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Genetically Encoded Bioluminescent Indicator for ERK2 Dimer in Living Cells

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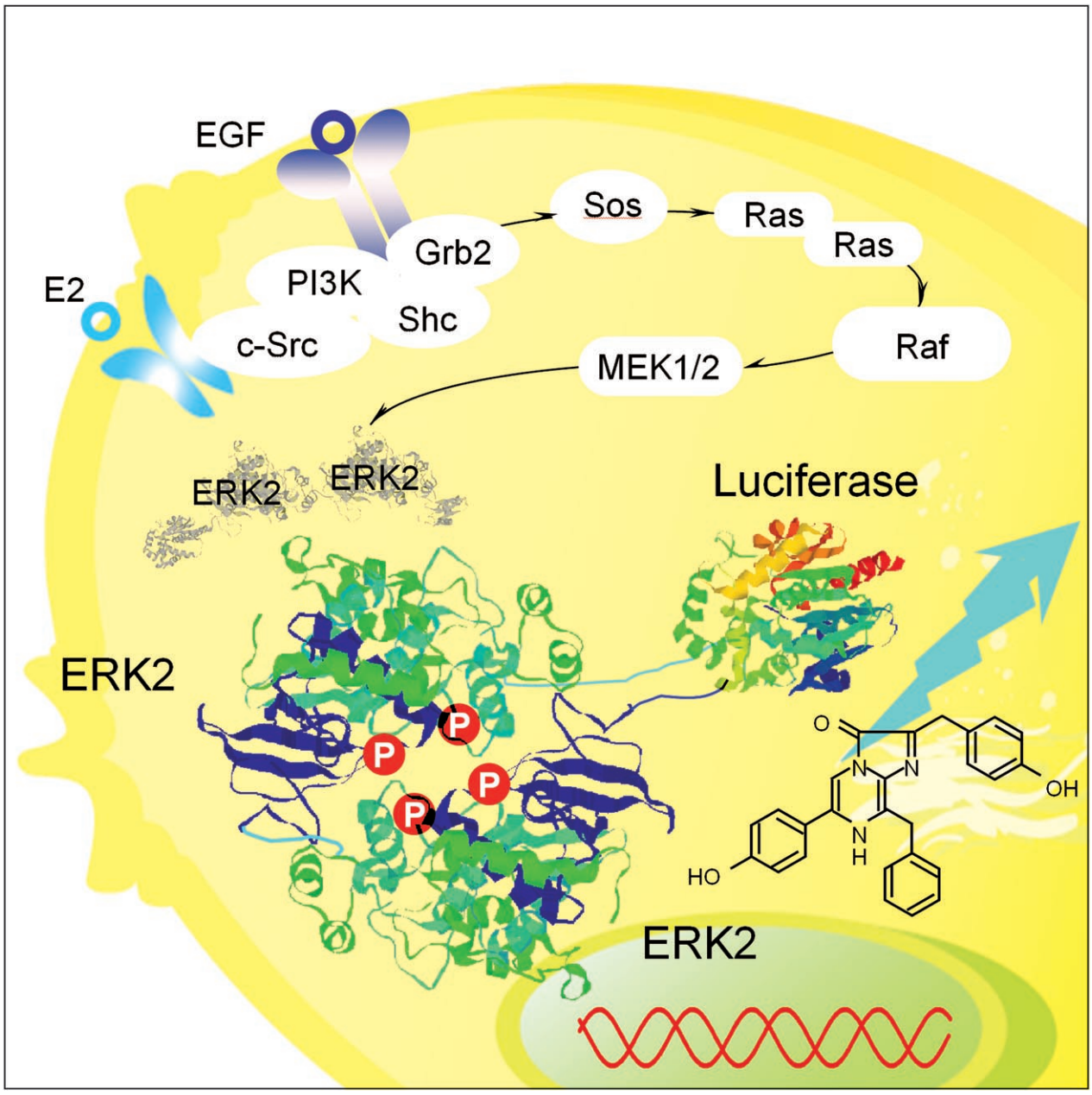

Abstract: In this study, a genetically encoded bioluminescent indicator for ERK2 dimer was developed with the split *Renilla* luciferase complementation method, in which the formation of ERK2 dimer induces a spontaneous emission of bioluminescence in living cells. In response to extracellular stimuli, such as epidermal growth factor (EGF) or 17β-estradiol (E2), extracellular signal-regulated kinase 2 (ERK2) is phosphorylated by its upstream kinase MEK, and also phosphorylates its substrates in various regions of the cell, including the nucleus. Phosphorylated ERK2 is led to form its dimer, thereby transporting itself into the nucleus. We demonstrated with the indicator that stimulation with EGF or E2 induces the formation of ERK2 dimer in living MCF-7 cells. The dynamics of this dimer formation was examined and discussed.

Keywords: biosensors · dimerization · ERK · luminescence · phosphorylation

Introduction

Protein–protein interactions are well known to play key roles in the structural and functional organization of living cells. Interactions between the same proteins result in dimer formation. However, despite cell-biological evidence of the importance of protein-dimer formation, protein dimers cannot be detected in living cells owing to the lack of conventional methods for such detection.

Extracellular signal-regulated kinases (ERKs) are some of the most characterized mitogen-activated protein kinases (MAPKs). The two isoforms of ERKs, ERK1 and ERK2, with molecular weights of 44 and 42 kD, are significant signals in breast cancer.[1,2] Both tyrosine and threonine residues of ERK2 are phosphorylated by its upstream kinase MEK.[3–6] The phosphorylation of ERK2 and its release from MEK results in its self-activation to phosphorylate a large number of its substrates that are localized in different regions of the cell.[3,5–12] Besides the phosphorylation of membrane and cytosolic proteins, ERK2 is capable of moving into the nucleus to regulate gene expression by phosphorylating transcription factors either directly or indirectly. The active transport of phosphorylated ERK2 into the nucleus is driven by the formation of its dimer. It has been demonstrated with exogenous recombinant ERK2 that the dimer formation of ERK2 requires its prior phosphorylation.[13] The crystal structure of phosphorylated ERK2 shows that its phosphorylation causes conformational changes in two flexible regions of ERK2: the activation loop and the C-terminal extension. With this conformational change, two ERK2 molecules bind through a hydrophobic zipper complemented by two ion pairs, one on either side of the zipper.[3,13–16]

In this study, for the temporal analysis of the formation of the ERK2 dimer in living cells, we developed a novel bioluminescent indicator to detect the ERK2 dimer in living cells by using split *Renilla* luciferase and its complementation. The split *Renilla* luciferase complementation method was developed earlier for locating insulin-induced protein–protein interactions in living cells.[17] Complementation here means that the N- and C-terminal halves of split *Renilla* luciferase are brought into close proximity and folded without forming the original peptide bond between the two. With this method, the presence of a protein–protein interaction leads to the complementation of the two halves of split *Renilla* luciferase in living cells, which results in the spontaneous and simultaneous emission of bioluminescence with a cell-membrane-permeable substrate of *Renilla* luciferase, coelenterazine. We previously confirmed that the intracellular Ca^{2+}-dependent interaction between calmodulin and M13 also induces the intramolecular complementation of split *Renilla* luciferase, which results in the spontaneous and simultaneous emission of bioluminescence (unpublished data).

The principle of the method for detecting the formation of ERK2 dimer is shown in Figure 1a. The two ERK2 molecules connected in tandem were inserted between the N- (1-

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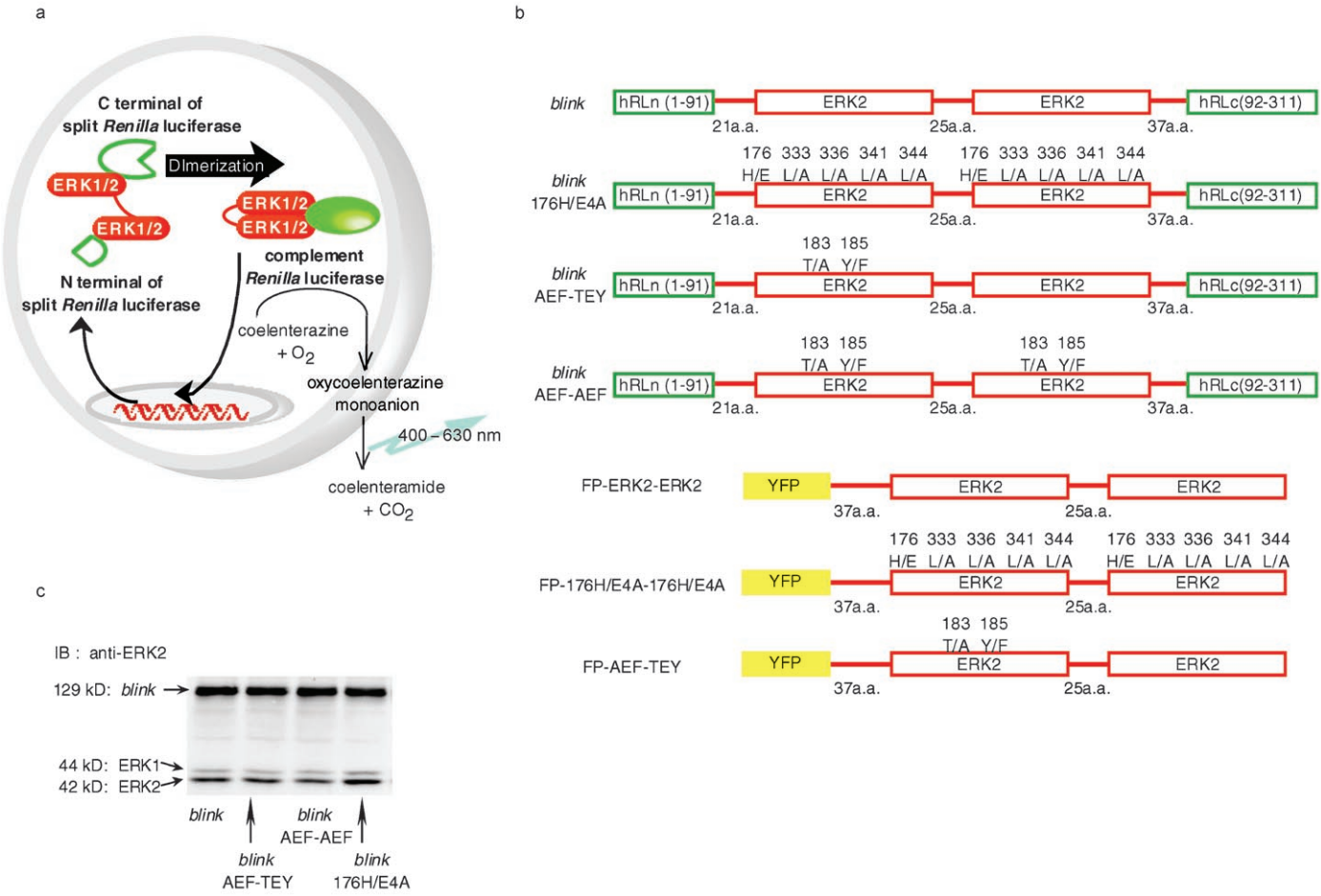

Figure 1. a) Diagram showing the principle of the bioluminescent indicator *blink*. After stimulus-dependent phosphorylation of ERK2, dimer formation of phosphorylated ERK2 induces spontaneous emission of luminescence upon complementation of split *Renilla* luciferase with a cell-membrane-permeable substrate, coelenterazine, in situ in living mammalian cells. b) Schematic representation of the plasmid constructs. All these plasmids have a CMV-promoter sequence in the upstream of the start codon. All the "hRLn"s contain the 1-91 amino acid of hRL124C/A. All the "hRLc"s contain the 92-311 amino acid of hRL124C/A. All the flexible amino acid linkers contain N-G amino acid repeating units. c) Immunoblot analysis for the expression of bioluminescent indicators. The bioluminescent indicators, *blink*, *blink* AEF-TEY, *blink* AEF-AEF, and *blink* 176H/E 4A were expressed in MCF-7 cells. The expression of these bioluminescent indicators and endogenous ERK2 were detected with anti-phospho-ERK2 antibody.

91 AA) and C-terminal (92-311 AA) halves of split *Renilla* luciferase. The dimer formation of such connected ERK2 molecules causes the complementation of the N- and C-terminal halves of split *Renilla* luciferase. This *Renilla* luciferase complementation induces a spontaneous emission of bioluminescence, which is a measure of the extent of the presence of ERK2 dimers in living cells. Using this bioluminescent indicator, we analyzed the dimer formation and the phosphorylation of ERK2 upon stimulation with epidermal growth factor (EGF) or 17β-estradiol (E2) in living MCF-7 cells.

Results

Construction and Characterization of Bioluminescent Indicator for ERK2 Dimer

The principle of the bioluminescent indicators for ERK2 dimer is shown in Figure 1 a. The formation of ERK2 dimer induces complementation of split *Renilla* luciferase to emit bioluminescence spontaneously with its substrate, coelenterazine. The dissociation of the ERK2 dimer causes the N- and C-terminal halves of the split *Renilla* luciferase to come apart. Plasmid constructs for the indicators are shown in Figure 1 b. The indicator for the ERK2 dimer was named *blink* (bioluminescent indicator for ERK2 dimer). So as not to obstruct dimer formation of ERK2 and thus allow it to occurs in the bioluminescent indicator *blink*, three optimized flexible amino acid/peptide linkers were used. A flexible linker of 21 amino acid/peptide-containing Asp–Gly repeating units was inserted between the N terminal of split *Renilla* luciferase and ERK2. A flexible linker of 25 amino acid/peptide-containing Asp–Gly repeating units was inserted between two ERK2 molecules connected in tandem. A flexible linker of 37 amino acid/peptide-containing Asp–Gly repeating units was inserted between ERK2 and the C terminal of split *Renilla* luciferase. The *Renilla* luciferase mutant hRL124C/A, in which the 124-cysteine residue in

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hRL was replaced with alanine, was used to increase luminescence activity.[17]

We confirmed the expression of *blink* in living MCF-7 cells by western blot analysis (Figure 1c).

The response of *blink* was evaluated with a luminometer. MCF-7 cells were expressed with *blink* and were starved of fetal bovine serum for 3 h to dephosphorylate the phosphorylated ERK2. Furthermore, to dephosphorylate ERK2 absolutely, the *blink*-expressing cells were incubated with U0126, an inhibitor for the kinase activities of both Raf and MEK. The cells were then stimulated with 100 ng mL^{-1} EGF, one of major activators for ERK2. The luminescence intensities of the cells were measured before and 10 min after EGF stimulation. The results are shown in Figure 2a. The luminescence intensity of the *blink*-expressing serum-starved cells was higher than that with U0126 without EGF stimulation. EGF stimulation increased the luminescent intensity of the *blink*-expressing MCF-7 cells by 1.3 times. These results indicate that ERK2 in the serum-starved MCF-7 cells forms the dimer with or without EGF. EGF stimulation increased the amount of ERK2 dimer in the living MCF-7 cells.

We then confirmed that the EGF-dependent increase in luminescence through the complementation of split *Renilla* luciferase is induced as a result of dimer formation of ERK2. We constructed *blink* 176H/E 4A with a mutant of ERK2, 176H/E 4A (Figure 1b). 176H/E 4A, in which the 176-histidine residue is replaced with glutamic acid, and the 333-, 336-, 341-, and 344-leusine residues are replaced with alanines, is known not to form the dimer.[15] Several previous studies have shown that the formation of ERK2 dimer does not affect its kinase activity, because the dimer-formation-defective ERK2 mutant (176H/E 4A) has an activity comparable to wild-type ERK2.[3] Furthermore, the crystal structure of phosphorylated ERK2 shows that its dimer formation does not interfere with the active sites of the kinase.[13] The cells that express *blink* 176H/E 4A did not exhibit any significant increase in luminescent intensity upon EGF stimulation (Figure 2a).

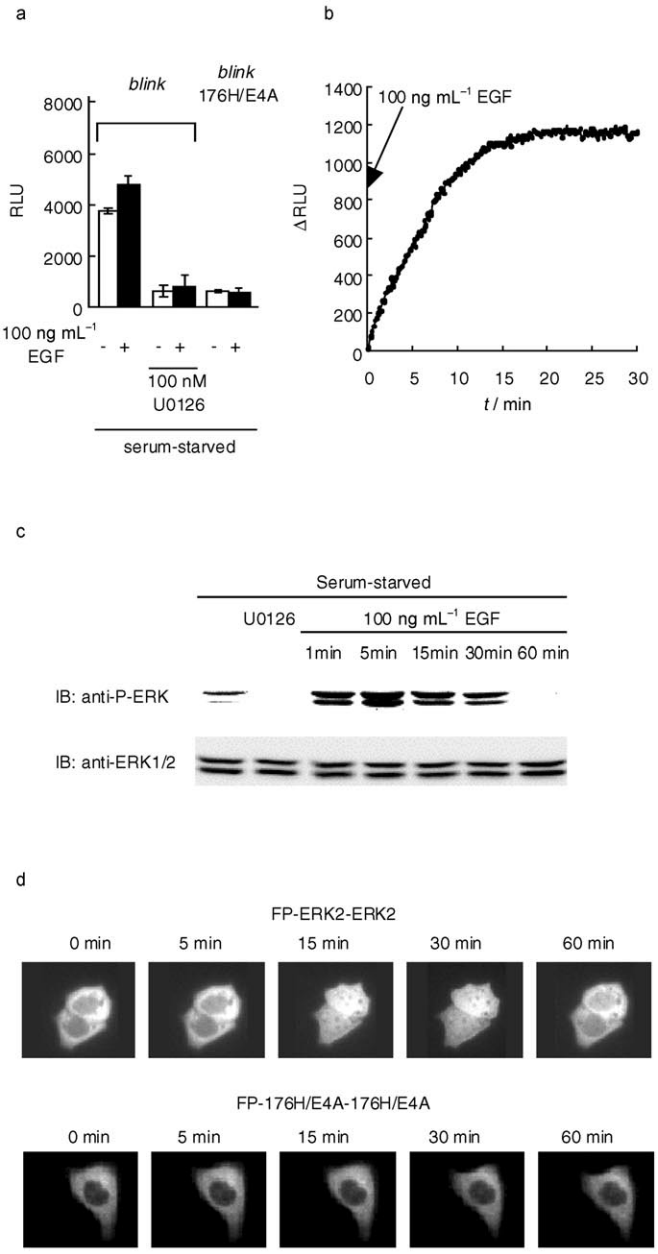

Figure 2. a) Luminescent intensities upon dimer formation of ERK2. The luminescence intensities of the cells expressing *blink* and *blink* 176H/E 4A were assessed with and without 100 ng mL^{-1} EGF. b) Time course of EGF-dependent dimer formation of ERK2. The luminescence intensities of the serum-starved MCF-7 cells expressing *blink* were monitored with and without 100 ng mL^{-1} EGF. The difference in the two (Δ = EGF(+)−EGF(−)) is shown. c) Immunoblot analysis of ERK2 phosphorylation. The phosphorylation of threonine and tyrosine in endogenous ERK1/2 were detected with anti-phospho-ERK1/2 antibody. The expressions of endogenous ERK1/2 were detected with anti-ERK1/2 antibody. d) Distribution of ERK2 dimer. The fluorescence images of the serum-starved MCF-7 cells expressing FP-ERK2-ERK2 and FP-176H/E4A-176H/E4A were acquired upon stimulation with 100 ng mL^{-1} EGF.

Time Course of EGF-Dependent Formation of ERK2 Dimer

Next, the time course of the EGF-dependent formation of ERK2 dimer was observed with *blink*. The luminescence intensity of the serum-starved MCF-7 cells expressing *blink* was measured upon stimulation with 100 ng mL^{-1} EGF. The result is shown in Figure 2b. The luminescence intensity of the cells gradually increased for 15 min and reached a plateau.

To examine if the EGF-dependent formation of ERK2 dimer occurs spontaneously with the phosphorylation of ERK2, a western blot analysis was performed. The phosphorylation of endogenous ERK2 with and without stimulation with 100 ng mL^{-1} EGF in living serum-starved MCF-7 cells was analyzed. The results are shown in Figure 2c. The ERK2 was phosphorylated immediately after the stimulation of the cells with 100 ng mL^{-1} EGF. The ERK2 was phosphorylated for 5 min and then gradually dephosphorylated by serine/threonine phosphatases and/or dual-specificity phosphatase. Comparing the time course of the phosphor-

ylation and that of dimer formation, we concluded that EGF stimulation induces the formation of ERK2 dimer approximately 10 min after the phosphorylation of ERK2.

To confirm if the formation of ERK2 dimer leads to self-translocation into the nucleus, yellow fluorescent protein-fused tandem-connected ERK2 molecules (FP-ERK2-ERK2) and mutants 176H/E 4A (FP-176H/E4A-176H/E4A) were constructed and respectively expressed in MCF-7 cells. The MCF-7 cells were starved of fetal bovine serum for 3 h. The distribution of these proteins upon stimulation with 100 ng mL^{-1} EGF was observed with fluorescence microscopy (Figure 2d). Without EGF stimulation, FP-ERK2-ERK2 was distributed throughout the cell except in the nucleus. 15 min after EGF stimulation, FP-ERK2-ERK2 was translocated into the nucleus. This time course agrees well with that of dimer formation upon EGF stimulation. As expected, FP-176H/E4A-176H/E4A did not translocate into the nucleus regardless of EGF stimulation. The results indicate that the formation of ERK2 dimer leads to self-translocation into the nucleus.

Next, dimer formation between phosphorylated and unphosphorylated ERK2, and between two unphosphorylated ERK2 molecules, was assessed. It was reported that phosphorylated ERK2 forms dimers with both phosphorylated and unphosphorylated ERK2.[13] The dissociation constant (K_d) for the dimer between phosphorylated ERK2 molecules is 7.5 nm, about 3000 times lower than that for the dimer between unphosphorylated ERK2 molecules in vitro.[13]

To detect EGF-dependent dimer formation between phosphorylated ERK2 and unphosphorylated ERK2, and between unphosphorylated ERK2 molecules in living cells, bioluminescent indicators *blink* AEF-TEY and *blink* AEF-AEF, respectively, were constructed (Figure 1c). The mutant with 183 T/A and 185 Y/F for ERK2, AEF, cannot be phosphorylated by MEK2. In the indicator *blink* AEF-TEY, tandem-fused ERK2 mutant AEF and wild-type ERK2 were connected between the N- and C-terminal halves of split *Renilla* luciferase. In *blink* AEF-AEF, two tandem-connected AEF molecules were fused between the N- and C-terminal halves of split *Renilla* luciferase. *Blink* AEF-TEY and *blink* AEF-AEF were expressed in MCF-7 cells. The cells were stimulated with 100 ng mL^{-1} EGF after serum starvation. As shown in Figure 3a, the luminescence intensities of the cells that express *blink* AEF-TEY increased upon EGF stimulation. This increase was roughly half that of the *blink*-expressing cells (Figure 3b). The luminescence intensities of the cells that express *blink* AEF-AEF did not increase after EGF stimulation as expected. This result indicates that EGF stimulation induces heterodimerization between phosphorylated ERK2 and unphosphorylated ERK2, as well as homodimerization between phosphorylated ERK2 molecules in living cells.

The distribution of the heterophosphorylated ERK2 dimer was analyzed with yellow fluorescent protein-fused tandem-connected ERK2 mutant AEF and wild-type ERK2 (FP-AEF-TEY) (Figure 3c). Without EGF stimulation, FP-AEF-TEY was distributed throughout the cell except in the nucleus. 15 min after EGF stimulation, it was translocated into the nucleus. This time course agrees well with that of dimer formation upon EGF stimulation. This distribution of ERK2 heterophosphorylated dimer is similar to the distribution of ERK2 homophosphorylated dimer.

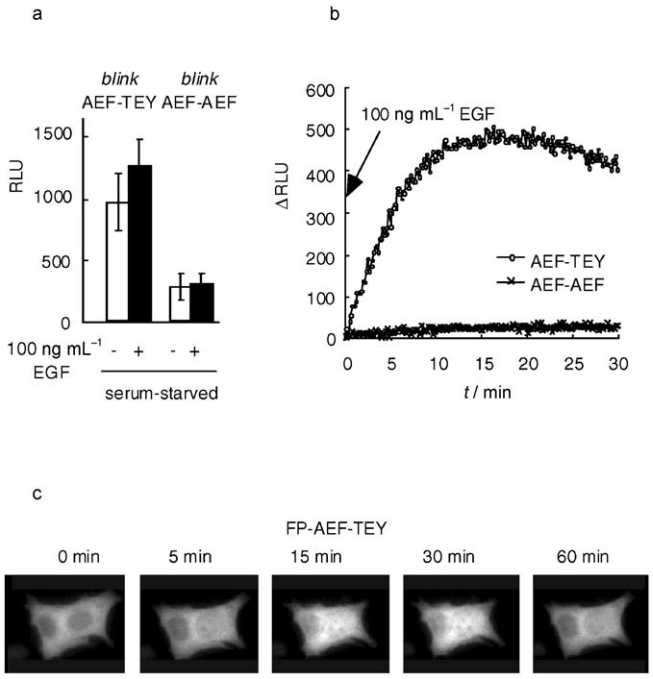

Figure 3. a) Dimer formation of phosphorylated ERK2 with unphosphorylated ERK2. The luminescence intensities of the cells expressing *blink* AEF-TEY and *blink* AEF-AEF were assessed with and without stimulation with 100 ng mL^{-1} EGF in the serum-starved MCF-7 cells. b) Time course of dimer formation of phosphorylated ERK2 with unphosphorylated ERK2. The luminescence intensities of the serum-starved MCF-7 cells expressing *blink* AEF-TEY and *blink* AEF-AEF were monitored upon stimulation with and without 100 ng mL^{-1} EGF. The difference in the two (Δ=EGF(+)−EGF(−)) is shown. c) Distribution of ERK2 heterodimer. The fluorescence images of the serum-starved MCF-7 cells expressing FP-AEF-TEY were acquired upon stimulation with 100 ng mL^{-1} EGF.

Formation of ERK2 Dimer upon Stimulation with 17β-Estradiol

17β-Estradiol (E2), one of the major sex steroid hormones, has attracted interest as an activation factor for ERK2 through the "nongenomic pathway" in MCF-7 cells, and is known to induce the phosphorylation of ERK2 rapidly.

To understand the effects of E2 on ERK2, the present bioluminescent indicator was used to detect E2-dependent formation of the phosphorylated ERK2 dimer in living MCF-7 cells. The luminescence intensities of the MCF-7 cells that express *blink* cultured in serum-starved and 10% serum-supplemented medium, respectively, were measured for 1 h after 100 nM E2 stimulation (Figure 4a). In the serum-starved MCF-7 cells, the luminescence intensity gradually increased for 30 min just after E2 stimulation and then

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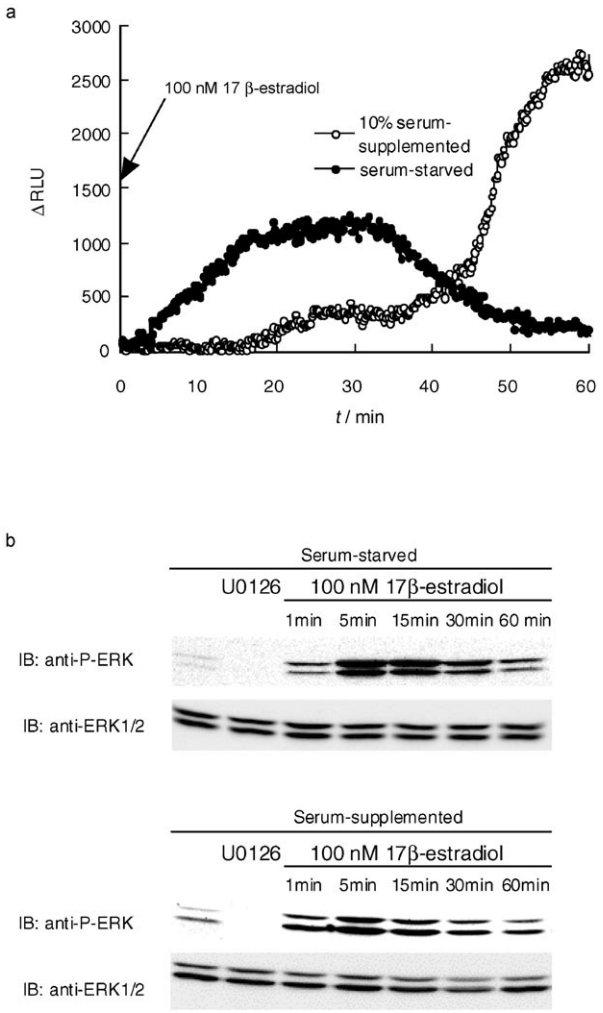

Figure 4. a) 17β-Estradiol-dependent dimer formation of ERK2. The luminescence intensities of MCF-7 cells expressing *blink* were monitored upon stimulation with 100 nM 17β-estradiol. The difference in the two (Δ = E2(+) − E2(−)) is shown. b) Immunoblot analysis of ERK2 phosphorylation. The phosphorylation of threonine and tyrosine in endogenous ERK1/2 were detected with anti-phospho-ERK1/2 antibody. The expressions of endogenous ERK1/2 were detected with anti-ERK1/2 antibody.

decreased. This result indicates that E2 stimulation induced the formation of ERK2 dimer for 30 min, and the dissociation of the dimer occurred afterward in the serum-starved MCF-7 cells. On the other hand, in MCF-7 cells cultured with the 10% serum-supplemented medium, the increase in luminescence intensity started 15 min after stimulation, and a remarkable increase in luminescence was observed afterward. The luminescence intensity continued to increase for 1 h. The time course of ERK2 dimer formation in MCF-7 cells cultured with the 10% serum-supplemented medium therefore differs from that in cells cultured with the serum-starved medium.

Next, we examined whether the above-described difference between the time courses of E2-dependent ERK2 dimer formation with and without the serum is induced by the difference in the time courses of ERK2 phosphorylation. A western blot analysis was performed for the phosphorylation of endogenous ERK2 with and without stimulation with 100 nM E2 in MCF-7 cells. The cells were cultured with the serum-starved medium or the 10% serum-supplemented medium. The results are shown in Figure 4b. For the cells cultured with the serum-starved medium, E2 stimulation induced the phosphorylation of ERK2 within 5 min of stimulation, and the phosphorylation of ERK2 continued for nearly 30 min. For the cells cultured with the serum, the phosphorylation of ERK2 increased within 5 min after E2 stimulation in a similar manner to that in the serum-starved cells.

Discussion

In the footsteps of earlier standard techniques such as co-immunoprecipitation, cross-linking, and cofractionation by chromatography, several methods that use split enzymes have been developed for detecting protein–protein interactions, including the split ubiquitin system,[19] the β-galactosidase system,[20] and the split GFP/luciferase system based on the protein-splicing system.[21,22] We previously developed the split *Renilla* luciferase complementation method for locating protein–protein interactions in living cells.[7]

In the present study, we investigated a genetically encoded bioluminescent indicator for ERK2 dimer with the split *Renilla* luciferase complementation method and named the indicator *blink* (Figure 1a). It was confirmed that luminescence is emitted with *blink* as a result of dimer formation of ERK2 in living cells, and we thus concluded that *blink* is capable of reporting ERK2 dimer as bioluminescence in living cells.

According to the previous structural analysis for ERK2 dimer, dimer formation is induced by the phosphorylation-dependent conformational change of ERK2.[3] The formation of ERK2 dimer induces the active transport of ERK2 into the nucleus, as opposed to the passive diffusion of monomer ERK2 into the nucleus.[3,13–15] In previous studies, although various stimuli-dependent phosphorylations occurred within a few minutes of stimulation and gradually dephosphorylated afterward, translocations of ERK2 into the nucleus were observed at 10–30 min after stimulation.[13,15,18] The present bioluminescent indicators demonstrate that the phosphorylation of ERK2 does not spontaneously lead to the formation of its dimer, and that ERK2 does not form the dimer while it is phosphorylated (Figure 2b and c). The phosphorylated ERK2 continues to form dimers with both phosphorylated and unphosphorylated ERK2 within 15 min, and these dimers continue to exist for 30 min after EGF stimulation (Figures 2b and 3b). Both the ERK2 homodimer and the heterodimer translocate themselves into the nucleus in living MCF-7 cells (Figures 2d and 3c). These results indicate that the phosphorylated ERK2 does not form its dimer immediately after phosphorylation. Therefore, the phosphorylated ERK2 does not transport rapidly into the

nucleus but exists outside the nucleus. The phosphorylation of ERK2 cytosolic substrates are attributed to this monomeric phosphorylated ERK2.

Furthermore, the present indicator showed that ERK2 forms its dimer in a different manner from its phosphorylation upon E2 stimulation in MCF-7 cells cultured with serum (Figure 4a). The phosphorylation of ERK2 was induced just after E2 stimulation and continued for nearly 30 min. The phosphorylated ERK2 was gradually dephosphorylated afterward. Meanwhile, phosphorylated ERK2 started to form its dimer 15 min after the E2-dependent phosphorylation and continued for over 1 h (Figure 4a and b).

The development of a novel bioluminescent indicator for ERK2 dimer therefore enabled us to analyze the correlation between phosphorylation and dimer formation of ERK2 in living cells.

Conclusions

We have devised a genetically encoded bioluminescent indicator for ERK2 dimer, which allows the temporal analysis of ERK2 dimer formation in living cells. Using this novel indicator, we showed that ERK2 forms its dimer upon stimulation with either EGF or E2 in living MCF-7 cells. This study first shows the difference between the time course of ERK2 phosphorylation and that of dimer formation. The ERK2 dimers formed, both homo- and heterophosphorylated, were found to translocate into the nucleus.

Experimental Section

Materials

Restriction enzymes, modification enzymes, and ligases were purchased from Takara Biomedicals (Tokyo, Japan). A synthetic *Renilla* luciferase gene vector (hRL-CMV) that codes *Renilla* luciferase with the most frequently used codons in mammals and a *Renilla* luciferase assay system were purchased from Promega Co. (Madison, WI). Mammalian expression vectors pcDNA3.1(+) were obtained from Invitrogen (Groningen, Netherlands) and Clonetech (Palo Alto, CA), respectively. Minimum essential Eagle's medium (MEME), fetal bovine serum (FBS), and LipofectAMINE 2000 were obtained from Gibco BRL (Rockville, MD). Anti-Phospho-p44/42 MAP kinase antibody, anti-ERK1/2 antibody, and anti-ERK2 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alkaline-phosphatase-labeled antirabbit and antimouse antibodies were purchased from Jacson ImmunoResearch Lab., Inc. (Pennsylvania, PA). U0126 was purchased from Promega, Co. (Madison, WI).

Plasmid Construction

The *Escherichia coli* strain DH5α was used as a bacterial host for all plasmid construction subcloning. All plasmids were verified by sequencing with a genetic analyzer ABI prism310 (PE Biosystems, Tokyo, Japan). Plasmid constructs are shown in Figure 1b. Two tandem-connected ERK2 molecules were fused between the N- (1-91 AA) and C-terminal (92-311 AA) halves of split *Renilla* luciferase. Mouse ERK2 was cloned in the p-Blue Script vector as a template. The dimer-deficient mutant of ERK2, 176H/E 4A, was made with a Quick-Change Mutagenesis kit (Strategene, La Jolla, CA). All plasmids were subcloned into the expression vector pcDNA 3.1(+) (Invitrogen).

Cell Culture and Transfection

MCF-7 cells were cultured in MEME medium supplemented with 10% heat-inactivated FBS, sodium pyruvate (1 mm), MEM nonessential amino acid solution (100 μm), penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}). Cells were maintained in 5% CO_2 at 37°C. Transfection was performed in the presence of LipofectAMINE 2000 reagent.

Luminescence Assay

Kinetic analysis of luminescence intensity was performed with a Mithras 940 (Berthold GmbH & Co. KG, Wildbad, Germany). The MCF-7 cells were seeded in 96-well microplates.

Immunoblot Analysis

Cells were stimulated with EGF (100 ng mL^{-1}) or 17β-estradiol (100 nM) at 37°C, and then lyzed with a 2×SDS-PAGE sample buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis and analyzed with the following antibodies: anti-ERK1/2 antibody (1:500) for endogenous ERK1/2 expression, and anti-phospho ERK antibody (1:500) for phosphorylation of endogenous ERK1/2. Antibody staining was visualized with an LAS-1000 plus image analyzer (Fujifilm Co., Tokyo, Japan).

Fluorescence Images

MCF-7 cells that express yellow fluorescent protein-fused tandem-connected ERK2 were placed onto glass-bottomed dishes and washed twice with Hank balanced salt solution. The cells were imaged at room temperature on a Carl Zeiss Axiovert 135 microscope with a cooled charged-coupled device Micro-MAX camera (Roper Scientific Inc., Tucson, AZ), controlled by a MetaFluor imaging system (Universal Imaging, West Chester, PA). The cells were excited at (425±30) nm for 100 ms, and fluorescence images were obtained through (535±12.5)-nm filters in a microscope with a ×40 oil-immersion objective lens (Carl Zeiss, Jena, Germany).

Acknowledgements

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