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Tadahide Furuno, Naohide Hirashima, Shinobu Onizawa, Noriko Sagiya and Mamoru Nakanishi

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Nuclear Shuttling of Mitogen-Activated Protein (MAP) Kinase (Extracellular Signal-Regulated Kinase (ERK) 2) Was Dynamically Controlled by MAP/ERK Kinase After Antigen Stimulation in RBL-2H3 Cells

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The mitogen-activated protein kinase (MAPK) cascade consists of MAPK and its activator, MAPK kinase (MAP/ERK kinase; MEK). However, the mechanisms for activation of ERK2 have not been defined yet in cells. Here, we used fluorescent protein-tagged ERK2 and MEK to examine the localization of ERK2 and MEK in living rat basophilic leukemia (RBL-2H3) cells. ERK2 was mainly in the cytoplasm in resting cells but translocated into the nucleus after the ligation of IgE receptors. The import of ERK2 reached the maximum at 6–7 min, and then the imported ERK2 was exported from the nucleus. MEK mainly resided in the cytoplasm, and no significant MEK translocation was detected statically after ligation of IgE receptors. However, analysis of the dynamics of ERK2 and MEK suggested that both of them rapidly shuttle between the cytoplasm and the nucleus and that MEK regulates the nuclear shuttling of ERK2, whereas MEK remains mainly in the cytoplasm. In addition, the data suggested that the sustained calcium increase was required for the optimal translocation of ERK2 into the nucleus in RBL-2H3 cells. These results gave a new insight of the dynamics of ERK2 and MEK in the nuclear shuttling of RBL-2H3 cells after the ligation of IgE receptors.

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Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan. E-mail address: mamoru@phar.nagoya-cu.ac.jp

Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan

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1 Address correspondence and reprint requests to Dr. Mamoru Nakanishi, Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan. E-mail address: mamoru@phar.nagoya-cu.ac.jp

2 Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; RBL-2H3, rat basophilic leukemia; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; CLSM, confocal laser scanning microscopy; fura 2-AM, fura 2-acetyl-methyl ester; [Ca2+]i, intracellular free calcium ion concentration; NES, nuclear export signal.

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Materials and Methods

Materials

GFP expression vector (pCMX-SAM/Y145F) was given by Professor K. Umesono (Kyoto University, Kyoto, Japan) (13). pEYFP-C1 and pECFP-N1 were obtained from Clontech Laboratories (Palo Alto, CA). Mouse ERK2 and human MEK (MKK1) genes were obtained from the American Type Culture Collection (Manassas, VA). Anti-ERK1/2, anti-phospho-ERK1/2, anti-MEK1/2, and anti-phospho-MEK1/2 Abs were obtained from New England Biolabs (Beverly, MA). Fura 2-acetoxymethyl ester (fura 2-AM) was obtained from Molecular Probes (Eugene, OR). PD98059 was obtained from Calbiochem (La Jolla, CA), and wortmannin was obtained from Wako Pure Chemicals (Osaka, Japan).

Construction of GFP-ERK2

Preparation of fluorescent protein-tagged ERK2 and MEK was as follows: ERK2 cDNA served as a template in PCR amplification using appropriate oligonucleotide primers, and GFP was conjugated to the N terminal of ERK2. For the generation of a GFP-ERK2 chimera protein, ERK2 cDNA was amplified with oligonucleotides such that Sall and Nhel restriction sites were introduced at the 5’ and 3’ ends, respectively. Sall and Nhel restriction sites, as described above, achieved fusion between GFP and N terminal of ERK2. Fusion between enhanced (E)YFP and N terminal of ERK2 was achieved at HindIII and BamHI restriction sites. HindIII and BamHI also did fusion between ECFP and C terminal of MEK restriction sites. Western blot analysis showed that the molecular mass of GFP-ERK2 (YFP-ERK2) and MEK-CFP were 68 and 72 kDa, respectively. They were well consistent with the calculated molecular mass of GFP-ERK2 (YFP-ERK2) and MEK-CFP.

Cell culture and electroporation

RBL-2H3 cells were cultured in MEM supplemented with 10% FCS from Boehringer Mannheim (Indianapolis, IN). The cells were electroporated in cold K+-PBS buffer with 20 μg of plasmid DNA at 300 V and 950 μF using Gene Pulser (Bio-Rad, Richmond, CA) (14). RBL-2H3 cells transfected with plasmid DNAs were cultured in the observation chamber (Elec- kon, Chiba, Japan) for a few days and were used for the experiments. Stable transfectant cells were obtained by the selection with antibiotic G418 (Life Technologies, Rockville, MD).

Western blot analysis

To prepare whole-cell lysate, collected RBL-2H3 cells were suspended in lysis buffer (20 mM HEPES (pH 7.3), 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 2.5 mM p-nitrophenyl phosphate, 1 mM Na3VO4, 10 μg/ml PMSF, 10 μg/ml leupeptin, and 10% glycerol) and allowed to stand on ice for 30 min (15). The suspension was clarified by centrifugation (150,000 × g for 20 min). After centrifugation, the resulting supernatants were solubi- lized by treatment with Laemmli buffer at 100°C for 3 min and separated by electrophoresis in 8% SDS-polyacrylamide gel. The electrophoresed proteins were transferred to polyvinylidene difluoride membrane with an electroblotter. After blocking with 0.5% casein, the membranes were probed with rabbit anti-ERK1/2 Ab (1:1000 dilution), anti-phospho-ERK1/2 Ab (1:1000 dilution), anti-MEK1/2 Ab (1:1000 dilution), or anti-phospho-MEK1/2 Ab (1:1000 dilution) and treated with 1:1000 dilution HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 0.4 μg/ml) (14). The amount of HRP-conjugated IgG bound to each protein band was determined by LAS-1000 (Fuji Film, Tokyo, Japan) and was analyzed by Image Gauge (Fuji Film).

Microscopic measurements

Fluorescence microscopic measurements were performed by the previous procedure (14, 16, 17). The transfected RBL-2H3 cells were harvested from culture dishes and transferred to an observation chamber. After that, the cells were treated with HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl2, and 1 mM CaCl2 (pH 7.2)) with mouse anti-DNP IgE. In our present experiments, seven 2,4-DNP groups, on average, were conjugated with BSA (DNP7-BSA). Fluorescence microscopic images were observed with confocal laser scanning microscopes (CLSM) (LSM-410 and LSM-510; Zeiss, Oberkochen, Germany) with argon ion lasers (458 and 488 nm). GFP and YFP fluorescence was excited at 488 nm, and its emission was observed through a long-path filter (above 515 nm). EYFP and fura red fluorescence were excited at 488 nm, and their emissions were observed through a band filter (505–530 nm) and a long-path filter (above 585 nm), respectively. ECFP and EYFP fluorescence was excited at 458 nm, and their emissions were observed through one band filter (475–490 nm) and another (560–615 nm), respectively. The temper-ature of the observation chambers was maintained at 37°C during experiments. Conventional fluorescence microscopy was done using Argus 50CA (Hamamatsu Photonics, Hamamatsu City, Japan). The excitation wavelengths were 340 nm for fura 2 and 490 nm for GFP. A long-path filter (above 530 nm) was used to observe their fluorescence emission.

Immunocytochemical experiments were conducted as described previously (16). RBL-2H3 cells were harvested from culture dishes and transferred to an observation chamber. After Ag stimulation RBL-2H3 cells were fixed by 3% formaldeyde in PBS for 15 min and were permeabilized by 0.1% triton X-100 in PBS for 15 min. Subsequently, anti-MEK1/2 Ab and FITC-labeled anti-rabbit IgGs were added to the cell.

Results

Characterization of the fluorescent protein-tagged ERK2 and MEK

First, we examined whether the fluorescent protein-tagged ERK2 and MEK could be activated as well as endogenous kinases after ligation of IgE receptors. Results by Western blot analysis are shown in Fig. 1. Here, we used Abs against kinases and phosphorylated kinases. Fig. 1 shows that the induction pattern of the kinase activation for YFP-tagged ERK2 (YFP-ERK2) and CFP-tagged MEK (MEK-CFP) was consistent with that for the endogenous ERK2 and MEK. GFP-tagged ERK2 (GFP-ERK2) gave the pattern of the kinase activation similar to that shown in Fig. 1a.

Subcellular localization of ERK2 and MEK

We studied the subcellular localization of the GFP-ERK2 chimera proteins by CLSM (Fig. 2, a-c). GFP-ERK2 resided mainly in the
cytoplasm before Ag stimulation, and, in some cases, the expression of excess amounts of ERK2 resulted in its nuclear accumulation. After Ag (DNP-BSA) stimulation, GFP-ERK2 translocated to the nucleus with a dose-dependent manner (5–500 ng/ml DNP-BSA) similar to the endogenous ERK2. Thus, the GFP functioned as a chromophore in essentially all of the expressing ERK2 in RBL-2H3 cells, as described above.

Upon the stimulation with Ag, translocation of GFP-ERK2 into the nucleus occurred mostly in the fluorescing RBL-2H3 cells. A typical example of time courses of GFP-ERK2 fluorescence in the nucleus and cytoplasm of an RBL-2H3 cell is shown in Fig. 3a. After a few minutes of a lag time, ERK2 translocated from the cytoplasm to the nucleus in the RBL-2H3 cell, as shown in Fig. 3a (○). The fluorescence intensity of GFP-ERK2 increased first, and it decreased again in the nucleus of the RBL-2H3 cell. On the contrary, the fluorescence of GFP-ERK2 decreased first in the cytoplasm with a similar extent of the lag time and increased again, as shown in Fig. 3a (●). The translocation of ERK2 was dependent on the concentration of Ag (5–500 ng/ml). Five nanograms per milliliter DNP-BSA was able to induce the import of ERK2 to the nucleus; however, it took a longer lag time for translocation of ERK2 to the nucleus. Five hundred nanograms per milliliter DNP-BSA was saturated enough to induce the import of GFP-ERK2 from the cytoplasm to the nucleus. The import of ERK2 reached the maximum at ~6–7 min, and the export to the cytoplasm almost finished at ~15 min after Ag stimulation (500 ng/ml DNP-BSA). The average of the fluorescence intensities in the nucleus and cytoplasm was not changed during 20 min, as shown in Fig. 3a (×). This indicated that photobleaching did not affect on the time courses shown in Fig. 3a. The results indicated that GFP-ERK2 was shuttling between the cytoplasm and nucleus after stimulation with Ag. Quite similar results were also observed in the nuclear shuttling of YFP-ERK2. These kinetic studies on ERK2 translocation were consistent with the phosphorylation patterns of transfected and endogenous ERK, as shown in Fig. 1.

It is demonstrated that ERK2 is activated both by MEK and by Raf-1. ERK2 is translocated from the cytoplasm to the nucleus, where the activated ERK2 phosphorylates and regulates nuclear proteins including transcriptional factors. When RBL-2H3 cells were pretreated with the inhibitor of MEK (PD98059, 20 μM), the translocation of fluorescent protein-tagged ERK2 (GFP-ERK2, YFP-ERK2) was completely blocked, as shown in Fig. 3b (also see CLSM images shown in Fig. 2, d–f). Western blot analysis showed that the phosphorylation of endogenous MEK was blocked completely by the pretreatment of RBL-2H3 cells with PD98059 and that, subsequently, the phosphorylation of endogenous ERK2 and YFP-tagged ERK2 was blocked. However, the nuclear shuttling of ERK2 was not blocked by the pretreatment with wortmannin (an inhibitor of phosphatidylinositol 3-kinase, 100 nM), although degranulation in RBL-2H3 cells was blocked after Ag stimulation.

To study the movements of ERK2 and MEK at the same time, we cotransfected plasmids of YFP-ERK2 and MEK-CFP into a single RBL-2H3 cell. Fluorescence intensities of ERK2 and MEK were observed mainly in the cytoplasm before Ag stimulation (Fig. 4, a and c). The fluorescence intensity of YFP-ERK2 increased in the nucleus of the cotransfected RBL-2H3 cell after Ag stimulation, but that of MEK-CFP did not increase (Fig. 4, b and d). Time courses of the fluorescence intensity changes of YFP-ERK2 and MEK-CFP in the nucleus of the RBL-2H3 cell after Ag stimulation are shown in Fig. 4c. The import of ERK2 reached the maximum at 6–7 min, and then the imported ERK2 was exported from the nucleus. MEK mainly resided in the cytoplasm, and no significant MEK translocation was detected after the ligation of IgE receptors. Furthermore, we checked the translocation of endogenous MEK proteins by immunocytochemistry. We confirmed that endogenous MEK as well as MEK-CFP remained mainly in the cytoplasm after Ag stimulation (data not shown).

Effects of calcium ions on the nuclear shuttling of ERK2

Next, we studied the effects of calcium ions on the nuclear shuttling of ERK2 in RBL-2H3 cells. Here, we measured both the intracellular free calcium ion concentration ([Ca2+]i) and the distribution of GFP-ERK2 in RBL-2H3 cells by a conventional fluorescence microscope. In the presence of the external calcium ions (1 mM), the [Ca2+]i increased after stimulation with Ag (500 ng/ml DNP-BSA), and it was sustained at a higher level in the cytoplasm for >20 min (Fig. 3d; ○). Simultaneously, GFP-ERK2 translocated from the cytoplasm to the nucleus after the removal of the stored calcium ions (Fig. 3e; ○). In the absence of the external calcium ions, the transient increase of the [Ca2+]i was observed; however, the sustained increase of the [Ca2+]i disappeared, as shown in Fig. 3d (●). The CLSM images in Fig. 5 also showed the effect of calcium ions on the translocation of ERK2 into the nucleus. These results indicated that the sustained calcium increase was required for optimal ERK2 translocation. We further checked by Western blot analysis whether the calcium increase affects MEK activation and subsequently affects ERK2 translocation or whether it has direct effect on ERK2 translocation. Results of

FIGURE 2. Fluorescence microscopic images of GFP-ERK2 in a single RBL-2H3 cell after Ag stimulation. GFP-ERK2 was expressed in RBL-2H3 cells. Fluorescence pseudo-color images of GFP-ERK2 were observed before and after Ag stimulation (500 ng/ml DNP-BSA). a–c, Fluorescence images of GFP-tagged ERK2 before (a), at 6 min after (b), and at 11 min after (c) Ag stimulation in an RBL-2H3 cell. d–f, Fluorescence images of GFP-tagged ERK2 before (d), at 6 min after (e), and at 11 min after (f) Ag stimulation in an RBL-2H3 cell pretreated with an inhibitor of MEK (PD98059, 20 μM) for 30 min.
Western blot analysis using Abs against phosphorylated kinase are shown in Fig. 6. The data shows that the difference in calcium levels did not affect the expression of ERK and MEK. However, the difference in calcium levels affected the expression of the phosphorylated ERK and the phosphorylated MEK. These results suggested that the calcium increase affects MEK activation and subsequently affects ERK2 translocation.

Discussion
We showed here that GFP-ERK2 (YFP-ERK2) in living RBL-2H3 cells was able to shuttle rapidly between the cytoplasm and the nucleus after Ag stimulation depending on the sustained increase of $[\text{Ca}^{2+}]_i$. As described in the previous paper, ERK2 has neither nuclear localization signal nor nuclear export signal (NES) sequence, although MEK has an NES sequence in the N-terminal region (residues 33–44) (18). Thus, it seemed that the NES sequence of MEK was used to export ERK2 from the nucleus and retain it in the cytoplasm. There are at least three explanations for the translocation of MAPKs from the cytoplasm to the nucleus. One explanation proposed by Seger’s group is that the MEK-MAPK complex translocates upon serum stimulation to the nucleus, where only MEK is rapidly excluded via its active NES sequence following dissociation (19). Another explanation proposed by Fukuda et al. (20) and Lenormand et al. (21) is a rapid dissociation of MAPK in the cytoplasm after retrophosphorylation.
of the anchoring complex via MAPKs (ERK1 and ERK2). Subsequent to this dissociation, MAPKs could easily translocate to the nucleus via simple diffusion (18), although a cotransport involving association of MAPKs with their nuclear localization signal-containing substrates cannot be excluded. This explanation is not in contradiction with the previous results, in which nonphosphorylatable mutants of MAPKs translocated to the nucleus upon mitogen stimulation (22, 23). A third explanation proposed by Khokhlatchev et al. is that phosphorylation of ERK2 promotes its homodimerization and nuclear translocation. The explanation was based on microinjection studies in fibroblasts (24). In contrast, it was suggested that the export of MAPK from the nucleus to the cytoplasm was mediated by the dephosphorylation of MAPK and/or by the NES sequence of MAPKK (20, 24).

The present results indicated that there was no significant appearance of MEK-CFP in the nucleus of the cotransfected RBL-2H3 cells before and after Ag stimulation (Fig. 4). This did not exclude the model that MEK might shuttle between the cytoplasm and the nucleus (19, 20, 24). It rather suggested that the translocation of MEK favors the cytoplasm; in other words, the equilibrium constant of the translocation of MEK to the nucleus \( K_{\text{MEK}} \) is much smaller than that of ERK2 \( K_{\text{ERK2}} \) that was detected in the nucleus. \( K_{\text{MEK}} \) can be expressed as a ratio of rate constants of the export and import of MEK from/to the nucleus, \( k_{\text{MEK}(\text{ex})}/k_{\text{MEK}(\text{im})} \), and \( k_{\text{MEK}(\text{ex})} \) was greater than \( k_{\text{MEK}(\text{im})} \), \( k_{\text{ERK2}(\text{ex})} \), and \( k_{\text{ERK2}(\text{im})} \), because only MEK has NES sequence. However, it is possible that the addition of CFP may have affected nuclear transport of MEK-CFP.

Then, our results showed that the import of ERK2 reached the maximum several minutes after Ag stimulation, and thereafter it went back to the cytoplasm again within \( \approx 15 \) min. The previous authors supposed that the NES sequence of MEK might play a role for the export of ERKs from the nucleus to the cytoplasm for several minutes. Our results showed that both import and export of ERK2 to/from the nucleus occurred in several minutes after Ag stimulation. However, the results in the present paper were not able to explain that the export rate of MEK was as low as the previous authors supposed. Because, if so, we can observe the appearance of MEK-CFP in the nucleus, and/or the NES sequence of MEK does not play any role for the export of ERK2. In short, the present experiments suggested that the nuclear shuttling, especially export, of MEK be much faster than that of ERK2, shown in Fig. 3c. A schematic representation of the dynamics in the nuclear shuttling of MAPK (ERK2) and MAPKK (MEK) is shown in Fig. 7. In a previous paper, Jaaro et al. described that 5% of MEK...
translocated into the nucleus (19). However, they stressed in conclusion that they were not able to determine whether MEK translocated into the nucleus because the amount of the nuclear translocation of MEK was very low. Thus, we think the model shown in Fig. 7 did not contradict the previous experimental results.

Last, we must explain that the time courses for the import and the export of ERK2 were due to the rate-limiting steps of the biochemical reactions in the cytoplasm and in the nucleus, respectively. Thus, the nuclear shuttling of MEK itself should be much faster than the time courses shown in Fig. 3c.

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