Processive phosphorylation of ERK MAP kinase in mammalian cells

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The mitogen-activated protein (MAP) kinase pathway is comprised of a three-tiered kinase cascade. The distributive kinetic mechanism of two-site MAP kinase phosphorylation inherently generates a nonlinear switch-like response. However, a linear graded response of MAP kinase has also been observed in mammalian cells, and its molecular mechanism remains unclear. To dissect these input-output behaviors, we quantitatively measured the kinetic parameters involved in the MEK (MAPK/ERK kinase)-ERK MAP kinase signaling module in HeLa cells. Using a numerical analysis based on experimentally determined parameters, we predicted in silico and validated in vivo that ERK is processively phosphorylated in HeLa cells. Finally, we identified molecular crowding as a critical factor that converts distributive phosphorylation into processive phosphorylation. We proposed the term quasi-processive phosphorylation to describe this mode of ERK phosphorylation that is operated under the physiological condition of molecular crowding. The generality of this phenomenon may provide a new paradigm for a diverse set of biochemical reactions including multiple posttranslational modifications.

Mitogen-activated protein (MAP) kinase cascades are evolutionarily conserved signaling pathways that are involved in the control of physiological and pathological cellular processes including cell proliferation, survival, differentiation, apoptosis, and tumorigenesis (1–4). Each MAP kinase pathway contains a three-tiered kinase cascade consisting of a MAP kinase kinase, a MAP kinase kinase, and the MAP kinase, which are sequentially activated in this order. MAP kinases, which are activated by dual phosphorylation of conserved threonine and tyrosine residues within the activation loop, phosphorylate their targets on serine or threonine residues (1, 3, 5). Five distinct groups of MAP kinases have been characterized in mammals. Among the five groups, the most studied is the Raf/MEK/ERK MAP kinase cascade (hereafter called the ERK MAP kinase cascade), which is activated by mitogenic ligands such as growth factors, cytokines, and phorbol esters.

Ferrell and coworkers found that in Xenopus oocytes the ERK MAP kinase pathway responds to increasing levels of progesterone in an all-or-none or “switch-like” manner, in which individual cells in the population exhibit either “on” or “off” status (6, 7). This property of ERK MAP kinase system benefits to determine all-or-none irreversible responses including cell-cycle progression, neuronal differentiation, and T cell selection (6–10). The switch-like responses can arise from both positive feedback via protein synthesis and dual phosphorylation steps of the MAP kinase, which is called the “distributive phosphorylation model” (11–15) (Fig. 1A). The distributive model of ERK phosphorylation results in an increase in the cooperativity of this system, and consequently contributes to a switch-like input-output response.

In different cellular contexts, however, the ERK MAP kinase cascade exhibits “graded” response, in which all cells in the population uniformly gain their signal output proportionally in response to the level of input stimulus. This input-output response is observed in the pheromone-stimulated Saccharomyces cerevisiae (16–18) and growth factor stimulated-HeLa cells and fibroblasts (19, 20). These graded responses can suit the physiological property of signaling, including the reversibility and the activation by different inputs with a wide range of threshold doses (18). However, MAP kinase cascades should inherently induce the switch-like response behavior due to the three-tiered enzyme cascade. To answer this question, it has been theoretically proposed that scaffold proteins might convert the switch-like response into the graded response (21). Here, the scaffold protein holds MAP kinase kinase and MAP kinase, thereby inducing dual phosphorylation at a single collision, which is called the “processive phosphorylation model” (11, 21–25). However, it is not known whether the scaffold proteins in mammals contribute to the processive phosphorylation of MAP kinase (10). Hence, the mechanism by which MAP kinase activity adopts a graded response remains unclear in mammalian cells.

In this study, we demonstrate that ERK is phosphorylated in a processive manner by employing quantitative simulation model of the ERK MAP kinase cascade. Furthermore, a condition that mimics physiological molecular crowding has been shown to convert the mode of ERK phosphorylation from distributive to processive. Under this condition, MEK and ERK do not form a stable complex as proposed in the processive phosphorylation model. Therefore, we propose that ERK is phosphorylated in a “quasi-processive” manner under the physiological condition of molecular crowding.

Results

Kinetic Parameters That Determine the Dynamics of the MEK-ERK Signaling Module. Our goal is to comprehend the systems behavior of the MEK-ERK signaling module. To this end, we attempted to determine most, if not all, of the kinetic parameters required for the kinetic simulation model, using HeLa cells as a model system. The model requires four classes of parameters: protein concentrations, association/dissociation rates, nuclear import/export rates, and phosphorylation/dephosphorylation rates. We experimentally determined approximately 30 parameters (SI Appendix: Fig. S1–7 and Tables S1 and S2). Notably, this study focused on the phosphorylation kinetics of the MEK-ERK module in the early phase of the signaling event. Thus, we neglected the role of ERK-induced gene expression of negative regulators such as dual specificity phosphatases, which shape the dynamics of intracellular signaling starting from 30 min after ligand addition (26).

In Vitro Phosphorylation Rates of ERK Measured by Phos-Tag Gel Electrophoresis. All parameters used in this study are determined in

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immunoblotting with specific antibodies (SI Appendix: Fig. S5) and constitutively active mutant of MEK1 (MEK1-SDSE). As previously demonstrated (11), pY-ERK was initially accumulated, followed by pTpY-ERK (Fig. 1 B and C). pT-ERK could not be detected under this condition. Importantly, the amount of the intermediate product, pY-ERK, exceeded that of the enzyme, MEK1-SDSE (3.0 × 10⁻⁷ M in Fig. 1 C), indicating that ERK was phosphorylated by a manner consistent with the distributive phosphorylation model in vitro (11). In this way, we obtained the first and second reaction rates with linear assumption (kcat/Km) of 3.9 × 10⁻⁸ [M/sec] and 2.1 × 10⁻⁸ [M/sec], respectively (SI Appendix: Fig. S5 and Table S2). The reason why pT-ERK was not detected may be because the first step of the reactions from np-ERK to pT-ERK is markedly slower than the second step from pT-ERK to pTpY-ERK in vitro. Therefore, the phosphorylation reaction rate (kcat/Km) from pT-ERK to pTpY-ERK was examined in vitro using pT-ERK as a substrate, and was found to be 2.0 × 10⁻⁸ [M/sec] (SI Appendix: Table S2). Thus, pT-ERK production from np-ERK could be ignored at least in the phosphorylation reactions (SI Appendix: Fig. S5G).

Validation of the Distributive Phosphorylation Model. Based on these parameters determined experimentally (SI Appendix: Tables S1 and S2), we built a distributive phosphorylation model of the MEK-ERK signaling module (Fig. 2A, SI Appendix: Fig. S8A), and numerically solved the ERK phosphorylation (Fig. 2 B and C). The input signal, i.e., the turnover rate of MEK phosphorylation by cRaf, was initially set to 1.0 × 10⁻⁶ [sec⁻¹] to reproduce the experimental data, and was varied with a broad range (SI Appendix: Fig. S9). Upon stimulation in silico, pY-ERK was steeply increased, followed by a gradual increase in pTpY-ERK and pT-ERK (Fig. 2B). pY-ERK was predominant over pTpY-ERK in low input strength, and vice versa for high input strength at 10 min after stimulation (Fig. 2C). These data were consistent with the distributive phosphorylation model demonstrated in vitro (Fig. 1) and in Xenopus oocytes (6, 11).

We next examined whether the phosphorylation of ERK2 in HeLa cells follows the distributive model. Against the prediction in silico, the increase in pY-ERK2 did not precede to, but rather accompanied the increase in pTpY-ERK2 upon EGF stimulation (Fig. 2D and E). Furthermore, pY-ERK2 increased in a dose-dependent manner as the EGF input increased (Fig. 2 F and G), but did not increase in a bell-shaped manner as predicted in silico (Fig. 2C). Of note, we could not detect the endogenous pT-ERK2 quantitatively due to the low abundance and to the signals of comigrating pY-ERK1.

Prediction of a Processive Phosphorylation Model In Silico. Because we determined all parameters in the cells except for the phosphorylation reaction rates, which were obtained in vitro, we speculated that the discrepancy between the experimental and in silico data of the MEK-induced ERK phosphorylation might have arisen from parameters related to the phosphorylation rates of ERK. Hence, we built a processive model by changing one re-action pathway of the distributive model (Fig. 3A, SI Appendix: Fig. S8B, Tables S1 and S2). Here, activated MEK does not dissociate from pY-ERK but processively phosphorylates threonine of pY-ERK to generate pTpY-ERK. Therefore, this model contains two species of pY-ERK, i.e., pY-ERK in complex with MEK and pY-ERK generated by the dephosphorylation of pTpY-ERK.

With this processive model, the experimental observation (Fig. 2 D–G) was reproduced successfully (Fig. 3 B and C, SI Appendix: Fig. S9). Namely, the increase in pY-ERK paralleled the increase in pTpY-ERK both in the time course analysis and
**Experimental Validation of the Processive Model 1: pY-ERK Dynamics.**

By employing computer-assisted kinetic simulation, we designed an experimental condition that distinguishes between the distributive model and the processive model. When cells are stimulated in the presence of phosphatase inhibitors, pY-ERK will be increased in the distributive model (Fig. 4), but reduced in the processive model. When cells are stimulated with EGF in the absence or presence of inhibitors for tyrosine- and threonine-phosphatase, bpV and Calyculin A, respectively. As expected in the processive model, phosphatase inhibitors substantially reduced the accumulation of pY-ERK, arguing for the processive phosphorylation model (Fig. 4 C and D).

**Experimental Validation of the Processive Model 2: Input-Output Response.** Previous studies supported the distributive phosphorylation model primarily because it explains the nonlinear switch-like response of MAP kinase upon stimulation. Therefore, to understand the contribution of the MEK-ERK signaling module to the switch-like response of ERK to EGF, we examined the input-output behavior of ERK phosphorylation in our experimental conditions. We quantified the amount of pTpY-ERK1/2 in individual cells by immuno-staining with anti-pTpY-ERK1/2 antibody. As reported previously in HeLa cells and fibroblasts stimulated with EGF and TPA, respectively (19, 20), this approach could provide quantitative relationship between the input signal and output response (Fig. 5 A and B). The Hill coefficient obtained by this method was 2.09 (Fig. 5B). Next, we confirmed this observation by Western blotting, which also demonstrated a mild nonlinearity of pY-ERK, having 1.18 Hill coefficient (Fig. 5C).

However, when we examined the correlation of the fraction of active MEK with the fraction of pTpY-ERK, we observed almost linear input-output response of ERK, having 1.18 Hill coefficient (Fig. 5D). Importantly, this Hill coefficient value determined experimentally was nearly identical to the Hill coefficient calculated at 1.19 by the processive model (Fig. 5F), but significantly smaller than the Hill coefficient calculated at 1.61 by the distributive model (Fig. 5E). Notably, the Hill coefficients might be slightly overesti-
Numerical simulations were performed based on the distributive model (A) and the processive model (B) in the absence (left column) or presence (right column) of the inhibition of all phosphatase reactions. Time series of np-ERK (blue), pY-ERK (green), pTPy-ERK (red), and pTP-ERK (orange) concentrations are plotted at Raf kphos value = 1.0 × 10^7 /M/sec. Maximal levels of pY-ERK without inhibition are indicated by gray dashed lines. (C and D) After serum starvation, HeLa cells were treated with mock or 1.0 × 10^-7 M Calyculin A and 1.0 × 10^-4 M bpV for 2 min, followed by 1.0 × 10^-5 g/L EGF for the indicated time period. (C) Cell lysates were separated by electrophoresis in SDS-polyacrylamide gels containing Phos-tag and probed with an anti-ERK antibody. (D) Time series of npERK2 (blue), pY-ERK2 (green), and pTPy-ERK2 (red) concentrations without (left) or with (right) phosphatase inhibitors are plotted after EGF stimulation with SD (n = 4).

Numerical Validation of the Processive Model. To examine which of the distributive and processive models is the plausible mechanism to account for the observed data (Fig. 2E and Fig. 4D), we calculated residual sum of squares (RSS) values, Akaike information criterion (AIC) values, and Hill coefficients for both models after parameter fitting (28) (SI Appendix: Fig. S11). This in silico validation was performed under three conditions of different numbers of variables. Condition 1: all phosphorylation rates were multiplied by a common constant value, which was optimized to fit the experimental data. Condition 2: the phosphorylation rates were individually fitted to the experimental data. Condition 3: in addition to the phosphorylation rates, the dephosphorylation rates were also fitted individually to the experimental data. In all conditions, experimentally determined parameters were used as the initial values for the fitting. In Conditions 1 and 2, the processive model was more plausible than the distributive model because the AIC of the former model was smaller than the AIC of the latter model. Although both distributive and processive models exhibited the lowest RSS in Condition 3, the AIC values were higher than those in the other conditions, which indicated overfitting in Condition 3. Importantly, the Hill coefficients calculated by the processive model were closer to the experimental value than the Hill coefficients calculated by the distributive model (Fig. 5F). Taken together, these analyses strongly argued that ERK is processively phosphorylated by MEK in HeLa cells.

Molecular Crowding as a Determinant for Processive Phosphorylation. What kind of factor(s) is a critical determinant for changing the distributive nature of ERK phosphorylation into the processive one in the cells? A critical difference between the environment in vitro and that within cells is the effect of molecular crowding (29, 30). To evaluate the effect of molecular crowding, we examined whether or not ERK is phosphorylated in a processive manner in diluted cytoplasmic cell extract. Similarly to the results in vitro ERK phosphorylation, ERK is phosphorylated in a distributive manner in the cytoplasmic extract, which was manifested by the initial accumulation of pY-ERK (SI Appendix: Fig. S12).
Conversely, we mimicked the physiological molecular crowding by adding 15% (wt/vol) polyethylene glycol (PEG) to in vitro kinase reaction solution as a crowder (31) (Fig. 6 A and B). The addition of PEG reduced the production rate of pY-ERK without changing the net production rate of pTpY-ERK, indicating an increase in the "processivity" of ERK phosphorylation (Fig. 6 C and D). Lastly, we built a partial processive model by combining the distributive and processive models and fitted it to the experimental data (Fig. 6E). For this purpose, we introduced two additional parameters: Processivity, $p$, as the probability of pTpY-ERK production in the processive manner, and crowding factor, $c$, as the effect of the crowder or viscosity on the net reaction rates. By the fitting we found that the presence of 15% PEG increased in the processivity of ERK phosphorylation of 47%, and decreased the reaction rates to 27% by viscosity (Fig. 6 C and D). Of note, the amount of pY-ERK at the zenith exceeded the net amount of MEK (Fig. 6D, dashed line), indicating that pY-ERK was not accumulated due to the sequestration by MEK binding. Thus, the molecular crowding within the cells could convert the distributive phosphorylation in vitro to the processive phosphorylation as seen in the cells.

**Discussion**

In this study, we have demonstrated that the amino acid residues in the activation loop of ERK, threonine, and tyrosine, are processively phosphorylated in HeLa cells, and have identified molecular crowding as one possible factor in determining whether the mode of phosphorylation will be distributive or processive. The term "processive phosphorylation" is used to describe reactions in which the substrate-enzyme complex is stable during the first and second phosphorylation reactions, which is not the case for the phosphorylation of ERK (Fig. 6D). We therefore proposed the term "quasi-processive phosphorylation" to describe two sequential phosphorylation reactions that are controlled by molecular crowding (Fig. 6F). In this model, MEK first phosphorylates ERK at the tyrosine residue and dissociates from the product, pY-ERK. Under the physiological conditions within cells, the diffusions of both MEK and pY-ERK are considerably restricted by the molecular crowding effect; therefore, MEK rebinds to and phosphorylates pY-ERK with high probability. Consequently, MEK phosphorylates ERK in a processive manner. Another MAP kinase, p38, also exhibited typical hallmarks of the processive phosphorylation (SI Appendix: Fig. S13), indicating the generality of the quasi-processive phosphorylation of MAP kinase within cells. In contrast to the phosphorylation step, this quasi-processive phosphorylation model could not be applicable to the dephosphorylation step of pTpY-ERK, because the premise of quasi-processive phosphorylation is sequential reactions operated by the identical enzyme. The dephosphorylation reactions of phospho-threonine and phospho-tyrosine were mediated probably by different classes of phosphatase at least in our experimental conditions (SI Appendix: Fig. S6).

The quasi-processive phosphorylation is critically different from the previously proposed processive phosphorylation model, in which scaffold proteins anchor MEK and ERK in order to cause sequential phosphorylation reactions by a single collision (21). The scaffold proteins are known to regulate assembly of two or more components of signaling pathways in many signaling cascades (21–25). As far as we have examined, however, none of the scaffold proteins for MEK and ERK contributed significantly to cause the processivity of ERK phosphorylation (SI Appendix: Fig. S14), consistent with the previous study on KSR (10). Therefore, at this moment, we failed to obtain any evidence that scaffold proteins cause processive phosphorylation in mammalian cells. Importantly, Takahashi and Pruciaik have concluded that sole expression of Ste5 scaffold did not induce linear response of MAP kinase phosphorylation in yeast, and instead the recruitment of the scaffold to the plasma membrane resulted in linear response of MAP kinase phosphorylation (18). This model seems to fit very well with our findings, in that the effect of Ste5 could be due to molecular crowding or confinement, rather than scaffolding per se. Recently, Takahashi et al., demonstrated by particle simulation that rapid re-binding of enzyme molecules to the substrate after modification of the first site can turn a distributive model into a processive model (32). Their simulation model has predicted that molecular crowding will make enzyme-substrate rebindings more likely due to sub-diffusion, strongly providing the theoretical basis of our findings.

It has been suggested that the high cooperativity of Xenopus oocytes in reaction to progesterone stimulation is at least partly accomplished by the distributive phosphorylation of ERK (6, 7).

**Fig. 6.** Effect of molecular crowding on processive phosphorylation. (A and B) ERK was phosphorylated by MEK in the absence (A) or presence (B) of 15% (w/v) polyethylene glycol (PEG) in vitro and were subjected to Phos-tag Western blotting analysis. (C and D) The concentrations of phospho-isofoms of ERK in (A and B) are plotted as dots against time. Concentration of constitutively active mutant of MEK (1.0 × 10^{-7} M) used in these experiments is indicated by gray dashed lines. The same experiments were repeated four times with reproducible results, and representative results are shown. Lines represent the concentration of phospho-isofoms of ERK obtained by fitting the processive model in (E) with the experimental data. The fitted parameters of processivity ($p$) and crowding factor ($c$) are represented. (E) Schematic representation of processive phosphorylation. $p$ and $c$ indicate processivity ($0 < p < 1$) and crowding factor, respectively. (F) Schematic view of quasi-processive model under the condition of molecular crowding.
Some degree of molecular crowding probably exists in Xenopus oocytes, indicating an important point of disagreement between these previous studies and our present findings. However, it should be noted that the phosphorylation dynamics of ERK phospho-isofoms has only been investigated in vitro, and has never been investigated experimentally in Xenopus oocytes or cells. Therefore, it is possible that ERK is processively phosphorylated in Xenopus oocytes as in HeLa cells. There may exist nonlinear reactions in the signaling pathway upstream of MEK, e.g., receptor activation or Mos activation, in Xenopus oocytes. Actually, the input-output relationship between EGF concentration and pTyr-ERK demonstrated higher cooperativity; namely, the Hill coefficient was almost two (Fig. 5 A–C). In contrast, the Hill coefficient for active MEK and pTyr-ERK phosphorylation was approximately one, implying the presence of a nonlinear reaction(s) in the signaling cascade upstream of MEK. Consistent with this assumption, it has been reported that EGFR phosphorylation responded in a highly amplified and switch-like manner by coupling EGFR activation and PTP inhibition (33).

As has been described in this study, the discrepancy between the data acquired from the cells and the data predicted by the kinetic simulation model led to the finding that ERK is processively phosphorylated within cells. Therefore, our results underscore the importance of simulation models based on experimentally determined kinetic parameters. Another important message from this study is that, in some cases, the physiological condition of molecular crowding significantly affected the mode of phosphorylation. Recent proteomic data demonstrates that proteins in a eukaryotic cell harbor multiple phosphorylation sites, each of which regulates different functions of their proteins (24). Therefore, molecular crowding, which is ubiquitous in biological systems, introduces additional complexity in their regulation. Thus, the finding that molecular crowding changes the mode of successive reactions to a processive type provides a new paradigm for a diverse set of biochemical reactions.

Materials and Methods

Plasmids, Cells, Reagents, and Antibodies. The plasmids used here were constructed according to the standard methods. Cells, reagents, and antibodies are detailed in SI Appendix.

Purification of Recombinant Proteins, Cytoplasm Extract, and In Vitro Kinase Assay. Details of purification of recombinant proteins, cytoplasmic extract and in vitro kinase assay are provided in SI Appendix.

Phos-Tag Polyacrylamide Gel Electrophoresis. Phos-tag polyacrylamide gel electrophoresis was performed essentially as described previously (27). For Phos-tag Western blotting of ERK and p38, 5.0 × 10⁻⁵ and 1.5 × 10⁻⁴ M Phos-tag and 1.0 × 10⁻⁴ and 3.0 × 10⁻⁵ M MnCl₂ were added to conventional SDS-polyacrylamide separation gels according to the manufacturer’s protocol, respectively. Proteins were detected by using an Odyssey infrared imaging system (Li-COR).

Imaging. Live cell imaging was performed essentially as described (34).

Kinetic Modeling and Numerical Simulation. Numerical simulation and parameter search were implemented by MATLAB software with ode23 and fminsearch function, respectively. The details are described in SI Appendix.

Immunostaining. Details of pTyr-ERK immunostaining are provided in SI Appendix.

Data Analysis. For the determination of kinetic parameters, curve fitting were performed by the solver function of Microsoft excel (Microsoft).

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