Dynamics of the Ras/ERK MAPK Cascade as Monitored by Fluorescent Probes*

Aki Fujioka1, Kenta Terai1, Reina E. Itoh1, Kazuhiro Aoki1, Takeshi Nakamura1, Shinya Kuroda1, EiSuke Nishida2, and Michiyuki Matsuda1,3

From the 1Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita-shi, Osaka 565-0871, Japan, the 2Undergraduate Program for Bioinformatics and Systems Biology, Graduate School of Information Science and Technology, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan, and the 3Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

To comprehend the Ras/ERK MAPK cascade, which comprises Ras, Raf, MEK, and ERK, several kinetic simulation models have been developed. However, a large number of parameters that are essential for the development of these models are still missing and need to be set arbitrarily. Here, we aimed at collecting these missing parameters using fluorescent probes. First, the levels of the signaling molecules were quantitated. Second, to monitor both the activation and nuclear translocation of ERK, we developed probes based on the principle of fluorescence resonance energy transfer. Third, the dissociation constants of Ras-Raf, Raf-MEK, and MEK-ERK complexes were estimated using a fluorescent tag that can be highlighted very rapidly. Finally, the same fluorescent tag was used to measure the nucleocytoplasmic shuttling rates of ERK and MEK. Using these parameters, we developed a kinetic simulation model consisting of the minimum essential members of the Ras/ERK MAPK cascade. This simple model reproduced essential features of the activation and nuclear translocation of ERK. In this model, the concentration of Raf significantly affected the levels of phospho-MEK and phospho-ERK upon stimulation. This prediction was confirmed experimentally by decreasing the level of Raf using the small interfering RNA technique. This observation verified the usefulness of the parameters collected in this study.

The Ras/ERK3 MAPK cascade has been highly conserved throughout evolution and plays a pivotal role in many aspects of cellular events, including proliferation, differentiation, and survival (reviewed in Refs. 1–5). The principal components of this Ras/ERK MAPK cascade include H-Ras, N-Ras, K-Ras, c-Raf, B-Raf, A-Raf, MEK1, MEK2, ERK1, and ERK2. In addition to these, a number of proteins regulate this signaling pathway either positively or negatively: A few examples include phosphatases and scaffold proteins that have been shown to play critical roles in the spatiotemporal regulation of ERK MAPK (reviewed in Refs. 6–9). Furthermore, there are many positive and negative feedback loops that modulate the activity of each signaling component, rendering this signal transduction cascade ever more complicated.

To comprehend the Ras/ERK MAPK cascade, many research groups have attempted to reconstruct this cascade in silico (10–17). These systems analyses are used to propose mechanisms to explain the ultra-sensitivity of ERK to the input signal (18), the stable response of the Ras/ERK signaling cascade to a wide range of epidermal growth factor (EGF) concentrations (15), and the different responses of PC12 pheochromocytoma cells upon stimulation by EGF or nerve growth factor (17). Each kinetic simulation model reported previously recapitulates the stimulus-induced ERK activation very nicely. Nevertheless, the parameters used therein are sometimes astonishingly different from each other. One apparent reason for this discrepancy is that many studies set parameters to fit experimental data using different algorithms (11–13). Another reason may be ascribed to the fact that most parameters are derived from in vitro experiments: the parameters obtained in vitro might vary significantly depending on each experimental condition. In any event, the remarkable differences in the parameters used in each model clearly show that some aspects of the Ras/ERK MAPK cascade can be reproduced in silico, even when the model is based on non-physiological parameters.

Recent advances in fluorescent protein technology have accelerated the trend to study protein dynamics in living cells. For example, the development of probes based on the principle of Förster/fluorescence resonance energy transfer (FRET) has enabled us to monitor activity changes in enzymes, protein-protein interactions, and distribution of phospholipids in living cells (reviewed in Refs. 19–25). Furthermore, a technique called “fluorescence recovery after photobleaching” in combination with fluorescent proteins is proving to be useful in characterizing the diffusion of proteins within the cells (reviewed in Refs. 26–28). More recently, Ando et al. (29) reported a fluorescent protein with the unique property of being able to be repeatedly highlighted and erased. This protein, named Dronpa, has been shown to be an extremely versatile tool for monitoring rapid protein dynamics such as the nucleocytoplasmic shuttling of ERK.

The aim of this study was to collect and evaluate the parameters for the development of a kinetic simulation model for Ras/ERK MAPK cascades. We prepared a FRET-based probe for ERK and Dronpa-tagged probes for Raf, MEK, and ERK. Using the parameters collected using these probes, we developed a kinetic simulation model consisting of the minimum number of signaling molecules. Even though this model lacks many important components and feedback loops that are known to regulate the Ras/ERK MAPK cascade, it could reproduce the essential features of the activation and nuclear translocation of ERK.
observed using FRET-based probes, verifying the usefulness of the collected parameters.

**MATERIALS AND METHODS**

**FRET Probes**—The FRET probes for ERK (named Miu2 for MAPK indicator unit ERK2) were constructed essentially as described previously (30). From the N terminus, Miu2 comprised the following: yellow fluorescent protein (YFP; amino acids 1–239), a spacer (Leu-Asp), Xenopus ERK2, a spacer (Gly-Gly-Arg), and cyan fluorescent protein (CFP; amino acids 1–237). Full-length and mutant cDNAs of Xenopus ERK2 (amino acids 1–362) were all amplified by PCR-based methods, followed by sequence verification. Ala was substituted for both Thr188 and Tyr190 in Miu2-TAYA. Arg was substituted for Lys87 in Miu2-KR.

**Plasmids**—cDNAs of wild-type Xenopus MEK1 and mutants were generated by PCR-based methods and subcloned into pCAGGS-FLAG. Ser was substituted for Lys87 in the MEK-KS mutant. Ala was substituted for both Ser218 and Ser222 in the MEK-SASA mutant. Asp and Glu were substituted for Ser218 and Ser222, respectively, in the MEK-SDSE mutant. pCAGGS-FLAG-H-RasV12 and pCAGGS-3HA-m1CFP-c-Raf-S621A-pm have been reported previously (30). For visualization of recombinant proteins, we used monomeric versions of YFP, CFP, and red fluorescent protein (RFP) (31, 32) or Dronpa, a fluorescent protein that can be highlighted by photoactivation (29). The plasmids fused to these fluorescent proteins were pCXN2-m1CFP-ERK2, pCAGGS-FLAG-ERK2-m1CFP, pCAGGS-FLAG-c-Raf-m1YFP, pCAGGS-FLAG-MEK1-m1YFP, pCAGGS-FLAG-ERK2-m1YFP, pCAGGS-FLAG-MEK1WT-mRFP-pm, pCAGGS-Dronpa-c-Raf, pCAGGS-FLAG-MEK1-Dronpa, and pCAGGS-FLAG-ERK2-Dronpa.

**Cells**—HeLa cells were purchased from the Human Science Research Resources Bank (Sennan-shi, Japan). The COS-7 cells used in this study were COS-7/E3, a subclone of COS-7 cells established by Y. Fukui (University of Tokyo). The cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum. 293F cells were purchased from Invitrogen and maintained according to the manufacturer’s protocol. Cell imaging was performed in phenol red-free Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (Invitrogen).

**Reagents**—Inhibitors against the EGF receptor and MEK (AG1468 and U0126, respectively) were purchased from Calbiochem. EGFr was purchased from Sigma. Rabbit anti-green fluorescent protein serum was purchased from Sigma. Rabbit anti-green fluorescent protein serum was purchased from Sigma. Anti-MEK1 monoclonal antibody were obtained from BD Transduction Laboratories (University, CA) and anti-MEK1 polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-phosphotyrosine monoclonal antibody Tyr204, anti-FLAG monoclonal antibody, anti-phospho-Y204, anti-phospho-MEK1/2, anti-phospho-MEK1/2 (Ser217, Ser222) monoclonal antibody, and anti-phospho-MEK1/2 monoclonal antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti-phosphotyrosine monoclonal antibody Tyr204, anti-FLAG monoclonal antibody, and anti-phospho-MEK1/2 monoclonal antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti-phosphotyrosine monoclonal antibody Tyr204, anti-FLAG monoclonal antibody, and anti-phospho-MEK1/2 monoclonal antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti-phosphotyrosine monoclonal antibody Tyr204, anti-FLAG monoclonal antibody, and anti-phospho-MEK1/2 monoclonal antibody were purchased from Cell Signaling Technology (Beverly, MA).

**Imaging with Intramolecular FRET Probes**—HeLa cells expressing the Miu2 probes were imaged every 30 s using an Olympus IX71 inverted microscope equipped with a cooled CCD CoolSNAP HQ camera (Roper Scientific, Trenton, NJ) and controlled by MetaMorph software (Universal Imaging, West Chester, PA) as described previously (30). For the dual-emission ratio imaging of the probe, we used a 440AF21 excitation filter, a 455DRLP dichroic mirror, and two emission filters (480AF30 for CFP and 535AF26 for YFP) (Omega Optical, Inc., Brattleboro, VT). After background subtraction, the ratio image of YFP/CFP was created with MetaMorph software, and the results were used to represent the level of FRET.

**Quantitation of Endogenous Ras, Raf, MEK, and ERK**—The protein concentrations of the endogenous Ras, Raf, MEK, and ERK proteins in HeLa cells were determined using glutathione S-transferase (GST)-fused recombinant proteins as standards. GST-fused Ras was prepared in our laboratory (36). GST-c-Raf (truncated), GST-MEK1, and GST-ERK2 were purchased from Upstate Biotechnology (Lake Placid, NY). The concentrations of these GST fusion proteins were verified by SDS-PAGE, followed by Coomassie Brilliant Blue staining. Total cell lysates were prepared by the addition of SDS sample buffer directly to the culture dishes. Serial dilutions of total cell lysates and GST-fused recombinant proteins were applied to the same SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and probed with specific antibodies. Bound antibodies were detected using the ECL chemiluminescence detection system (Amersham Biosciences) and quantitated with an LAS-1000 image analyzer (Fujifilm). Calibration curves were obtained from the luminescence intensities of the standards and were used to quantitate the endogenous proteins. In some experiments, we also used FLAG-tagged proteins as standards.

**Calculation of the Fraction of Phosphorylated Miu2**—The fraction of phosphorylated Miu2 was calculated as follows. Total cell lysates of Miu2-expressing cells were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with anti-ERK antibody. The intensities of slower migrating phosphorylated ERK2 (pERK/ERK) and total Miu2 (pMiu/total Miu) were detected, including both phosphorylated and non-phosphorylated forms, were quantitated as described above. The same filter was reprobed with anti-phospho-ERK antibody, followed by measurement of the intensities of phosphorylated ERK2 (pERK/total ERK) and phosphorylated Miu2 (pMiu/total Miu). The fraction of phosphorylated Miu2 (pMiu/total Miu) was obtained using the following equation: F = [pMiu/total Miu] / [pERK/total ERK] / [pERK/total ERK].

**Morphometric Analysis**—HeLa and COS-7 cells expressing RFP fused to a nuclear localization signal were trypsinized, suspended in phosphate-buffered saline, and observed under an inverted microscope.
Thirty cells for each were imaged for differential interference contrast and fluorescence to measure the diameters of cells and nuclei, respectively. The volumes of cells and nuclei were calculated assuming that they were spheres. The nuclear and cytoplasmic volumes of HeLa cells were 0.22 and 0.94 pl, respectively. Those of COS-7 were 0.14 and 2.4 pl, respectively.

**Nucleocytoplasmic Shuttling Analysis**—Nucleocytoplasmic shuttling of the Dronpa-tagged proteins was analyzed essentially as described previously (29). The cells expressing Dronpa-fused proteins were observed under an Olympus Fluoview FV500 confocal microscope equipped with an argon laser and a diode laser (405 nm). The fluorescence of Dronpa was erased at 488 nm for 20 s (20% laser power), followed by its photoactivation at the predetermined region by excitation at 405 nm for 400 ms (10% laser power). A decrease in the fluorescence of Dronpa at the predetermined region was monitored by weak excitation (1% laser power) at 488 nm using a BA505IF band-pass filter. The nuclear export rate was calculated from the initial decrease rate of the fluorescence. Here, we defined the export rate as the fraction exported from the nucleus to the cytoplasm/s. After the time-lapse experiment, Dronpa was highlighted in the whole cell area, and its fluorescence images were obtained by Z-sectioning to create a stacked image. Using the stacked image, the ratio of the molecules of nuclear Dronpa and cytoplasmic Dronpa was calculated.

**Numerical Simulation of Biochemical Reactions and Block Diagram**—All reactions including nucleocytoplasmic shuttling were represented by molecule-molecule interactions and enzymatic reactions. All steps are considered as taking place in either the cytosolic or nuclear compartment. GENESIS simulator (version 8.0) with a Kinetikit interface was used for solving the ordinary differential equations with a time step of 100 ms as described previously (14, 17). The GENESIS script of our in silico model is provided as supplemental material.

**RESULTS**

**Quantitation of Components of the ERK Signaling Cascade in HeLa Cells**—Because molecule numbers used as parameters differ considerably among simulation models, we started our study by quantitating the concentrations of H-Ras, N-Ras, K-Ras, c-Raf, MEK1, MEK2, ERK1, and ERK2 in the HeLa and COS-7 cells used in this study. The concentrations of these proteins were determined using either GST- or FLAG-tagged proteins as standards (supplemental Figs. 1 and 2). The volumes for the whole cells and nuclei were calculated from the diameters of the suspended cells. These volumes were used to obtain the concentrations of the signaling molecules in the nucleus and cytoplasm (supplemental Table 1). The results are summarized in Table 1 with reference to previous reports. The concentrations of Ras, MEK, and ERK were in the range of 0.1–3 μM, whereas the concentration of Raf was as little as 0.013 μM.

**Development of a Probe for ERK2 Binding to MEK**—Next, we developed a FRET-based probe for ERK to monitor its spatiotemporal regulation. The Miu2 probe comprised YFP, ERK2, and CFP from the N terminus and the YFP fluorescence acceptor. The level of FRET increases when Miu2 adopts the closed conformation upon binding to MEK. A probe was localized to the Miu2 with and without MEK in HeLa cells. C, YFP and YFP images of HeLa cells expressing the Miu2 probes and MEK1 as indicated were acquired and used to calculate the FRET level (YFP/CFP) (upper panel). The average CFP fluorescence intensities of the nucleus and cytoplasm adjacent to the nuclear membrane were calculated and used to obtain the relative fluorescence intensity of the cytosol/nucleus (middle panel). The net fluorescence intensity of YFP was measured to compare the expression levels of the probe (lower panel). Data for each cell (n > 30) are represented in the histogram. The wild-type (WT), T188A/Y190A mutant (Miu2-TAYA), and K57R mutant (Miu2-KR) Miu2 probes were used in combination with wild-type MEK1 and the S218D/S222E mutant (MEK5D5S) with anti-Flag or anti-GFP (IP) with anti-green fluorescent protein serum. Immunoprecipitates (IP) and total cell lysates (TCL) were analyzed using the antibodies indicated to the left.

**TABLE 1**

| TABLE 1 Concentrations of Ras, Raf, MEK, and ERK |
|-----------------------------------------------|
| **Concentration (μM)** | **this study** | **Huang and Ferrell** | **Bhalla et al.** | **Schoeberl et al.** | **Sasagawa et al.** | **Brightman and Fell** | **Hatakeyama et al.** |
|------------------------|---------------|----------------------|------------------|---------------------|---------------------|----------------------|------------------------|
| **HeLa**               | **COS-7**     | **(18) (oocytes)**   | **(44) (ND)**    | **(15) (HeLa)**     | **(17) (PC12)**     | **(13) (PC12)**       | **(12) (CHO)**         |
| Ras                    | 0.40          | 0.53                | ND               | 0.20                | 1.6                 | 0.10                 | 0.033                  |
| Raf                    | 0.013         | 0.0054              | 0.0030           | 0.20                | 0.0057              | 0.50                 | 0.017                  |
| MEK                    | 1.4           | 1.8                 | 1.2              | 0.18                | 3.1                 | 0.68                 | 0.6                    |
| ERK                    | 0.96          | 0.81                | 1.2              | 0.36                | 2.1                 | 0.26                 | 1.25                   |

* The concentrations were calculated as the number of molecules/cell, assuming that the cell volume was 1.0 pl, as described in Ref. 15.

**FIGURE 1** A FRET-based probe for monitoring ERK2 activity in living cells. A, shown is a schematic representation of Miu2. ERK2 is sandwiched between the CFP fluorescence donor and the YFP fluorescence acceptor. The level of FRET increases when Miu2 adopts the closed conformation upon binding to MEK. B, shown is the localization of Miu2 with and without MEK in HeLa cells. C, CFP and YFP images of HeLa cells expressing the Miu2 probes and MEK1 as indicated were acquired and used to calculate the FRET level (YFP/CFP) (upper panel). The average CFP fluorescence intensities of the nucleus and cytoplasm adjacent to the nuclear membrane were calculated and used to obtain the relative fluorescence intensity of the cytosol/nucleus (middle panel). The net fluorescence intensity of YFP was measured to compare the expression levels of the probe (lower panel). Data for each cell (n > 30) are represented in the histogram. The wild-type (WT), T188A/Y190A mutant (Miu2-TAYA), and K57R mutant (Miu2-KR) Miu2 probes were used in combination with wild-type MEK1 and the S218D/S222E mutant (MEK5D5S). A.U., arbitrary unit. D, cells expressing the proteins indicated were lysed and immunoprecipitated (IP) with anti-green fluorescent protein serum. Immunoprecipitates (IP) and total cell lysates (TCL) were probed with the antibodies indicated to the left.
Dynamics of ERK Activation

FIGURE 2. Time courses for the phosphorylation of Miu2. A and B, HeLa cells mock-transfected or transfected with expression plasmids for Miu2 and MEK1-monomeric RFP (mRFP) were stimulated with EGF (50 ng/ml). Total cell lysates were prepared at the indicated time points, followed by immunoblot (IB) analysis with the antibodies indicated to the left. C, a portion of the cell lysates was immunoprecipitated with anti-phospho-Thr188 antibodies and analyzed in parallel with the total cell lysates after incubation with varying concentrations of EGF. The column of immunoprecipitates was used to calculate the fraction of phospho-MEK upon EGF stimulation. The relative intensities of the bands at 100 ng/ml EGF are noted at the bottom. D, the fraction of phospho-ERK was determined from the band shift of endogenous ERK shown in A. The intensities of endogenous ERK on anti-ERK and anti-phospho-ERK immunoblots were used to calculate the fraction of phospho-Miu2. E, shown is the time course for the increase in phospho-MEK.

either the absence or presence of MEK. We could not coexpress the wild-type probe with constitutively active MEK (MEK-SDSE) because of its severe cytotoxicity. However, we could do so using the Miu2-TAYA and Miu2-KR mutants, probably because these mutants could not be activated by the MEK-SDSE mutant. MEK-SDSE could increase the FRET efficiency of the Miu2-TAYA mutant, but not the Miu2-KR mutant. Notably, the increase in FRET efficiency correlated perfectly with the retention of the probe in the cytoplasm. Thus, these observations indicated that the phosphorylation of Thr188 and Tyr190 triggered the dissociation of MEK from the Miu2 probe and that the FRET level reflected its binding to MEK. This finding was further confirmed by the co-immunoprecipitation experiment (Fig. 1D).

Correlation of the Phosphorylation of the FRET Probe with That of Endogenous ERK2—To validate the use of the probe for monitoring ERK2 activation, we compared the time course of the phosphorylation of the probe and that of endogenous ERK2 in EGF-stimulated HeLa cells (Fig. 2, A, B, and D). The phosphorylation of Thr188 and Tyr190 of both the probe and endogenous ERK reached to its zenith in 5 min and returned slowly to the basal level in 30 min. The time course of MEK phosphorylation also correlated with this time course. Furthermore, we calculated the percentage of probe phosphorylation as described under “Materials and Methods.” The maximum phosphorylation level (<50%) and the overall time course were very similar between the Miu2 probe and endogenous ERK. Thus, we concluded that the phosphorylation of the probe and therefore the FRET efficiency of the probe could be used as an index of activation of endogenous ERK. We also examined the level of phosphorylated MEK using anti-phospho-MEK immunoprecipitates as a calibration marker (Fig. 2, C and 2E). In contrast to ERK, only 5% of MEK was phosphorylated, even in the presence of an excess of EGF.

FIGURE 3. FRET imaging of ERK activation by Miu2. A, HeLa cells expressing the Miu2 probe were replated onto glass-bottom dishes coated with collagen to observe the activation of the probe upon EGF stimulation. YFP and CFP images were recorded every 1 min for 40 min. A YFP/CFP ratio image was created to represent the FRET level. Shown here are representative pseudo-color images of the FRET (shown in intensity modulated display mode) and CFP images. Ratio ranges are shown to the right. Scale bar = 10 μm. B, CFP fluorescence intensities and FRET levels (YFP/CFP) in the nucleus and cytoplasm are plotted against time. A.U., arbitrary unit.

ERK Activation Analyzed Using the Miu2 Probe—Using the FRET probe described above, we visualized the activation of ERK in HeLa cells. Typical images and time courses of CFP intensity and FRET values of the probe are shown in Fig. 3. The data obtained from seven in depth-analyzed video images are summarized as follows. First, EGF stimulation immediately triggered a decrease in the FRET level in both the cytoplasm and nucleus. Probably because we needed 30 s to apply EGF to the culture dish, we could not detect a time lag between EGF application and the decrease in the FRET level. Second, there was a time lag (2.8 ± 1.3 min) between the initiation of FRET change and the onset of a detectable increase in the level of ERK in the nucleus. Third, the FRET level reached its nadir first in the cytoplasm (5.5 ± 2.2 min) and then in the nucleus (5.9 ± 2.1 min). Nuclear accumulation of the probe reached its zenith in 7.8 ± 2.1 min (Fig. 3B).

Interaction of ERK and MEK Visualized Using Intermolecular FRET Probes—To confirm the results obtained above and to examine the dynamics of MEK, we next visualized the interaction of ERK2 with MEK1 by the intermolecular FRET method. We first searched for an optimum pair of ERK and MEK tagged with monomeric CFP and monomeric YFP, respectively, by fluorescence-activated cell sorter analysis. In a preliminary experiment, we found that MEK, if fused to the N terminus of YFP, did not bind to ERK; therefore, we excluded this construct from further analyses. Using MEK-YFP, we compared the FRET levels of CFP-ERK and ERK-CFP. As shown in supplemental Fig. 3 (A–D), only CFP-ERK yielded significant FRET with MEK-YFP. This result might suggest that the N terminus of ERK is in close proximity to the C terminus of MEK. We then imaged the dynamics of MEK and ERK in cells expressing CFP-ERK and MEK-YFP in HeLa cells (supplemental
nucleus. The results were very similar to those shown in Fig. 3. Notably, we did not find a significant change in the distribution of MEK upon EGF stimulation. EGF-dependent phosphorylation of CFP-ERK and YFP-MEK was confirmed by immunoblotting (supplemental Fig. 3G). Of note, similar results were obtained when we used YFP-ERK and MEK-CFP, indicating that the uneven distribution of fluorescent proteins did not significantly affect the results (supplemental Fig. 3, H and I).

Kinetics of RasRaf-MEK-ERK Dissociation—Next, we attempted to measure the dissociation rates of protein-protein complexes in living cells. c-Raf tagged with Dronpa was detected mostly at the plasma membrane when coexpressed with constitutively active Ras (H-Ras-G12V). c-Raf-Dronpa was highlighted by a flash of light, and its dissociation was monitored (Fig. 4A). In the presence of H-Ras-G12V, the half-life ($\tau_o$) of c-Raf-Dronpa increased significantly, indicating that the dissociation of the Ras-Raf complex was significantly slower than the cytoplasmic diffusion of c-Raf-Dronpa. Thus, by measuring the decay of c-Raf-Dronpa in the presence of H-Ras-G12V, we could estimate the half-life of the Ras-Raf complex ($\tau_{1/2} = 14.1$ s) (Fig. 4B). To monitor the dissociation of the Raf-MEK complex, we used the c-Raf-S621A-pm mutant (30). For c-Raf binding to MEK, c-Raf must adopt the open conformation, which is induced by the binding of Ras in the physiological context. The S621A mutant is found to adopt the open conformation in the absence of Ras. The suffix pm indicates that the protein localizes at the plasma membrane by means of a K-Ras C box domain fused to c-Raf. In cells expressing c-Raf-S621A-pm, most of the MEK-Dronpa fusion protein localized at the plasma membrane. Under this condition, the dissociation of MEK-Dronpa from c-Raf-S621A-pm was monitored to calculate the half-life of the Raf-MEK complex ($\tau_{1/2} = 10.5$ s). Similarly, using MEK-pm and ERK-Dronpa, we calculated the half-life of the MEK-ERK complex ($\tau_{1/2} = 7.8$ s). From these half-lives, we estimated the dissociation rates (Table 2), assuming that

![FIGURE 4. Monitoring protein-protein complex dissociation using Dronpa-tagged proteins. HeLa cells expressing Dronpa-tagged proteins were analyzed in the presence or absence of their binding partners anchored to the plasma membrane. A typical example is shown in A. After erasure of the fluorescence, Dronpa was highlighted only in the encircled regions, followed by time-lapse imaging. Scale bar = 10 μm. The decrease in the fluorescence of the highlighted regions is plotted against time in B.]

### TABLE 2
Parameters for interactions: $k_f$ (per $s/\mu m$), $k_o$ (per s), and $K_d$ (μM)

| Step | Reaction | This study$^a$ | Schoeberl et al. (15)$^b$ | Sasagawa et al. (17) | Bhalla et al. (44) | Hatakeyama et al. (12) | Yamada et al. (11) |
|------|----------|---------------|-------------------------|---------------------|-------------------|------------------------|------------------|
| 1    | Ras + Raf→ Ras(p)Raf | 0.49, 0.049, 0.1 | 1.0, 0.0053, 0.0053 | 60.0, 0.0083 | 24.5, 0.02 | 1.8, 0.05, 0.028 |
| 2    | RasRaf + MEK→ RasRafMEK | 0.65, 0.065, 0.1 | 11.0, 0.018, 0.0016 | 15.6, 2.0, 0.13 | 3.3, 0.4, 0.12 | 9.2, 0.9, 0.098 |
| 3, 4, 13, 14 | (p)MEK + ERK→ (p)MEK-ERK | 0.88, 0.088, 0.1 | 0.11, 0.033, 0.03 | 16.6, 0.035 | 16.3, 0.6, 0.037 | 0.318, 0.9, 2.8 |

$^a$ $k_o$ was obtained from $\tau_o$, shown in Fig. 4. From the same imaging data, we approximated $K_d$ at 0.1, assuming that the concentration of the recombinant proteins was 10 μM each and that 90% of the proteins were associated at the plasma membrane. The $k_f$ values were calculated from $k_o$ and $K_d$.  

$^b$ This parameter set was also used in the model reported by Hatakeyama et al. (12).

$^c$ Assuming that the ratio $= 4$, $V_{max} = 0.5$, and $K_m = 0.16$.  

### TABLE 3
Parameters for kinase reactions: $V_{max}$ (per s)

| Step | Reaction | This study$^a$ | Schoeberl et al. (15) | Sasagawa et al. (17) | Bhalla et al. (44) | Hatakeyama et al. (12) | Yamada et al. (11) |
|------|----------|---------------|-------------------------|---------------------|-------------------|------------------------|------------------|
| 6    | RasRafMEK→RasRaf+pMEK | 0.18 | 2.9 | 0.5 | 0.105 | 3.5 | 1.7 |
| 5, 15 | pMEK+ERK→pMEK+pERK | 0.22 | 5.7 | 0.15 | 0.15 | 9.5 | 0.10 |

$^a$ $V_{max}$ was calculated as described in the supplemental material.

### TABLE 4
Phosphatase reactions: $k_p$ (per s)

| Step | Reaction | Present study$^a$ | Schoeberl et al. (15)$^b$ | Sasagawa et al. (17)$^b$ | Bhalla et al. (44)$^b$ | Hatakeyama et al. (12)$^b$ |
|------|----------|-------------------|-------------------------|---------------------|-------------------|------------------------|
| 16, 18 | pMEK→MEK | 0.010 | 0.064 | 0.046 | 0.086 | 0.011 |
| 17, 19 | pERK→ERK | 0.014 | 75 | 0.054 | 0.0036 | 0.011 |

$^a$ The phosphatase reaction was represented as a first-order reaction by integrating the concentration of phosphatases, assuming that the cell volume was $1.0 \times 10^{-12}$ liter.

$^b$ The original data were provided in the form of $K_m$ and $V_{max}$. The concentrations of phosphatases were integrated to obtain $k_p$ as described in supplemental Tables 2a and 2b.
the diffusion of the probe could be neglected and that the initial dissociation was well described by a single exponential function.

Estimation of the \( V_{\text{max}} \) Values of Raf and MEK—The \( V_{\text{max}} \) values of MEK phosphorylation by c-Raf and ERK phosphorylation by MEK were estimated as described in the supplemental material and summarized in Table 3.

Measurement of Phosphatase Activity—To measure the dephosphorylation rates of ERK by phosphatases, we treated the EGF-stimulated HeLa cells with the MEK inhibitor U0126 and determined the decreases in the phospho-MEK and phospho-ERK levels every 30 s (supplemental Fig. 4). The level of phospho-ERK decreased rapidly (\( \tau_{1/2} = 50 \) s). Similarly, we treated the EGF-stimulated HeLa cells with AG1478, an EGF receptor inhibitor, and followed the decrease in the phospho-MEK level. The half-life of phospho-MEK was slightly longer than that of phospho-ERK (\( \tau_{1/2} = 88 \) s). From these half-lives, we estimated the rate constants of phosphatase reactions (Table 4). Of note, these half-lives and rate constants must be underestimates because the time required for the penetration of the reagents was not considered.

Nucleocytoplasmic Shuttling of MEK and ERK—We examined the nuclear export rates of ERK and MEK by highlighting ERK-Dronpa and MEK-Dronpa in the nucleus (Fig. 5A and supplemental Fig. 5A). Using the slope of the initial decrease in the fluorescence intensity, we calculated the export rates of ERK and MEK (Table 5). The export rate of MEK was markedly faster than that of ERK, as was expected because of the presence of a nuclear export signal in MEK (38). To examine the export rate of activated ERK, we examined the export rate of ERK-Dronpa in the presence of the active MEK-SDSE mutant, which harbors phosphomimetic aspartate and glutamate at putative phosphorylation sites. Under this condition, >80% of ERK was phosphorylated when examined using anti-phospho-p44/42 ERK antibody (data not shown). We did not find a significant difference in the export rate of ERK-Dronpa upon the coexpression of MEK-SDSE, negating the effect of ERK phosphorylation on its export rate. To examine the export rate of activated MEK, we used MEK-SDSE-Dronpa. However, we did not find a significant difference between the export rates of MEK-Dronpa and MEK-SDSE-Dronpa, suggesting that the phosphorylation of MEK did not significantly affect the export rate of MEK-Dronpa. Notably, the export rate of ERK-Dronpa was remarkably increased upon the expression of wild-type MEK, in support of a previous report showing that MEK exports ERK from the nucleus to the cytoplasm (39).

We next examined the import rate of ERK by photoactivating ERK-Dronpa in the cytoplasm (Fig. 5B and supplemental Fig. 5B). Again, we did not find any remarkable differences in the presence or absence of active MEK. Notably, the nuclear import rates measured by this method were similar to those calculated from the export rate and relative molecule numbers in the nucleus and cytoplasm (Table 5, shown in parentheses). We could not measure the nuclear import rate of MEK because the fluorescence of nuclear MEK-Dronpa increased only modestly after photoactivation of cytoplasmic MEK-Dronpa. In this case, the nuclear import ratio was calculated from the export rate and the nuclear/cytoplasmic ratio of molecule numbers, assuming that the import and export were in equilibrium.

Construction of a Simulation Model of ERK Activation—The parameters obtained as described above are summarized in comparison with parameters used in previous studies in Tables 1–5. Using these parameters obtained with living cells, we constructed the kinetic simulation model shown schematically in Fig. 6. In step 1, Ras recruits and activates Raf at the plasma membrane. The Ras-Raf complex recruits and phosphorylates MEK (steps 2 and 6). Both non-phosphorylated MEK and phosphorylated MEK form heterodimers with ERK in either the cyto-
TABLE 5
Nucleocytoplasmic shuttling of MEK and ERK

| Step | Reaction (probes used to monitor) | Molecule no. ratio (cytoplasm/nucleus) | \( \tau_c \) (export, import) | Export rate (per s) | Import rate (per s) |
|------|----------------------------------|----------------------------------------|-------------------------------|--------------------|-------------------|
| 7    | MEKnuc ⇔ MEKcyt (MEK-Dronpa)     | 13 ± 3.7                                | 1.1 ± 0.22, ND                | 0.61               | 0.046*            |
| 10   | pMEKnuc ⇔ pMEKcyt (MEK-SDSE-Dronpa) | 13 ± 5.7                                | 1.3 ± 0.49, ND                | 0.54               | 0.040*            |
| 9    | ERKnuc ⇔ ERKcyt (ERK-Dronpa)     | 1.5 ± 0.2                               | 39 ± 14, 80 ± 28             | 0.018              | 0.0086 (0.012)*   |
| 8, 12| (p)MEK-ERKnuc ⇔ (p)MEK-ERKcyt (ERK-Dronpa + MEK) | 7.5 ± 2.0                               | 2.7 ± 1.3, ND                | 0.26               | 0.035*            |
| 11   | pERKnuc ⇔ pERKcyt (ERK-Dronpa + MEK-SDSE) | 1.1 ± 0.72                              | 55 ± 94, 98 ± 18             | 0.013              | 0.0070 (0.011)*   |

*The import rate was calculated as the export rate/molecular number ratio.

---

**Comparison of Parameter Sets**—The kinetic simulation program was next operated using parameter sets from previous studies (Fig. 8). When we considered the level of nuclear phospho-ERK as the output, significant differences were observed in the basal levels. When we used the parameters reported in Refs. 12 and 44, the basal levels of nuclear phospho-ERK were significantly higher than those of the others. Another interesting difference became evident when we plotted the concentrations of the maximum values of activated Ras, the Ras/Raf complex, phospho-MEK, and phospho-ERK (Fig. 8, lower right panel). In all cases, the concentration of the active molecule was least in Raf. The signals were amplified mostly at the level of MEK activation with parameter sets from Refs. 15 and 17, whereas the signals were amplified at the level of both MEK and ERK phosphorylation using the other parameter sets, including ours.

**Sensitivity to the Concentration of Raf**—During the analyses of the model, we noticed that the levels of phospho-MEK and phospho-ERK were particularly sensitive to the concentration of Raf (Fig. 9, A and B). To confirm this prediction experimentally, we decreased the amount of c-Raf in HeLa cells by the small interfering RNA method. Almost linear decreases in the levels of phospho-MEK (Fig. 9, C and D) and phospho-ERK (Fig. 9, C and E) were observed depending on the amount of c-Raf, as predicted by the model.

**DISCUSSION**

Because of technical difficulty, previous computational models of the Ras/ERK MAPK cascade were forced to contain parameters estimated without experimental bases. Such parameters that are optimized for each model sometimes differ significantly among different models. Thus, the principal object of this study was to provide the parameter set...
Dynamics of ERK Activation

FIGURE 8. Comparison of parameter sets. The parameter sets used in previous studies (Tables 1–4) were employed in our in silico model. The maximum values during the course of simulation are plotted in the lower right panel.

FIGURE 9. Sensitivity to the concentration of c-Raf. A and B, shown are computer simulations of growth factor-stimulated phosphorylation of MEK and ERK, respectively, at different concentrations of c-Raf. C, HeLa cells incubated with small interfering RNA (siRNA) for 24 or 43 h were subjected to immunoblot analysis. D and E, the concentrations of phospho-(p)-MEK, phospho-ERK, and c-Raf were calculated from the immunoblot (IB) images. The concentrations of cellular phospho-MEK and phospho-ERK were calculated provided that 5 and 65% of total MEK and ERK, respectively, were phosphorylated upon stimulation. The concentrations of signaling molecules in the cells in our experiments, we obtained from experiments or at least estimated from experimental data for the computational analysis of the Ras/ERK MAPK cascade.

Number of Signaling Molecules—Having first determined the concentrations of signaling molecules in the cells in our experiments, we then compared them with those used in previous simulation models (Table 1). The most conspicuous difference among the reports is the concentration of Raf. We found that the concentration of Raf was significantly lower than those of the other signaling molecules, which was predicted by three groups (13, 15, 18). In contrast, the three other studies set the level of Raf to a level similar to or higher than those of the other components. This discrepancy might be explained by differences between cell lines used for the model. Alternatively, the property of anti-c-Raf antibodies used in each study might have caused the difference. We tested three commercially available anti-c-Raf antibodies and found that one reacted preferably with endogenous c-Raf compared with exogenous c-Raf (supplemental Fig. 2). This could lead to an overestimation of endogenous c-Raf. The observation that overexpression of exogenous c-Raf readily enhances EGF-induced MEK activation seems to support the notion that the concentration of c-Raf is indeed significantly lower than those of the other components (30). Furthermore, decreasing the concentration of c-Raf linearly decreased both the basal and stimulated levels of ERK phosphorylation both in vivo and in our in silico model (Fig. 9). Thus, we believe that the concentration of c-Raf is indeed markedly lower than those of the other components.

In some models, the concentration of ERK exceeds that of MEK (Table 1). If we included this assumption in our in silico model, ERK accumulated in the nucleus even before stimulation (data not shown), which is apparently in contrast many reports showing that ERK is mostly cytosolic before stimulation. Notably, upon EGF stimulation, only 5% of MEK was phosphorylated, under the condition in which nearly 50% of ERK was phosphorylated. This observation suggested that a small fraction of MEK contributed to the phosphorylation of ERK and that most of the ERK in the MEK-ERK complex was not phosphorylated directly by the associated MEK. From another viewpoint, the excess of MEK might contribute primarily to exclude ERK from the nucleus to the cytoplasm.

Molecular Interaction—We did not find any critical differences between our parameters used to simulate the molecular interactions and those of previous reports (Table 2). Schoeberl et al. (15) adopted slow dissociation velocity ($k_d$) for the interactions of Ras and Raf; Ras, Raf, and MEK; and MEK and ERK. However, we found that the system was robust with respect to these parameters as far as the dissociation constant ($k_d/k_c$) remained the same (data not shown). In other words, the velocities of association and dissociation are fast enough when we consider the dissemination of growth signals from the plasma membrane to the nucleus.

Kinase Reactions—To obtain the $V_{\text{max}}$ of the kinase reactions from the imaging data, we assumed that all enzymes were fully activated and associated with the substrates at the time of stimulation. Furthermore, we did not consider the time lag between the application of a stimulus and the initiation of the reaction. Thus, the $V_{\text{max}}$ obtained must have been underestimated. Indeed, the $V_{\text{max}}$ values obtained in this study are similar to or smaller than those adopted in previous reports (Table 3). This may explain the slower kinetics and lower peak level of ERK phosphorylation in silico compared with that observed in vivo. An increase in the $V_{\text{max}}$ of either Raf or MEK accelerated ERK phosphorylation in silico (supplemental Fig. 7); however, a 16-fold increase in the $V_{\text{max}}$ of MEK or Raf resulted in a <2-fold increase in nuclear phospho-ERK at its zenith. When we increased the $V_{\text{max}}$ of both Raf and MEK, the increase in phospho-ERK was more obvious. However, a 16-fold increase in the $V_{\text{max}}$ of both Raf and MEK resulted in only a 2-fold increase in the level of phospho-ERK. Thus, it is likely that the $V_{\text{max}}$ values obtained in this study might be significantly lower than those in vivo. We need to...
develop a method to stimulate Raf and MEK more directly and rapidly within the cells to obtain more accurate $V_{\text{max}}$ values.

**Phosphatase Reactions**—To analyze the phosphatase reactions, we used inhibitors of the EGF receptor and MEK (supplemental Fig. 4). The $k_f$ was obtained from the half-lives of phosphorylated substrates, assuming that these inhibitors blocked the signaling immediately after application. Thus, the parameters used in our study must have been underestimated considering the time required for the penetration of the inhibitors. In agreement with this notion, the $k_f$ values obtained by our method are smaller than most of the parameters used in the previous studies (Table 4). However, it should be noted that the parameters used for phosphatases differ surprisingly in each study. This may be ascribable to the obscurity of the phosphatases responsible for the down-regulation of ERK and MEK.

In our *in silico* model, we assumed that the phosphatases distribute evenly both in the cytoplasm and nucleus. This is an unavoidable constraint simply because it is not possible to measure the phosphatase activities of the nucleus and cytoplasm separately. Some ERK-specific dual phosphatases that localize mostly in the nucleus are induced upon activation of Raf as a major bottleneck of the signal flow in our kinetic simulation program. A major reason for the discrepancy might be our assumption that Raf is active only in the Ras-bound form (30). In addition to the lowest molecule number, this restriction caused the activation of Raf as a major bottleneck of the signal flow in our kinetic simulation model (Fig. 8).

**Conclusion**—We have attempted to construct a simulation model of the Ras/ERK MAPK cascade consisting of quantifiable molecules and measurable processes in living cells. As a consequence, we neglected all scaffold proteins, feedback loops, inducible proteins, etc. Nevertheless, our simulation model reproduced the essential features of EGF-induced activation and nuclear translocation of ERK. This success has proved the versatility of fluorescence-based probes for the collection of spatial-temporal parameters of signaling molecules and encourages us to expand the simulation model based on the parameters collected in living cells.

**Acknowledgments**—We thank S. Sasagawa and Y. Ozaki for the introduction to the simulation program; A. Miyawaki and J. Miyazaki for plasmids; and N. Yoshida, N. Fujimoto, K. Fukuhara, and Y. Matsuura for technical assistance.

**REFERENCES**

1. Sturgill, T. W., and Wu, J. (1991) *Biochim. Biophys. Acta 1092*, 350–357
2. Nishida, E., and Gotoh, Y. (1993) *Trends Biochem. Sci. 18*, 128–131
3. Lewis, T. S., Shapiro, P. S., and Abl, N. G. (1998) *Adv. Cancer Res. 74*, 49–139
4. Chang, L., and Karin, M. (2001) *Nature 410*, 37–40
5. Pearson, G., Robinson, F., Beers, G. T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001) *Endocr. Rev. 22*, 153–183
6. O’Neill, E., and Kolch, W. (2004) *Br. J. Cancer 90*, 283–288
7. Tori, S., Nakayama, K., Yamamoto, T., and Nishida, E. (2004) *J. Biochem. (Tokyo) 136*, 557–561
8. Pouyssegur, J., Volmat, Y., and Lenormand, P. (2002) *Biochim. Pharmacol. 64*, 755–763
9. Ebiuwa, M., Kondoh, K., and Nishida, E. (2005) *J. Cell Sci. 118*, 2997–3002
10. Markevich, N. I., Hoek, J. B., and Khodolenko, B. N. (2004) *J. Cell Biol. 164*, 353–359
11. Yamada, S., Taketomi, T., and Yoshimura, A. (2004) *Biochem. Biophys. Res. Commun. 314*, 1113–1120
12. Hatakeyama, M., Kimura, S., Naka, T., Kawasaki, T., Yumoto, N., Ichikawa, M., Kim, J. H., Saito, K., Saecki, M., Shirouzu, M., Yokoyama, S., and Konagaya, A. (2003) *Biochem. J. 373*, 451–463
13. Brightman, F. A., and Fell, D. A. (2000) *FEBS Lett. 482*, 169–174
14. Bhalla, U. S., and Iyengar, R. (1999) *Science 283*, 381–387
15. Schoebler, B., Eichler-Jonsson, C., Gilles, E. D., and Muller, G. (2002) *Nat. Biotechnol. 20*, 370–375
16. Bhalla, U. S., Ram, P. T., and Iyengar, R. (2002) *Science 297*, 1018–1023
17. Sasagawa, S., Ozaki, Y., Fujita, K., and Kuroda, S. (2005) *Nat. Cell Biol. 7*, 365–373
18. Huang, C. Y., and Ferrell, J. E., Jr. (1996) *Proc. Natl. Acad. Sci. U. S. A. 93*, 10078–10083
19. Pollok, B. A., and Heim, R. (1999) *Trends Cell Biol. 9*, 57–60
20. Sekar, R. B., and Periasamy, A. (2003) *J. Cell Biol. 160*, 629–633
21. Miyawaki, A. (2003) *Dev. Cell 4*, 295–305
22. Zhang, J., Campbell, R. E., Ting, A. Y., and Tsien, R. Y. (2002) *Science 296*, 70–73
23. Bastiaens, P. I., and Squire, A. (1999) *Trends Cell Biol. 9*, 48–52
24. Hailey, D. W., Davis, T. N., and Muller, E. G. (2002) *Methods Enzymol. 351*, 34–49
25. Deutscher, M. P. (2000) *Trends Biochem. Sci. 25*, 1387–1395
26. Lippincott-Schwartz, J., and Patterson, G. H. (2003) *Science 300*, 87–91
27. Reits, E. A., and Neefjes, J. J. (2001) *Trends Cell Biol. 11*, E145–E147
28. Fujimoto, K., and Cinacchi, M. (2002) *Dev. Cell 3*, 223–228
29. Huang, C. Y., and Ferrell, J. E., Jr. (1996) *Proc. Natl. Acad. Sci. U. S. A. 93*, 10078–10083
30. Pollok, B. A., and Heim, R. (1999) *Trends Cell Biol. 9*, 57–60
31. Sekar, R. B., and Periasamy, A. (2003) *J. Cell Biol. 160*, 629–633
32. Miyawaki, A. (2003) *Dev. Cell 4*, 295–305
33. Zhang, J., Campbell, R. E., Ting, A. Y., and Tsien, R. Y. (2002) *Science 296*, 70–73
34. Bastiaens, P. I., and Squire, A. (1999) *Trends Cell Biol. 9*, 48–52
35. Deutscher, M. P. (2000) *Trends Biochem. Sci. 25*, 1387–1395
36. Lippincott-Schwartz, J., and Patterson, G. H. (2003) *Science 300*, 87–91
37. Reits, E. A., and Neefjes, J. J. (2001) *Trends Cell Biol. 11*, E145–E147
38. Day, R. N., and Schaufele, F. (2005) *Mol. Endocrinol. 19*, 1675–1686
39. Ando, R., Mizuno, H., and Miyawaki, A. (2004) *Science 306*, 1370–1373
40. Terai, K., and Matsuda, M. (2005) *EMBO Rep. 6*, 251–255
41. Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002) *Science 296*, 913–916
32. Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7877–7882
33. Ohba, Y., Kurokawa, K., and Matsuda, M. (2003) EMBO J. 22, 859–869
34. Kawai, T., Sato, S., Ishii, K. J., Coban, C., Hemmi, H., Yamamoto, M., Terai, K., Matsuda, M., Inoue, J. I., Uematsu, S., Takeuchi, O., and Akira, S. (2004) Nat. Immunol. 5, 1061–1068
35. Sorkin, A., McClure, M., Huang, F., and Carter, R. (2000) Curr. Biol. 10, 1395–1398
36. Ohba, Y., Mochizuki, N., Yamashita, S., Chan, A. M., Schrader, J. W., Hattori, S., Nagashima, K., and Matsuda, M. (2000) J. Biol. Chem. 275, 20020–20026
37. Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A., and Matsuda, M. (2001) Nature 411, 1065–1068
38. Fukuda, M., Gotoh, I., Adachi, M., Gotoh, Y., and Nishida, E. (1997) J. Biol. Chem. 272, 32642–32648
39. Adachi, M., Fukuda, M., and Nishida, E. (2000) J. Cell Biol. 148, 849–856
40. Pouyssegur, J., and Lenormand, P. (2003) Eur. J. Biochem. 270, 3291–3299
41. Torii, S., Kusakabe, M., Yamamoto, T., Maekawa, M., and Nishida, E. (2004) Dev. Cell 7, 33–44
42. Riddick, G., and Macara, I. G. (2005) J. Cell Biol. 168, 1027–1038
43. Walker, S. A., Kupzig, S., Bouyoucef, D., Davies, L. C., Tsuboi, T., Bivona, T. G., Cozier, G. E., Lockyer, P. J., Buckler, A., Rutter, G. A., Allen, M. J., Philips, M. R., and Cullen, P. J. (2004) EMBO J. 23, 1749–1760
44. Bhalla, U. S. (2004) Biophys. J. 87, 745–753