleedthrough and cross-excitation were deduced to obtain the corrected FRET image (cFRET).

Ras-GTP Pulldown Assay

GTP-bound Ras proteins were detected by Bos' pulldown method with slight modification (Franke *et al.*, 1997; Ohba *et al.*, 2001).

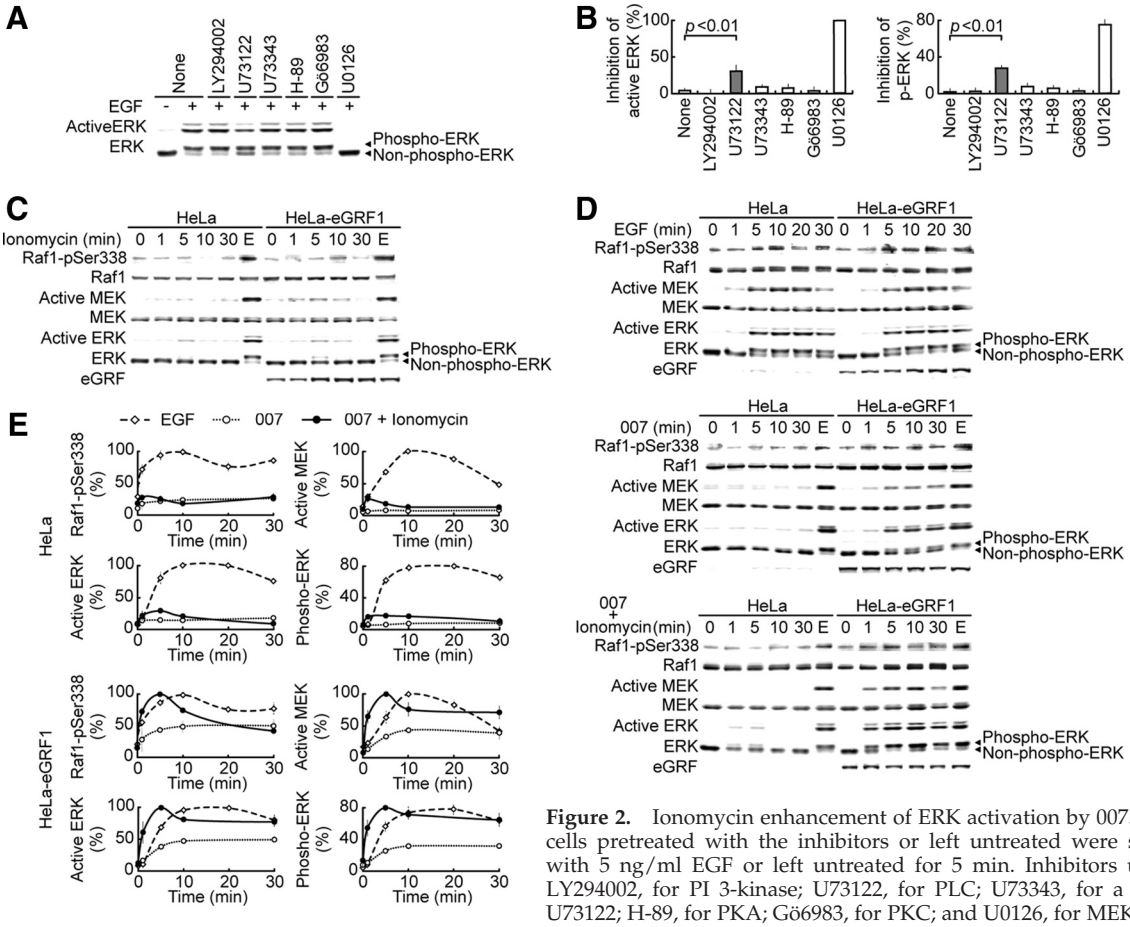

Figure 2. Ionomycin enhancement of ERK activation by 007. (A) HeLa cells pretreated with the inhibitors or left untreated were stimulated with 5 ng/ml EGF or left untreated for 5 min. Inhibitors used were LY294002, for PI 3-kinase; U73122, for PLC; U73343, for a control of U73122; H-89, for PKA; Gö6983, for PKC; and U0126, for MEK. The cells were lysed and subjected to SDS-PAGE and immunoblotting analysis with the antibodies against the proteins depicted on the left. (B) The levels of active ERK and phospho-ERK are quantified as in Figure 1D. The difference was evaluated by Student's *t* test ($p < 0.01$, $n = 3$). (C) HeLa and HeLa-eGRF1 cells were stimulated with 1 μM ionomycin for the indicated periods and subjected to immunoblotting analysis. For the controls (shown as E), the cells were also stimulated with 5 ng/ml EGF for 5 min. (D) HeLa and HeLa-eGRF1 cells were stimulated with 5 ng/ml EGF, 100 μM 007, or 1 μM ionomycin and 100 μM 007 for the indicated periods and subjected to immunoblotting analysis. For the controls (shown as E), the cells were also stimulated with 5 ng/ml EGF for 5 min. (E) The levels of active Raf1, active MEK, active ERK, and phospho-ERK were quantified as in B. Results of three independent experiments were averaged; error bars, ±SD ($n = 3$).

RESULTS

Ras Activation Is Not Sufficient for the Activation of Raf1, MEK, and ERK

There is ample evidence for the essential role of Ras in the EGF-mediated ERK activation. However, it has been uncertain whether Ras activation is sufficient for the full activation of ERK in EGF-stimulated cells, mainly because there has been no tool for activating Ras directly and specifically. To overcome this problem, we took advantage of a recombinant GEF named eGRF, which consists of the regulatory domain of Epac1, cAMP-responsive Rap1GEF, and the catalytic domain of RasGRF1 (Ohba *et al.*, 2003). eGRF can be activated by 007, an Epac-specific cAMP agonist, without perturbing the PKA pathway. We stably transfected the eGRF expression plasmid into HeLa cells and established two cell lines, HeLa-eGRF1 and -eGRF2. Because the expression of Epac (Epac1 and Epac2) is mostly restricted to neuronal cells (Kawasaki *et al.*, 1998; de Rooij *et al.*, 1998) and is not detected in HeLa cells, 007 activates only the Ras protein in these cell lines. Administration of 007 activated Ras in HeLa-eGRF1 and -eGRF2 to the same degree as did administration of EGF (Figure 1A). ERK activation was measured by either reactivity to anti-active-ERK antibody or phosphorylation-dependent band-shift in the SDS-PAGE gel. To discriminate these two species of ERK, we refer them active ERK and phospho-ERK, respectively. We found ERK activation was significantly lower in 007-stimulated HeLa-eGRF cells than EGF-stimulated HeLa-eGRF cells (Figure 1B). As expected, neither Ras nor ERK was significantly activated by 007 in the parent HeLa cells. We further characterized HeLa-eGRF1 in the following studies. As shown in Figure 1, C and D, the time course and the strength of Ras activation by 007 were almost comparable to those by EGF. In contrast, the activations of Raf1, MEK, and ERK were markedly lower in 007-stimulated cells than in EGF-stimulated cells. In addition, we did not find any effect of 007 in EGF-stimulated cells, negating the possibility that 007 inhibited Raf1 activation independent of Ras (data not shown). The results clearly demonstrated that a certain quantitative level of Ras activation does not warrant subsequent full activation of Raf1, MEK, and ERK, suggesting the presence of a signal enhancer of Ras.

It is possible that the difference between EGF and 007 arises from the specificity of GEFs toward the Ras isoforms, HRas, KRas, and NRas. To exclude this possibility, we examined the levels of Ras-GTP with isoform-specific antibodies. As shown in Supplementary Figure 1, we could detect KRas and NRas, but not HRas in HeLa cells. Both KRas and HRas were activated by 007 as efficiently as EGF. This observation agrees with the previous report showing that Ras isoforms are redundant for EGF-activated ERK activation and that HRas is a minor isoform in HeLa cells compared with KRas and NRas (Omerovic *et al.*, 2008). Thus, we used anti-pan Ras antibody in the following studies and did not discriminate the Ras isoforms, unless it is clearly stated.

Phospholipase-mediated Ca^{2+} Influx Enhances Ras-mediated ERK Activation

As an initial screening for the signal enhancer of Ras, we examined the contribution of phosphoinositide-3 kinase (PI3K), PKC, and phospholipase C (PLC) with specific inhibitors (Figure 2, A and B). Among them, we found that a

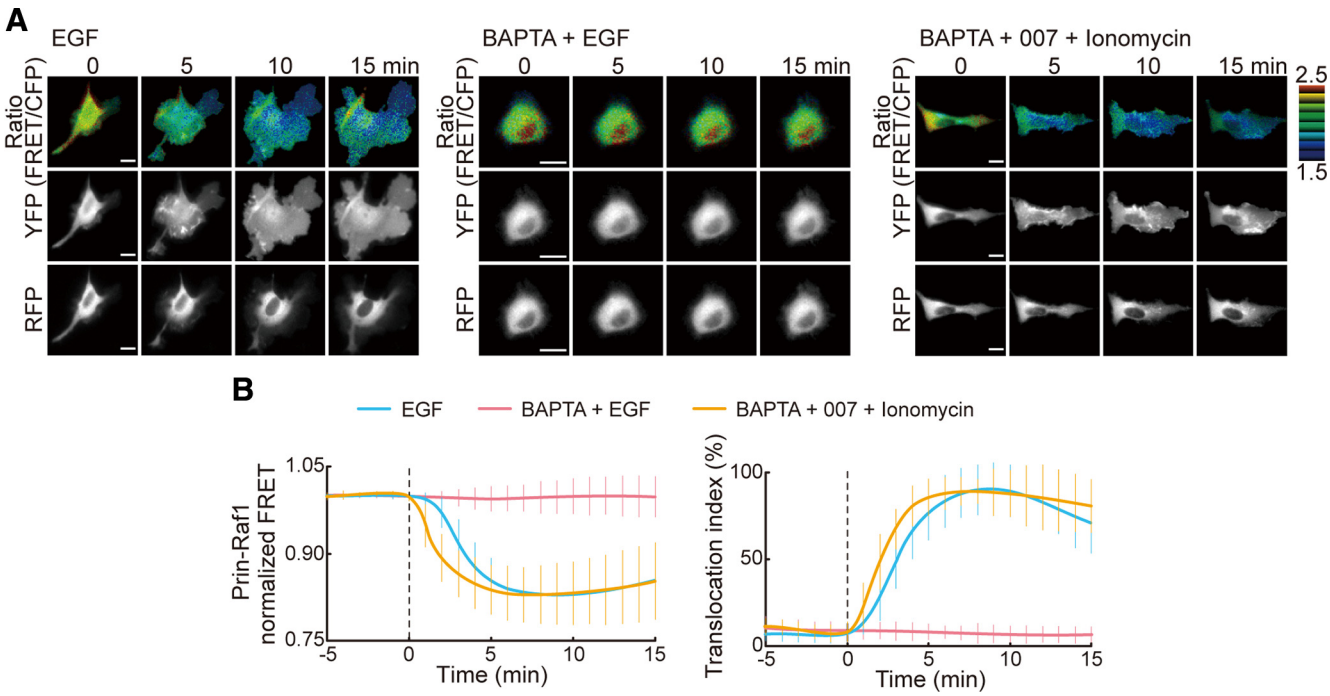

Figure 3. An essential role of Ca^{2+} in the plasma membrane recruitment and activation of Raf1. The activity of Raf1 was monitored with a FRET biosensor, Prin-Raf1. Prin-Raf1 adopts either a closed-inactive high-FRET or open-active low-FRET conformation. (A) HeLa-eGRF1 cells expressing Prin-Raf1, HRas, and mRFP were stimulated with 50 ng/ml EGF and time-lapse–imaged for CFP (ex. 440 nm/em. 480 nm) and FRET (ex. 440 nm/em. 530 nm). The ratio image of FRET/CFP is used to represent the FRET efficiency, and the FRET image is used to show the localization of the biosensor. The mRFP protein was used as a standard to calculate the translocation index as described previously (Aoki *et al.*, 2007). Representative images before and after EGF stimulation are shown. The color bars show the range of FRET/CFP. White scale bar, 10 μm. (B) The FRET/CFP ratio normalized to the values before stimulation and the translocation index are averaged and plotted over time. Bars, $\pm$SD (n $\geqq$ 15).

PLC inhibitor U73122 suppressed EGF-induced ERK activation/phosphorylation to ca. 50% of the control. Because a PKC inhibitor Gö6983 did not inhibit EGF-induced ERK activation, we speculated that a PLC-mediated increase in Ca^{2+} influx enhanced the Ras-mediated ERK activation in a PKC-independent manner. An ionomycin-induced increase in Ca^{2+} activated ERK, albeit much more weakly than did EGF (Figure 2C). In contrast, costimulation of Hela-eGRF1 cells with 007 and ionomycin induced ERK activation as early and strongly as did EGF in HeLa-eGRF1 cells (Figure 2, D and E). The ERK activation by 007 plus ionomycin was not suppressed by any of the inhibitors of PI3K, PKC, or PLC used in this study (data not shown). These observations strongly suggested that, in addition to the Ras activation, the PLC-mediated increase in Ca^{2+} concentration is essential for the full activation of Raf1, MEK, and ERK in HeLa cells.

Ca^{2+} Influx Promotes Raf1 Recruitment to Ras at the Plasma Membrane

To understand the mechanism by which Ca^{2+} activates Raf1, we used a FRET biosensor for Raf1, Prin-Raf1 (Terai and Matsuda, 2005). The FRET level of this biosensor is high in the closed inactive conformation and low in the active open conformation. As we reported previously, upon EGF stimulation Prin-Raf1 moved from the cytoplasm to the plasma membrane and adopted an open active conformation in a Ras-dependent manner (Figure 3A, top). Pretreatment with the Ca^{2+} chelator BAPTA almost completely inhibited Raf1 translocation and activation (Figure 3A, middle). This inhibitory effect of BAPTA could be overridden by costimulation with 007 and ionomycin (Figure 3A, bottom), probably because the ionomycin-induced increase in intracellular Ca^{2+} concentration exceeded the concentration of BAPTA in the cells. Quantification of images more convincingly showed that the increase in the intracellular Ca^{2+} concentration was essential for the Ras-mediated plasma membrane translocation and subsequent conformational change of Raf1 (Figure 3B).

Shoc2 Mediates Ca^{2+} Enhancement of Raf1 Activation

The observation that an increase in Ca^{2+} concentration was required for Raf1 translocation to the plasma membrane led us to examine the contribution of scaffold proteins, which provide the platform for Ras binding to Raf1 (Morrison and Davis, 2003). During the course of screening with siRNA-mediated knockdown of scaffold proteins, we found that knockdown of Shoc2 inhibited EGF-induced Raf1 activation most prominently in HeLa cells. As shown in Figure 4, A and B, knockdown of Shoc2 inhibited plasma membrane translocation and conformational change of Raf1 not only by EGF stimulation but also costimulation with 007 and ionomycin. In these Shoc2-knockdown cells, the strengths and the time courses of Raf1 activation by EGF and 007 plus ionomycin were similar to those by 007 alone. Importantly, Shoc2 knockdown did not inhibit the weak Raf1 activation induced by 007 alone. These observations strongly suggested that the Ca^{2+} enhancement of Raf1 activation was mediated primarily by Shoc2. We confirmed that the Ras activation was not inhibited by the knockdown of Shoc2 (Figure 4C). In fact, we observed an increase in the level of Ras activation in Shoc2-knockdown cells. This observation suggested that a negative feedback loop might be impaired in Shoc2-knockdown cells; however, we have not yet pursued this issue.

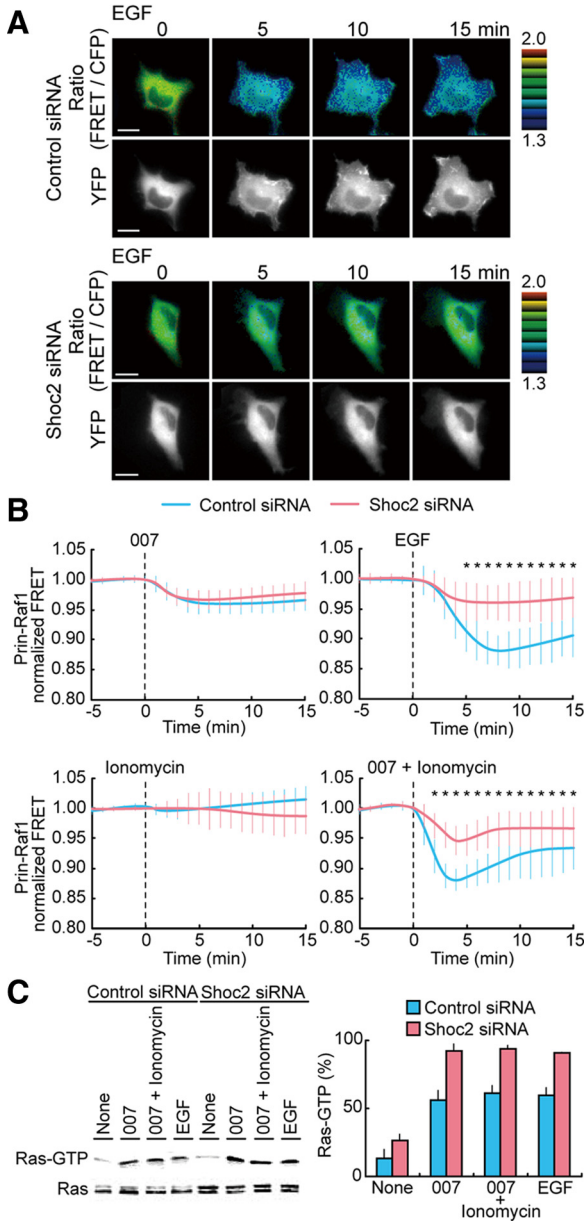

Figure 4. Effect of Shoc2 knockdown on Raf1 activation. (A) HeLa-eGRF1 cells stably expressing Prin-Raf1 were transfected with either Shoc2-specific siRNA or scrambled siRNA. Twenty-four hours after transfection, cells were further transfected with expression plasmids for HRas and mRFP. Time-lapse imaging was performed as in Figure 3. (B) HeLa-eGRF1 cells prepared as in A were stimulated with 50 ng/ml EGF, 100 μM 007, 1 μM ionomycin, or 100 μM 007 and 1 μM ionomycin. Bars, $\pm$SD ($n \geqq 15$). The asterisks show the time points in which difference between the control and Shoc2-knockdown cells was significant by Student's t test ($p < 0.00001$). (C) HeLa-eGRF1 cells were transfected with either Shoc2-specific siRNA or scrambled siRNA. Forty-two hours after transfection, cells were serum-starved for 6 h and stimulated with 5 ng/ml EGF, 100 μM 007, or 100 μM 007 and 1 μM ionomycin for 5 min, followed by Ras-GTP pulldown assay. Results of three independent experiments were averaged; error bars, $\pm$SD ($n = 3$).

Shoc2 Mediates Ca^{2+} Enhancement of Activation of MEK and ERK

In agreement with the suppression of Raf1 activation by Shoc2 knockdown, the activation of both MEK and ERK was

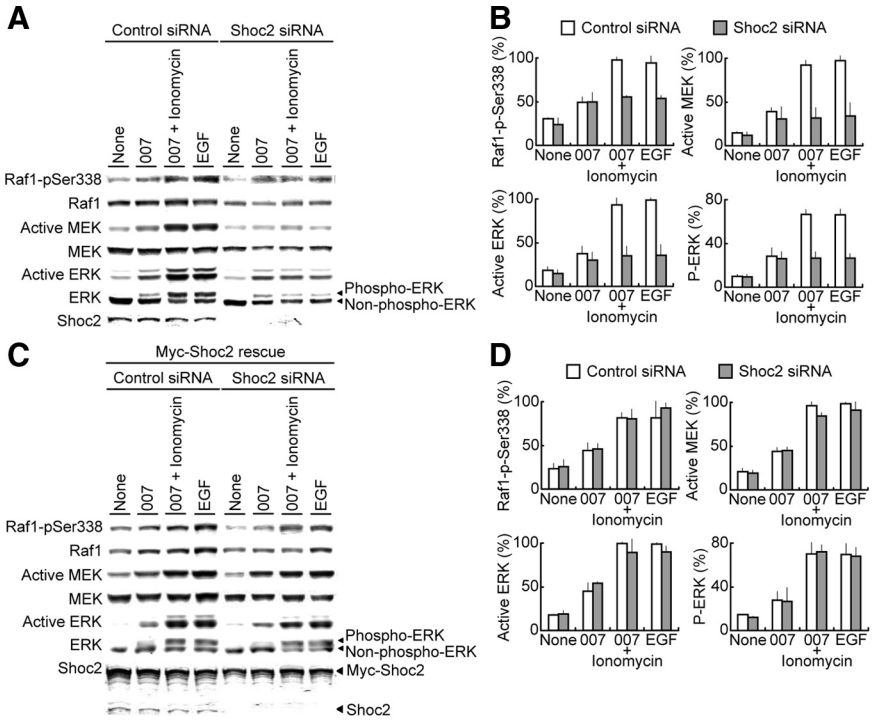

Figure 5. Reversal of the effect of Shoc2 knockdown by exogenous Shoc2 expression. (A) HeLa-eGRF1 cells were transfected with either Shoc2-specific siRNA or scrambled siRNA. Forty-two hours after transfection, cells were serum-starved for 6 h and stimulated with 5 ng/ml EGF, 100 μM 007, or 100 μM 007 and 1 μM ionomycin for 5 min, followed by immunoblotting analysis. (B) The activity of Raf1 and MEK as measured with the phospho-specific antibodies was normalized to the maximum values of scramble siRNA-transfected EGF-stimulated cells. The fraction of phospho-ERK was calculated from the ratio of phospho-ERK versus ERK shown in A. Averages are shown ± SD (n = 3). (C) HeLa-eGRF1 cells stably expressing siRNA-resistant Myc-tagged Shoc2 were transfected with either Shoc2-specific siRNA or scrambled siRNA. Forty-two hours after transfection, cells were serum-starved for 6 h and stimulated with 5 ng/ml EGF, 100 μM 007, or 100 μM 007, and 1 μM ionomycin for 5 min, followed by immunoblotting analysis. (D) The activities of Raf1, MEK, and ERK were measured as in C. Averages are shown ± SD (n = 3).

also suppressed by Shoc2 knockdown in cells stimulated with EGF or 007 plus ionomycin (Figure 5A and 5B). This effect of Shoc2 knockdown was counteracted by the expression of Shoc2 cDNA, which was resistant to the siRNA used in this experiment, validating the specificity of siRNA against Shoc2 (Figure 5, C and D).

A Calmodulin Inhibitor Enhances Raf1 Activation in a Shoc2-dependent Manner

To gain further insight into the role of Ca^{2+} and Shoc2 in Raf1 activation, we examined the effect of a competitive inhibitor of calmodulin, W-13, in HeLa-eGRF1 cells (Figure 6A). As reported previously (Moreto *et al.*, 2008), W-13 alone increased Raf1 activation, albeit weakly. When cells were costimulated with W-13 and 007, Raf1 was activated significantly. Importantly, this W-13–mediated enhancement of 007-induced Raf1 activation was abolished by the ablation of Shoc2 (Figure 6B). This observation strongly suggested that calmodulin inhibited Shoc2-mediated binding of Ras to Raf1 and that Ca^{2+} suppressed this calmodulin inhibition of Ras binding to Raf1.

To directly assess this possibility, we examined the Shoc2 binding to Ras upon W-13 treatment by the intermolecular FRET method with CFP-KRas and Shoc2-YFP (Figure 7). We found that the level of FRET increased significantly upon treatment with 007 and W-13 or 007 and ionomycin, indicating the Shoc2 association with KRas. In conclusion, the results shown here strongly argue that calmodulin inhibits Shoc2 association with Ras and thereby formation of the Ras-Shoc2-Raf1 complex and that Ca^{2+} enhances Ras to Raf1 signaling by suppressing calmodulin's inhibition of Shoc2 binding to Ras. We performed similar experiments by using CFP-HRas and obtained essentially the same results (data not shown).

DISCUSSION

Deciphering the role of Ca^{2+} on the growth factor-induced activation of Ras/Raf/MEK/ERK signaling cascades is extremely difficult because of the pleiotropic effects of Ca^{2+} on the activities and expression levels of a number of signaling molecules (Agell *et al.*, 2002; Cullen, 2006). Therefore, for a more precise understanding of the role of Ca^{2+} in each signaling step, the immediate upstream regulator must be activated to minimize the effect of signaling from the upstream regulators. Here, with the help of a synthetic GEF for Ras, eGRF, and its specific ligand, 007, we have shown that Ras requires additional signals from Ca^{2+} for the full acti-

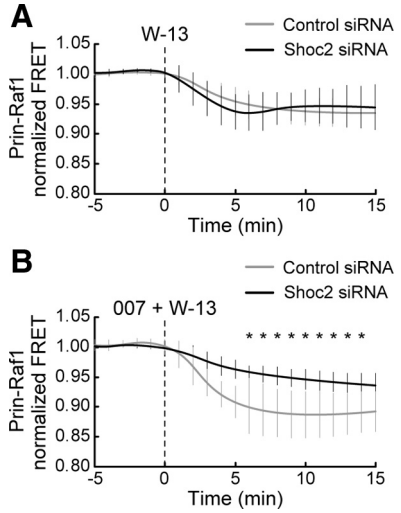

Figure 6. Raf1 activation by a competitive calmodulin inhibitor W-13 in a Shoc2-dependent manner. HeLa cells coexpressing eGRF, Prin-Raf1, and HRas were stimulated with 100 μM W-13 or 100 μM W-13 + 100 μM 007. The FRET/CFP ratio normalized to the values before stimulation was averaged and plotted over time (n = 12). The asterisks show the time points in which the difference between the control and Shoc2-knockdown cells was significant by Student's *t* test ($p < 0.0001$).

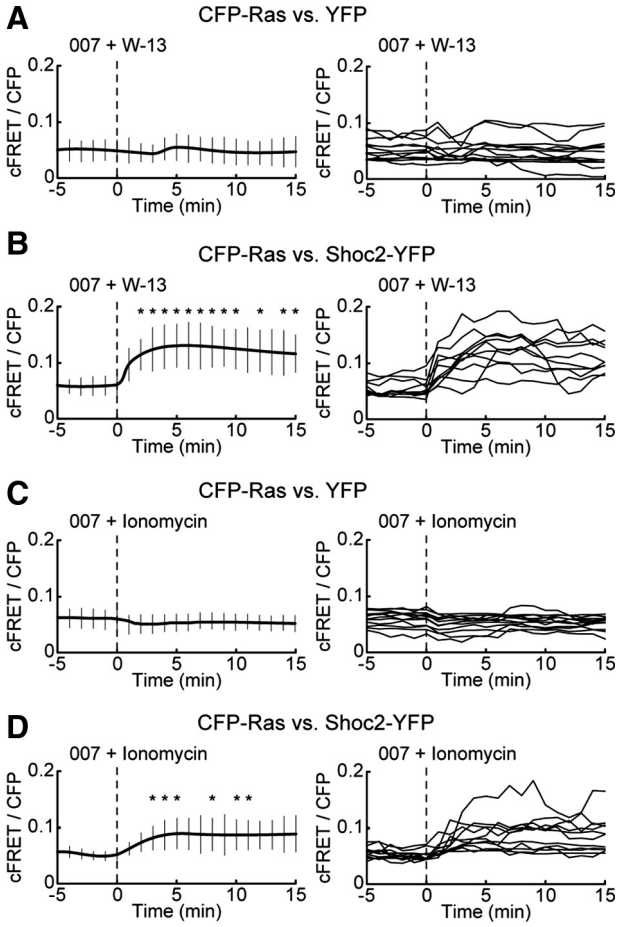

Figure 7. Shoc2 association with Ras by the calmodulin inhibitor or calcium ionophore. HeLa-eGRF1 cells expressing CFP-KRas and YFP (A and C) or Shoc2-YFP (B and D) were stimulated with 100 μM W-13 + 100 μM 007 (A and B) or 1 μM ionomycin + 100 μM 007 (C and D) and time-lapse–imaged as described in the text. The ratio of corrected FRET versus CFP was averaged over each cell and plotted against time. Shown are averages ± SD (left panel) and data of each cell (right panel; n = 11). The asterisks show the time points in which difference from time = 0 was significant by Student's *t* test ($p < 0.001$).

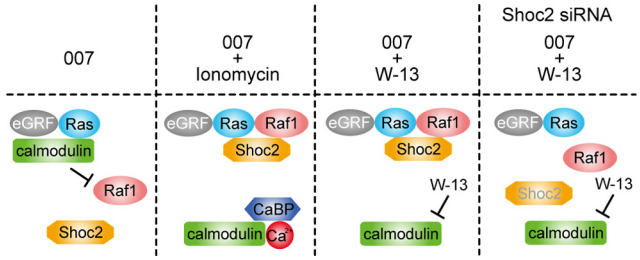

Figure 8. A model for the integration of Ca^{2+} signals by Shoc2. See the text for details.

vation of Raf1. We propose for the following reasons that Shoc2 mediates this Ca^{2+}-mediated enhancement of Raf1 activation. First, the level of Raf1 activation by 007 was similar to that by EGF in Shoc2-knockdown cells (Figure 4B). Second, the level of 007-mediated Raf1 activation could not be enhanced by ionomycin in Shoc2-knockdown cells (Figure 4B). Third, ionomycin could not enhance 007-induced activation of MEK and ERK in Shoc2-knock down cells (Figure 5). Fourth, W-13–mediated Raf1 activation was abolished by the ablation of Shoc2 (Figure 6). Finally, we found that both ionomycin and W-13 cooperated with 007 in the Shoc2 association with Ras (Figure 7).

The Shoc2- and Ca^{2+}-mediated enhancement of the plasma membrane recruitment of Raf1 seems to be related to the negative regulation of Ras signaling by calmodulin. Villalonga *et al.* (2001) reported that calmodulin bound both KRas and Raf1 in Swiss 3T3 fibroblasts. In their study, Ca^{2+}-bound calmodulin was suggested to inhibit KRas and thereby to suppress KRas-dependent ERK activation. Of note, they also observed that W-13–mediated inhibition of calmodulin has a positive effect on KRas signaling in the absence of any stimuli in quiescent Swiss 3T3 fibroblasts. This observation strongly suggests that the calcium concentration of the quiescent cells is sufficient to inhibit KRas in a calmodulin-dependent manner. Recently, Moreto *et al.* (2008, 2009) reported that a calmodulin inhibitor induced association of HRas and KRas with Raf1 in COS-1 cells. This observation also supports the idea that calmodulin binds to HRas and KRas and suppresses their binding to Raf1 at the basal calcium concentration. In the Ca^{2+}-gated K^+ channel, calmodulin was shown to associate with the channel constitutively, and Ca^{2+} triggered the conformational change of the calmodulin-K^+ channel complex, thereby opening the gate (Hoeflich and Ikura, 2002). In analogy to this, calmodulin may suppress spontaneous signaling from Ras to Raf1 at the basal calcium concentration and may allow Ras-GTP to bind to Raf1 only at a high calcium concentration.

Also taking into consideration our observations that Ca^{2+} could induce Raf1 binding to Ras in a Shoc2-dependent manner (Figure 4) and that the number of molecules of Ras-GTP is in a large excess of that of Raf1 even in quiescent cells (Fujioka *et al.*, 2006), we propose the following scenario for the role of Shoc2 and calmodulin in Ras regulation (Figure 8). In the absence of Ca^{2+}, calmodulin binds to Ras-GTP and inhibits it from binding to Shoc2. In the presence of a higher Ca^{2+} concentration, Ca^{2+}/calmodulin changes its conformation, thereby leaving Ras-GTP for binding with Shoc2. The complex of Shoc2 and Ras-GTP provides a platform for recruiting Raf1 to the plasma membrane, thereby triggering the MEK-ERK signaling cascade. W-13 inhibits calmodulin binding to Ras, thereby allowing Shoc2 to bind to Ras to enhance Ras binding to Raf1. In the absence of Shoc2, Ras binds to Raf1 with low efficiency, even if calmodulin is inhibited. In this scenario, 007 serves to increase the level of Ras-GTP and to increase the fraction of Shoc2 that associates with Ras-GTP. A potentially important player that we need to characterize in the future is M-Ras, which has been shown to bind to Shoc2 and PP1c-holoenzyme (Rodriguez-Viciana *et al.*, 2006). It is be possible that M-Ras is also suppressed by calmodulin and that Ca^{2+} may contribute ERK activation via PP1c-mediated Raf1 activation.

We have shown that Ca^{2+} induces dimerization of BRaf via the BRaf-specific N-terminal region of Raf1 (Terai and Matsuda, 2006). Interestingly, calmodulin binds to Raf1 but not BRaf (Egea *et al.*, 2000). This observation may suggest that the binding between Ras-GTP and Raf proteins requires additional reinforcement from the Ca^{2+} signaling pathway. The N-terminal BRaf-specific region and Shoc2 may play an equivalent role for BRaf and Raf1, respectively. In relation to this issue, we noted that the weak Raf1 activation by W-13 was independent of Shoc2, indicating that the Shoc2 enhancement of Raf1 binding to Ras-GTP could be manifested only in the presence of large amount of Ras-GTP (Figure 6).

This observation might lead to another hypothesis that Shoc2 may facilitate the formation of a hetero-tetramer consisting of two Raf1 and two Ras-GTP molecules. Of note, the numbers of Raf1 and BRaf molecules in HeLa cells used in this study are 3×10^4/cell and 2×10^3/cell, respectively. Therefore, in the HeLa cells used in this study, the Shoc2-Raf1 pathway is dominant over BRaf.

Another scaffold protein for the Raf proteins, IQGAP1, has recently been shown to be regulated by Ca^{2+}/calmodulin (Ren *et al.*, 2008). Addition of Ca^{2+}, which binds to both BRaf and IQGAP1, resulted in a twofold increase in their binding to each other in vitro. This interaction was completely abolished in the presence of Ca^{2+}/calmodulin. In agreement with this in vitro data, BAPTA increased the BRaf binding to IQGAP1 and concomitantly activated BRaf in mouse embryonic fibroblasts. EGF, which has been shown to increase the cytoplasmic concentration of Ca^{2+} (Jorissen *et al.*, 2003), also promoted BRaf association with IQGAP1. Ca^{2+} has been suggested to play a role in the negative feedback in this scheme; however, it is also possible that the substantial effect of calcium ionophores or chelators used in this study may reflect a change in the activity of Ras, which can be either positively or negative modulated by Ca^{2+} (Cullen, 2006).

We have shown that inhibition of PLC suppressed EGF-induced ERK activation to ca. 50% of the full activation (Figure 2, A and B). This observation may contradict the previous report negating the role of PLCγ1 in EGF-induced ERK activation in PLCγ1-deficient mouse embryonic fibroblasts (Ji and Carpenter, 2000). The reason for this discrepancy may be simply ascribable to the cell type difference or to the use of mouse embryonic fibroblasts, in which knockout of a gene often induces another gene to compensate for the defect.

In conclusion, we have identified a role played by Shoc2 and Ca^{2+}/calmodulin in the signaling from Ras to Raf1. Needless-to-say, Ca^{2+} modulates EGF signaling to ERK in many ways. For the future systemic analysis of this signaling cascade, it would be extremely important to establish methodology for the analysis of each step by insulating the effect of upstream regulators. Here, by adducing Ca^{2+}-dependent activation of Raf1, we have shown that the synthetic GEFs could be versatile tools for this purpose.

ACKNOWLEDGMENTS

We thank Michael White, A. Miyawaki (Brain Science Institute, RIKEN), Tsuyoshi Akagi (KAN Research Institute), and J. Miyazaki (Osaka University School of Medicine) for provision of the plasmids and A. Nishiyama-Abe and Y. Kasakawa for their technical assistance. We are grateful to the members of the Matsuda laboratory for their helpful discussions. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas and Innovative Techno-Hub for Integrated Medical Bio-imaging Project of the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, and Science of Japan; Sagawa Cancer Research Grant; and by the Kyoto University Global COE Program Center for Frontier Medicine.

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