Multiple Decisive Phosphorylation Sites for the Negative Feedback Regulation of SOS1 via ERK

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EGF-induced activation of ERK has been extensively studied by both experimental and theoretical approaches. Here, we used a simulation model based mostly on experimentally determined parameters to study the ERK-mediated negative feedback regulation of the Ras guanine nucleotide exchange factor, son of sevenless (SOS). Because SOS1 is phosphorylated at multiple serine residues upon stimulation, we evaluated the role of the multiplicity by building two simulation models, which we termed the decisive and cooperative phosphorylation models. The two models were constrained by the duration of Ras activation and basal phosphorylation level of SOS1. Possible solutions were found only in the decisive model wherein at least three, and probably more than four, phosphorylation sites decisively suppress the SOS activity. Thus, the combination of experimental approaches and the model analysis has suggested an unexpected role of multiple phosphorylations of SOS1 in the negative regulation.

Dynamic control of the extent and kinetics of this EGF-ERK signaling is governed by positive and negative feedback loops. One example of the positive feedback regulation is a Ras-mediated increase in SOS GEF activity by an allosteric mechanism (5). A few examples of the negative feedback regulation include EGF receptor internalization mediated by Grb2, EGF receptor degradation induced by Cbl (for a review, see Ref. 6), and suppression of SOS GEF activity by ERK-mediated phosphorylation (7–9).

Several research groups have studied the role of growth factor-induced SOS phosphorylation but do not necessarily reach the same conclusion (7–12). Among the two SOS isoforms, SOS1 and SOS2, only SOS1 is phosphorylated by ERK (9). Growth factor-induced phosphorylation of SOS1 is mediated mostly by ERK, which phosphorylates at least four serine residues in the C-terminal region of SOS1 (8, 9, 12). All these previous studies agree with the negative regulation by ERK phosphorylation of SOS1, but the mechanism is controversial. In some studies, the phosphorylation of SOS1 is suggested to induce disassembly of the Grb2-SOS1 complex, thereby terminating SOS1-dependent Ras activation (9–11). However, in others, it is reported that the phosphorylation of SOS1 does not affect the binding of SOS1 to Grb2 but does induce dissociation of the Grb2-SOS complex from the activated EGF receptor (8, 12). Notably, in these previous studies, the role of multiplicity of phosphorylation sites has not been studied.

Kinetic simulation models have been increasingly used to clarify this complex network of the EGF-ERK signaling pathway (for a review, see Ref. 13). Each kinetic simulation model reported previously recapitulates the stimulus-induced ERK activation fairly well. Nevertheless, the parameters used therein are sometimes astonishingly different from each other (14). One apparent reason for this discrepancy is that these studies often use different algorithms to fit the parameters to the experimental data (15–17). Another reason may be that some parameters are derived from in vitro experiments, which may not reflect in vivo conditions. For these reasons, development of a kinetic simulation model using parameters collected in living cells or under physiological conditions is awaited as a solid basis for future theoretical studies.

To collect and evaluate the parameters in living cells, we previously adopted fluorescent protein technologies and developed a kinetic simulation model based on the experimentally validated parameters (14). This simulation model contained only four signaling molecules, Ras, Raf, MEK, and ERK, but still
successfully reproduced the essential features of the Ras-ERK MAPK pathway and demonstrated the usefulness of the parameters collected in living cells.

In this study, we have extended this approach to include SOS in this model. First, we confirmed the role of SOS phosphorylation in the negative feedback loop and examined the dephosphorylation rate in the cells. Second, the role of multiple phosphorylation sites was studied with the kinetic simulation models based on the experimentally validated parameters. We found that the multiple phosphorylation sites must independently and decisively suppress SOS GEF activity to reproduce the transient Ras activation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—U73122, Gö6983, LY294002, SB203580, PP2, FR180204, and rapamycin were purchased from Calbiochem. EGF, U0126, puromycin, and blasticidin S were purchased from Sigma-Aldrich. Small interfering RNA (siRNA) oligonucleotide against human SOS2 (sense, 5'GCCUUUGCUAGAAAAUGCAGAAACU-3') was purchased from iGENE Therapeutics (Tsukuba, Japan). Stealth RNAi negative control duplex (Invitrogen) was used as a control siRNA. Anti-phospho-ERK (Ab-1) was purchased from Calbiochem. Anti-phospho-EGFR (2236) were purchased from Cell Signaling Technology (Danvers, MA). Anti-SOS1 (610096), anti-phosphotyrosine (610000), and anti-ERK (610123) were purchased from BD Transduction Laboratories.

**Cell Culture**—HeLa cells were obtained from the Human Science Research Resources Bank (Sennan-shi, Japan) and maintained in DMEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (Invitrogen). BOSC23 cells were purchased from ATCC (Manassas, VA) and maintained in DMEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (Invitrogen). BOSC23 cells were maintained in DMEM prepared in our laboratory.

**Stable Expression of FRET Probes by Retrovirus-mediated Expression**—We previously reported the FRET probes for Ras activity and tyrosine kinase activity, Raichu and Picchu, respectively (18, 19). Retroviral expression plasmids for Raichu and Picchu were constructed as follows: cDNA for monomeric TFP (Allele Biotechnology, San Diego, CA) was PCR-amplified and substituted for cyan fluorescent protein (CFP). The cDNA of the TFP version of FRET probes was inserted into a retroviral domain of FKBP12. The retroviral vector pCX4puro-LDR encodes a fusion protein consisting of the N-terminal myristoylation signal of Lyn and the FK506-rapamycin binding domain of mammalian target of rapamycin (mTOR). HeLa cells infected with retroviruses derived from pMCsbsr-cRaf-FKBp and pCX4puro-LDR were selected with 2.0 μg/ml puromycin and 10 μg/ml blasticidin S. Cells were stimulated with 50 nM rapamycin for 10 min and subjected to immunoblotting analysis.

**Immunoblotting and Ras-GTP pulldown Assay**—siRNA oligonucleotides were transfected with RNAi MAX (Invitrogen) according to the manufacturer's protocol. Other plasmids were transfected with 293Fectin (Invitrogen). Lysed cells were analyzed by SDS-PAGE followed by immunoblotting. Precast SDS-polyacrylamide gels were purchased from Wako Pure Chemical Industries or System Instruments (Tokyo, Japan). Immunoblotting was performed according to the protocols of each manufacturer of the antibodies. Bound antibodies were detected with secondary antibodies conjugated with IRDye680 or IRDye800 and analyzed with an Odyssey Imager system (LI-COR, Lincoln, NE). For detection of Ras-GTP, Bos and co-workers’ (22) pulldown assay with the GST-tagged Ras-binding domain of cRaf was performed as described previously.

**Quantification of EGFR and SOS1**—Protein concentrations of the endogenous EGFR and SOS1 in HeLa cells were determined as described previously (14). First, serial dilutions of total HeLa cell lysates expressing EGFP-tagged EGFR and SOS1 and GST-tagged YFP were applied to the same SDS-polyacrylamide gels, transferred to PVDF membrane, and probed with anti-GFP antibody. The bound antibody was quantified with an Odyssey Imager system to plot a calibration curve. Second, the EGFP-EGFR and EGFP-SOS1 were used as the standard to quantify the endogenous EGFR and SOS1. Recombinant GST-tagged YFP was prepared in our laboratory (23). Expression plasmids for EGFR-EGFP and EGFP-mSOS1 were also prepared in our laboratory.

**Imaging with FRET Probes**—HeLa cells expressing FRET probes were plated on 35-mm glass-based dishes (Asahi Techno Glass, Tokyo, Japan), which were coated with collagen type I (Nitta Gelatin Inc., Osaka, Japan), and maintained in phenol red-free DMEM (Invitrogen). Before imaging, the medium was replaced with phenol red-free M199 medium (Invitrogen) containing 0.1% BSA and overlaid with mineral oil (Sigma-Aldrich) to prevent evaporation. The HeLa cells expressing FRET probes were imaged every 1 or 2 min with an Olympus IX81 inverted microscope equipped with a light-emitting diode light source PreciseExcite (CoolLED, Hampshire, UK), a CoolSNAP K4 cooled charge-coupled device (CCD) camera (Roper Scientific, Trenton, NJ), a laser-based autofocus system (IX2-ZDC, Olympus), and an automatically programmable XY stage (MD-XY30100T-Meta, Sigma Koki, Tokyo, Japan), which allowed us to obtain time lapse images of several view fields in a single experiment. The imaging system was controlled by MetaMorph software (Universal Imaging, West Chester, PA). The filters used for the dual emission imaging were obtained from Omega Optical and consisted of an XF1071 (440AF21) excitation filter, an XF2034 (455DRLP) dichroic mirror, and two emission filters (XF3075 (480AF30) for CFP and XF3079...
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(535AF26) for FRET. Cell images were acquired with a 60× oil immersion objective lens (PlanApo 60×/1.4, Olympus). The binning of the charge-coupled device camera was 8. The exposure time was 100 ms for the TFP and FRET images. After background subtraction, FRET/TFP ratio images were created with MetaMorph software. Quantification of the time course of the FRET images was conducted according to Aoki and Matsuda (24).

**Determination of Dephosphorylation Rate of SOS1**—Upon EGF stimulation, SOS1 is phosphorylated at multiple serine residues, which is manifested by an electrophoretic mobility shift in SDS-PAGE (7–9). We quantified this band shift as follows. First, HeLa cells were starved for 3 h and stimulated with EGF for 10 min to maximize the SOS1 phosphorylation by ERK. Second, MEK activity was acutely ablated by U0126 treatment. After predetermined periods of incubation, cells were lysed for the SDS-PAGE and immunoblotting analysis. FLAG-tagged DOCK180 protein was added to the cell lysates as an internal molecular size marker (25). After separation in a 5% SDS-polyacrylamide gel, proteins were transferred to PVDF membrane and probed with anti-FLAG and anti-SOS1 primary antibodies and fluorescent dye-labeled secondary antibodies. The distance between FLAG-DOCK180 and SOS1 was measured for the band shift analysis. Third, we calculated the phosphorylation rate assuming that the level of band shift was linearly correlated with the multiplicity of phosphorylation. Here, we postulated that the concentration and activity of phosphatase(s) remained constant during the course of simulation. Thus, the production rate of unphosphorylated SOS1 was

\[
\frac{d[SOS]}{dt} = \frac{k_{cat,\text{phosphatase}_pSOS} \times [\text{phosphatase}] \times [pSOS]}{K_{m,\text{phosphatase}_pSOS} + [pSOS]}\quad (\text{Eq. 1})
\]

where \(k_{cat,\text{phosphatase}_pSOS}\) and \(K_{m,\text{phosphatase}_pSOS}\) are the rate and the Michaelis constant of this phosphatase reaction, respectively. Assuming that phosphatase activity is constant and that \(K_{m,\text{phosphatase}_pSOS}\) is greatly larger than \([pSOS]\), this reaction can be approximated by the first-order kinetics

\[
\frac{d[SOS]}{dt} = \frac{V_{max,pSOS} \times [pSOS]}{K_{m,\text{phosphatase}_pSOS}} = k_{\text{phos}_pSOS\_SOS} \times [pSOS]\quad (\text{Eq. 2})
\]

where \(V_{max,pSOS}\) and \(k_{\text{phos}_pSOS\_SOS}\) are the maximum rate and the first-order rate constant, respectively. We obtained this first-order rate constant by fitting the dephosphorylation time course of SOS1 with a single exponential decay function using Excel Solver.

**Simulation**—The EGF-ERK pathway simulation implemented here consists of 27 kinetic reactions involving 10 different molecules and 33 parameters. Kinetic reactions were based on Michaelis-Menten kinetics, but many reactions were described as the first-order kinetics when reactions could be approximated to decrease the number of parameters. The biochemical reactions and the rate constants used in this study are shown in supplemental Tables S1 and S2 and supplemental Fig. S2. The simulation program described by using Cell Designer (Systems Biology Institute, Tokyo, Japan) was transferred to and run on MATLAB (MathWorks, Natick, MA).

**RESULTS**

**Attenuation of EGF-induced Ras Activation by ERK in HeLa Cells**—To analyze the negative feedback loop from ERK to Ras at the single cell level, we established a cell line expressing a FRET probe for Ras, Raichu-HRas (19). In this cell line, the concentration of the probe was 0.27 \(\mu M\), which is about two-thirds that of the endogenous Ras proteins, 0.40 \(\mu M\). As reported previously (13, 15, 30), EGF-induced Ras activation was transient due to feedback suppression but was sustained by pretreatment with an MEK inhibitor, U0126 (Fig. 1A), or ERK inhibitor, FR180204.4 This observation was confirmed by a pulldown assay with the GST-tagged Ras-binding domain of cRaf (Fig. 1, B and C). On the other hand, the activity of EGFR tyrosine kinase, which was monitored with another FRET probe, Picchu (18), was not affected by U0126 pretreatment (Fig. 1D). Thus, the ERK-mediated negative feedback loop was found to enter the signaling cascade downstream of EGFR.

To exclude the possible off-target effect of U0126, we adopted two approaches. First, we knocked down MEK1 and MEK2 by siRNA (supplemental Fig. S1, A–C). Knockdown of MEK1 and MEK2 suppressed the rapid and transient activation of Ras within 5 min of EGF application. Notably, however, the weak Ras activation was prolonged as observed in U0126-treated cells. Although the cause of the suppression of Ras activation is unknown, this observation also supported that the presence of MEK and ERK mediated the negative feedback loop to Ras. We also examined the effect of another MEK inhibitor, PD184352, and obtained similar results (supplemental Fig. S1D).

**Essential Role of SOS1 in EGF-dependent Ras Activation**—We focused on SOS as the target of negative regulation. There are two isoforms of SOS, SOS1 and SOS2, in human cells. We depleted SOS1 and/or SOS2 and measured the EGF-induced Ras activation (Fig. 2). Depletion of SOS1 inhibited the EGF-induced Ras activation almost completely irrespective of the presence of SOS2, indicating that SOS1 was the primary GEF for Ras in EGF-stimulated HeLa cells. In contrast, the effect of SOS2 depletion was negligible. Therefore, we further focused on SOS1 in the following study. Notably, Ras activity was decreased rapidly and transiently in the SOS1 and SOS2 knockdown cells, suggesting EGF stimulation of GTPase-activating protein(s) for Ras, for example GAP1m (26). Because this decrease in Ras activity continues less than 4 min, we neglected this potential activation of GTPase-activating protein(s) for Ras in this study, which focuses on the role of SOS1 phosphorylation in the later phase, i.e. from 10 to 30 min after EGF stimulation.

**ERK-dependent Phosphorylation of SOS1**—It has been shown that SOS1 phosphorylation causes retardation of electrophoretic mobility in an SDS-polyacrylamide gel (7–9). First, we examined the contribution of several serine/threonine kinases and tyrosine kinases to this band shift with specific inhibitors against phospholipase C (U73122), PKC (Gö6983), PI3K

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Among them, only U0126 could suppress the band shift of SOS1 induced by EGF (Fig. 3A). Assuming that the amount of the band shift correlates with the quantity of SOS1 phosphorylation, we compared the time courses of phosphorylation of EGFR, SOS1, and ERK (Fig. 3, B–D). We observed that phosphorylation of ERK increased rapidly, reached its zenith at 3 min, and decreased to less than 40% of the maximum activity within 30 min. In contrast, phosphorylation of SOS1 reached its zenith at 10 min after EGF stimulation and continued for 30 min. We calculated the dephosphorylation rate of SOS1 to be 0.0025/s by approximating the decay of phosphorylated SOS1 after acute ablation of MEK activity (Fig. 3E and F) to the first-order kinetics as described under “Experimental Procedures.” This half-life of phosphorylation, 4.6 min, is markedly faster than that used in the previous model (15) in which the dephosphorylation velocity and molecule number of SOS1 were 75 molecules/cell/min and 4 × 10^4 molecules/cell, respectively.

Suppression of EGF-induced Ras Activation and SOS1 Phosphorylation by Preactivated ERK—To confirm that ERK activation was sufficient for both suppression of Ras activity and phosphorylation of SOS1, we used a rapamycin-inducible cRaf translocation system (Fig. 4A) (21). The rapamycin-induced plasma membrane translocation of cRaf caused ERK activation and SOS1 phosphorylation as expected (Fig. 4B). This rapamycin-induced activation of cRaf markedly attenuated EGF-dependent Ras activation (Fig. 4C). Activation of EGFR as measured by the Picchu probe was not affected by the rapamycin-induced ERK activation (Fig. 4D). These results indicated that ERK activation was sufficient for the SOS1 phosphorylation and resulting inhibition of EGF-induced Ras activation. This result also showed that SOS1 could be phosphorylated by...
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ERK in the absence of association with EGFR at the plasma membrane, which is a phosphotyrosine-dependent process.

Prediction of Role of Multiple SOS1 Phosphorylation Sites on Ras Activation—Based on these observations, we developed a kinetic simulation model of the ERK signaling pathway and examined the role of SOS1 phosphorylation in silico. We reported previously a kinetic simulation model of Ras-induced activation of ERK based on parameters determined experimentally (14). In the present study, we added EGFR and SOS1 to this model (supplemental Fig. S2). The concentrations of EGFR and SOS1 were determined by quantitative immunoblotting (supplemental Fig. S3). All parameters, including those related to SOS1 phosphorylation, are listed in supplemental Tables S1 and S2. In this model, four ERK-dependent phosphorylation sites in SOS1 were included based on the findings of a previous report (9).

The first question addressed in this work is whether phosphorylation of multiple sites is required to suppress SOS1 activity or a single phosphorylation of only one of the four sites is sufficient to suppress SOS1 activity. For simplicity, we classified phosphorylation sites into two types: a “relevant site” that regulates the SOS1 activity and an “irrelevant site” that does not regulate SOS1 activity. For the sake of simplicity, each SOS1 molecule was assumed to exhibit 100 or 0% activity depending on the phosphorylation status. With these simplifications, we built two models. In the first “cooperative” model, two to four relevant sites have to be phosphorylated to suppress SOS1 activity, whereas in the second “decisive” model, phosphorylation of any of the relevant phosphorylation site(s) is sufficient to suppress SOS1 activity.

The possible combinations of the SOS1 phosphorylation and the ratio of active and inactive SOS1 proteins in each model are schematically shown in Fig. 5. We offer triphosphorylated SOS1 as an example (Fig. 5, shown as p3). In the four-cooperative site model shown in Fig. 5, top column, all triphosphorylated SOS1 are active. In the three-cooperative site and two-cooperative site models, 75 and 50% of triphosphorylated SOS1 are active, respectively (the second and third columns). Meanwhile, 25% of triphosphorylated SOS1 is active in one-decisive site model (the fourth column), and none of the triphosphorylated SOS1 is active in the other decisive models.

We constrained these models by two of the experimental observations schematically shown in Fig. 6A. First, the duration of the EGF-induced activation of Ras was limited to less than 30 min to reproduce the transient Ras activation (Fig. 6A). Second, the basal level of phosphorylated SOS1 (pSOS) was assumed to be less than 25% (Fig. 6A). Unlike previous studies that contain only one species of GEF, this constraint became very important in our study because the basal activity or a single phosphorylation of only one of the four sites is sufficient to suppress SOS1 activity. For simplicity, we classified phosphorylation sites into two types: a “relevant site” that regulates the SOS1 activity and an “irrelevant site” that does not regulate SOS1 activity. For the sake of simplicity, each SOS1 molecule was assumed to exhibit 100 or 0% activity depending on the phosphorylation status. With these simplifications, we built two models. In the first “cooperative” model, two to four relevant sites have to be phosphorylated to suppress SOS1 activity, whereas in the second “decisive” model, phosphorylation of any of the relevant phosphorylation site(s) is sufficient to suppress SOS1 activity.

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Next we tested decisive models with one to four relevant phosphorylation sites; i.e. in these models, phosphorylation of any of the critical sites shut off the SOS1 activity. In each panel of Fig. 6B, the yellow region denotes the condition that phos-
phorylation of basal SOS1 is less than 25%, and the blue region denotes the condition that the duration of EGF-stimulated Ras activation is less than 30 min. We found that these two regions overlapped in the very limited area shown in green in Fig. 6B. In particular, if we assume that $K_m$ is over 1 μM as reported previously (27–29), only the model in which all of the four phosphorylation sites were critical could account for the observed combination of $K_m$ and $k_{cat}$. Notably, the $k_{cat}$ values expected in this model are much smaller than the $k_{cat}$ values determined in vitro (supplemental Table S3), suggesting that the slow kinetics may be important for the negative feedback loop.

The role of the four critical phosphorylation sites in the decisive model was more intuitively illustrated by holding the $K_m$ and $k_{cat}$ values constant (Fig. 7). As the number of critical phosphorylation sites in the decisive model increased, the strength of the negative regulation increased as well. Importantly, the number of phosphorylation sites did not affect the rate or the strength of any of the signaling components in the activation phase.

**Evaluation of Decisive Phosphorylation Model by Ablation of Negative Feedback Loop**—To validate the model, we compared the dynamics of Ras activation between the in silico and the tissue culture conditions (Fig. 8). In the absence of the MEK inhibitor U0126, transient activation of Ras as observed in tissue culture was reproduced in silico with a wide range of EGF concentrations (Fig. 8, A and C). When the negative feedback loop was ablated by the MEK inhibitor, Ras activation was sustained both in tissue culture and in silico (Fig. 8, B and D). Interestingly, at high EGF concentrations, the suppression of Ras activity could not be completely restored as expected from the model (Fig. 8, B and D). This observation suggests the presence of another mechanism that attenuates the Ras activity after 10 min. It is likely that EGFR activity may be suppressed by endocytosis at high EGF concentration. This inability to reproduce the effect of U0126 at high EGF concentration is a flaw of the present model; however, in other words, this observation clearly indicates that our model based on the experimentally verified parameters could let us know that a critical regulator is missing in our knowledge.

**DISCUSSION**

In this study, we attempted to incorporate a negative feedback loop from ERK to SOS into our previously developed kinetic simulation model based on the experimentally verified parameters (14). In the EGF-ERK signaling cascade, there are many negative feedback loops, the effects of which sometimes depend on the cell type and/or cellular context (13, 30). In the early phase within 1 h after EGF stimulation, ERK-dependent phosphorylation of SOS1 plays a major role in the negative feedback regulation of this signaling cascade in many cell types (7–12, 31) as well as in the HeLa cells used in this study (Figs. 1–3).

Previous tryptic phosphopeptide map analyses have revealed that EGF induces phosphorylation of several serine/threonine residues of SOS1. Notably, the rapamycin-inducible cRaf translocation system were serum-starved for 3 h, incubated with DMSO or 50 nm rapamycin for 10 min, and stimulated with 50 ng/ml EGF or left unstimulated. Ras and tyrosine kinase activities were measured as in Fig. 1.
residues in the C-terminal region of human or murine Sos1 (8, 12). This C-terminal region contains multiple proline-rich motifs that bind to Grb2 (8); however, the phosphorylation of Sos1 does not affect Sos1 binding to Grb2 but perturbs Sos1 binding to Shc and EGFR (8, 12). Interestingly, serum- or insulin-induced phosphorylation of Sos1, which is also ERK-dependent, dissociates Sos1 from Grb2 (9–11). To accommodate this discrepancy in the role of Sos1 phosphorylation, we regarded SOS1 as representative of the SOS1-Grb2-Shc complex, which is dissociated from EGFR in an SOS1 phosphorylation-dependent manner in the present simulation model.

A specific question addressed in the present study is the role of the cluster of phosphoserine residues in the EGF-stimulated SOS1 protein. Corbalan-Garcia et al. (9) have reported that mutation of these phosphorylation sites renders the SOS1 protein insensitive to phosphorylation-dependent dissociation from Grb2. The same phosphorylation-defective mutant of SOS1 has been shown to be insensitive to ERK-mediated negative regulation in basic fibroblast growth factor-stimulated NIH-3T3 cells (32). However, to the best of our knowledge, there is no report examining the role of each phosphorylation site of SOS1. In addition, it is unknown whether SOS1 activity is

FIGURE 5. Cooperative and decisive models for role of multiple phosphorylation sites on activity of SOS1. To understand the role of multiple phosphorylations, we first classified phosphorylation sites into two types: a relevant site that regulates the SOS1 activity and an irrelevant site that does not regulate SOS1 activity. These two types of phosphorylation site are discriminated by round and rectangular concave symbols in this figure. For the sake of simplicity, each SOS1 molecule was assumed to exhibit 100 or 0% activity. The activities of SOS are depicted by either red or blue color of the symbols. Two models, the cooperative and decisive models, are considered according to the role of the phosphorylation sites. In the cooperative models, all critical phosphorylation sites have to be phosphorylated to suppress SOS1 activity, whereas in the decisive models, phosphorylation of any of the relevant sites is sufficient to suppress SOS1 activity. In both models, irrelevant phosphorylation sites do not affect the activity of SOS1 in any way. The pink and blue background colors show the ratio of active and inactive SOS1 proteins in each phosphorylation status (unphosphorylated (np), monophosphorylated (p1), diphosphorylated (p2), triphosphorylated (p3), or tetraphosphorylated (p4)) when the probability of the phosphorylation is equal among the four phosphorylation sites.

binding to Shc and EGFR (8, 12). Interestingly, serum- or insulin-induced phosphorylation of Sos1, which is also ERK-dependent, dissociates Sos1 from Grb2 (9–11). To accommodate this discrepancy in the role of Sos1 phosphorylation, we regarded Sos1 as representative of the Sos1-Grb2-Shc complex, which is dissociated from EGFR in an Sos1 phosphorylation-dependent manner in the present simulation model.

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FIGURE 6. Range of kinetic parameters constrained by duration of Ras activation and level of basal phosphorylation of SOS1. A, schematic representation of the constraint for the parameters. The duration, which is defined as the period during which Ras activity is at least 30% of maximum, is restricted to less than 30 min to reproduce the transient activation in vivo (left panel). Phosphorylated SOS1, which is caused by basal ERK activity, is less than 25% of total SOS1 (right panel). B, range of Km and kcat of the ERK phosphorylation of SOS1 that fulfills the two constraints. The Km and kcat values are varied from 0.1 to 10 μM and from 0.001 to 1/s, respectively. The kcat/Km value is plotted against Km. The regions that fulfill the constraints of transient Ras activation and basal ERK activity in A are indicated by blue and yellow, respectively. The overlapped regions are shown in green. AU, arbitrary units.
regulated by each phosphorylation site in a decisive manner or by multiple phosphorylation sites in a cooperative manner.

There are several previous examples of protein functions that are regulated by a cluster of serine and/or threonine phosphorylation within a relatively small region. The *Drosophila* clock protein Period is heavily phosphorylated in the per-short domain and per-short downstream domain, which together encompass some 60 amino acids in length. This hyperphosphorylation is associated with short period circadian rhythms and the stability of Period (33). Another example is the retinoblastoma tumor suppressor protein (RB). In its hypophosphorylated state, RB sequesters a subset of E2F complexes. Cdk-dependent hyperphosphorylation of RB dissociates E2F from RB, thereby triggering cell cycle-related genes (34, 35). In these previous studies, the contribution made by the phosphorylation of each specific amino acid residue was examined with mutants having a single amino acid substitution. However, as is the case with SOS1, it is largely unknown whether multiple phosphorylation sites regulate the protein function decisively or cooperatively in either Period or RB.

In this study, instead of experimentally examining the contribution of each phosphorylation site one by one, we built various kinetic simulation models and tested their probability *in silico* (Figs. 5 and 6). We constrained these models by two conditions. First, the amount of pSOS before stimulation was restricted to less than 25% of total SOS (Constraint 1). This condition was based on our own observation and also on previous tryptic mappings of pSOS before and after EGF stimulation (8, 12). Second, EGF-stimulated Ras activation was constrained to return to the near basal level within 30 min (Fig. 1). To our surprise, these two conditions rejected most of the models and left only one model in which all four phosphorylation sites play the decisive role in the negative regulation of SOS1 (Fig. 6). In other words, the model predicted that single phosphorylation of SOS1 was sufficient to dissociate SOS1 from EGFR. This role of multiple phosphorylation sites is similar to that of rhodopsin (36). Rhodopsin is phosphorylated at multiple sites in the C-terminal region upon photon absorption. Each phosphorylation site independently inactivates rhodopsin, thereby conferring reproducibility in single photon response. Therefore, the multiple decisive phosphorylation sites may be used in a variety of signal transduction modules to increase the fidelity of signaling.

Lastly, we should consider the reason why previous simulation models were able to reproduce the ERK-dependent suppression of SOS1 without taking the multiple phosphorylation sites into consideration. Three critical differences between these previous reports and the present study are schematically shown in *supplemental Fig. S4*. First, Brightman and Fell (15), whose model of the negative feedback loop from ERK to SOS has been adopted in many studies, postulated that ERK phosphorylates only SOS1 in complex with EGFR but not SOS1 in the cytoplasm (*supplemental Fig. S4B*). Because in this model ERK does not phosphorylate SOS1 in the basal state, the parameters that govern the phosphorylation of SOS1, $K_m$ and $k_{cat}$, or the dephosphorylation rate of pSOS can be selected without the constraint of less than 25% pSOS in the basal state (Constraint 1). This assumption, however, was negated by the direct activation of cRaf, which resulted in efficient SOS1 phosphorylation and inactivation, indicating that ERK phosphorylates SOS1 irrespective of the localization (Fig. 4).

Second and most importantly, the basal level of active ERK was neglected in all preceding models (15, 17, 37) (*supplemental Fig. S4C*). This assumption also clears the constraint of less than 25% pSOS in the basal state.

Third, the parameter values concerning the phosphorylation and dephosphorylation of SOS1 in the previous models were sometimes significantly different from ours. For example, the half-life of pSOS was about 3 h in the model of Brightman and
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Fell (15). In this way, negative feedback of SOS could be made almost irreversible in the time range used. However, as we show in Fig. 3, pSOS is rapidly dephosphorylated by phosphatases, indicating that the pSOS level is balanced by phosphorylation and dephosphorylation cycles.

The present model contained two major modifications, EGF-induced SOS1 activation and ERK phosphorylation of SOS1, and several updates of parameters in comparison with the previous model (14). To examine the effect of such modifications, we conducted sensitivity analysis (supplemental Fig. S5). The transient Ras activation (Constraint 1) was most sensitively influenced by the rate of ERK phosphorylation of SOS and also by the concentration of ERK. The basal low SOS1 phosphorylation (Constraint 2) was modestly affected by many parameters. Our simulation model consists of minimum number of reactions; therefore, perturbation in most parameters linearly affects the output such as SOS1 phosphorylation. Acquisition of experimentally verified parameters and the refinement of the model may yield a more robust model, or this system is inherently sensitive to many kinetic parameters.

In conclusion, with the help of experimentally verified parameters and a simulation model, we have found that multiple decisive phosphorylation sites contribute to the suppression of SOS1. Because phosphoproteomics has revealed a number of phosphorylation sites in many other signaling molecules, multiple decisive phosphorylation sites similar to that of SOS1 may be identified in other proteins.

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