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Nongenetic Control of Receptor Signaling Dynamics with a DNA-based Opto-chemical Tool

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ABSTRACT

Optochemical tools that can modulate activity of the target protein provide an opportunity for studying and regulating the related biological processes. Here we present a DNA-based nongenetic optochemical tool that can control dynamics of growth factor signaling. A photo-caged DNA aptamer for a growth factor receptor was designed to assemble into an active dimer form, depending on UV irradiation, thereby enabling optical control of the dimerization and subsequent activation of the receptor. This photo-caged mimicry of growth factor can be a promising tool for elucidating a linkage between dynamics of signaling and resulting biological outcomes, as well as for manipulating cellular functions and the fate of living cells.

MAIN TEXT

Growth factors (GFs) are polypeptide ligands that regulate various cellular activities such as proliferation, migration, and differentiation. Binding of GFs to receptor tyrosine kinases (RTKs) on the cell membrane induces dimerization and subsequent phosphorylation of the RTKs and initiates phosphorylation cascades of intracellular kinases¹. Intriguingly, the activation dynamics of kinases downstream of RTKs plays an important role in determining a cellular function and fate (Figure 1)². For example, in rat PC12 cells, both epidermal growth factor (EGF) and nerve growth factor (NGF) activate the Ras-Raf-Mek-Erk pathway via activation of their cognate receptors, but the dynamics of kinase activation and the resulting cellular outcomes show unique patterns³. EGF causes transient activation of Erk that leads to cell proliferation, while NGF causes sustained activation of Erk that leads to cell differentiation. To date, much attention has been focused on the mechanisms that initiate such dynamics of GF signaling, thereby regulating cellular functions. Therefore, a method capable of controlling the activity of RTKs with a precise temporal resolution is highly sought after for studying receptor-level mechanisms guiding

the dynamics of GF signaling and rewiring a cellular function of living cells by modulating the signaling dynamics⁴.

Optochemical tools that target the interaction between GF and RTK represent a promising approach for the regulation of the dynamics of GF signaling without any gene transfer process. Luebke *et al.* reported synthesis of EGF containing a photo-caged glutamine at the Glu40 position, which is involved in the interaction with the receptor of EGF, using solid phase peptide synthesis (SPPS)⁵. The photo-caged EGF showed reduced affinity toward the receptor, compared with that of native EGF. A light-irradiation liberated the caging group and restored the binding affinity to the receptor. One intrinsic issue with this approach is the difficulty to find an optimal modification site for the caging group. The photo-caged EGF showed only four-fold higher change in the effective concentration. Therefore, concentration of the ligand should be optimized to achieve low background signal at an expense of high off/on ratio. In addition, this strategy cannot be applied to the majority of GFs, as they consist of large and complex polypeptides with post-translational modifications, which render their preparation via SPPS, challenging and labor-intensive.

In this work, we present, for the first time, a nongenetic temporal patterning of GF signaling, using a synthetic surrogate of GF. We adopted DNA aptamer as a building block due to its synthetic accessibility and amenability to chemical modifications. Previously, we demonstrated that the aptamer to an RTK⁶ could be used as a synthetic switch, which can control dimerization state of the RTK, depending on external cues^{7a}. The RTK-binding aptamer was designed to form a “sandwich” with a target molecule by tethering the aptamer with an appropriate ligand molecule, thereby enabling receptor dimerization in the presence of the target molecule^{7a}. This concept has been extended to the design of aptamer-based tools for inducing receptor dimerization, responding to other external stimuli, including light irradiation^{7b,c}. For example, Chen *et al.* reported multi-component system that enables light-induced receptor dimerization using DNA strand exchange reactions, initiated by cleavage of a photo-labile linker^{7c}. However, none of these tools has realized nongenetic temporal patterning of the GF signaling.

To realize optical control of dynamics of the GF signaling, we designed a single-component system that can turn on its RTK-activating potential, depending on a deprotection event of just one photo-

Figure 1. Schematic representation of GF signaling and its implication in determining cell function and fate. (a) Binding of GF induces dimerization and subsequent phosphorylation of receptor tyrosine kinase (RTK) on the cell membrane. (b) The activated RTK triggers phosphorylation cascades of intracellular kinases. (c) The dynamics (*e.g.* amplitude, duration, and frequency) of kinase activity determine the biological outputs.

Figure 2. (a) DNA sequences used in the present study and schematic representation of light-induced receptor dimerization with a caged DNA aptamer. The Met-binding aptamer sequence (50-mer) and self-complementary linker sequence (10-mer) are shown in blue and red, respectively. The site of NPM-dC modification is denoted by X. (b) Scheme of the photo-deprotection reaction of NPM-dC.

caging group. As a proof-of-concept study, we selected Met, a receptor for hepatocyte growth factor (HGF), as the target⁸. As shown in Figure 2, we designed a single-stranded DNA sequence composed of two functional parts; first, a 50-mer DNA aptamer that binds to Met (blue)⁹ and a 10-mer self-complementary linker sequence (red), tethered to the 3' terminal of the aptamer sequence. In the linker sequence, 6-nitropiperonyl- α -methyl (NPM)-caged deoxycytidine (dC)¹⁰ was introduced to enable photo-induced dimerization of the aptamer upon irradiation of 365 nm light (Figure 2). The melting temperature (T_m) of the linker sequence with or without the photo-caging group was measured in the physiological conditions (in Dulbecco's phosphate buffered saline). While T_m value of the linker without the photo-caging was 39.0 °C, the transition in the melting curve was not observed in the case of the linker, which was modified with the NPM-caging group, indicative of significant destabilization of the DNA duplex (Figure S1). Importantly, a clear transition in the melting curve was observed (T_m = 40.7 °C) after an irradiation with 365 nm-peak UV light to the caged linker sequence, suggesting the light-dependent formation of duplex at the physiological conditions. In an assay using Met-expressing DU145 cells, the current system showed excellent off/on ratio in receptor activating potential, as the aptamer was designed to modulate its dimerization propensity, depending on a light irradiation instead of modulating the affinity to the receptor. The

Figure 3. (a) Met phosphorylation induced by HGF (1 nM) or aptamer (5, 20, and 50 nM). DU145 cells were stimulated with the ligand for 15 min and the resulting Met phosphorylation level was evaluated by ELISA. Error bars indicate SD (N = 3). (b) Western blotting of DU145 cell lysates. The cells were stimulated with HGF (1 nM) or aptamer (100 nM) for 15 min. The samples were irradiated with a 365-nm peak UV irradiation (8 mW) for the indicated time before the addition to the medium.

1 non-caged aptamer (Apt) exhibited increased Met activation level in a concentration dependent manner,
2 while the NPM-caged aptamer (cApt) showed significantly reduced Met activation potential (Figure 3a).

3 To understand whether a light irradiation can restore the Met activating potential of the cApt, the
4 photo-caged aptamer was irradiated with a 365-nm peak UV light (8 mW, 5–60 sec), before the addition
5 to the culture medium. The result indicated that the cApt showed increased Met-activating potential in
6 an irradiation time-dependent manner (Figure 3b), indicative of production of aptamer dimer upon the
7 liberation of photo-caging group. It was also confirmed that the aptamer could induce activation of the
8 downstream kinase, Erk, after the UV-irradiation.

9 Finally, we used the caged aptamer for optical control of the dynamics of GF signaling. In the pre-
10 sent study, we focused on the dynamics of Erk activity. Previous researches suggested that the dynamics
11 of Erk activity modulate cellular activities, such as proliferation and differentiation². To visualize Erk
12 activity in the living cells, we observed Met-expressing HeLa cells expressing Erk-kinase translocation
13 reporter (KTR)-Clover¹¹, a fluorescent reporter for the Erk activity, under time-lapse imaging using a
14 fluorescent microscopy (Figure 4a). This fluorescent reporter was designed to translocate from nucleus
15 to cytosol upon phosphorylation, caused by the activated Erk¹¹. Since basal Erk activity and the result-
16 ing nuclear localization propensity of the reporter may vary from cell to cell, the cells showing moderate
17 nuclear localization of the reporter at the beginning of the analysis were used for the analysis (Figure
18 S2). We confirmed that the addition of recombinant HGF to the medium induced a prolonged (>60 min)
19 translocation of the reporter from nucleus to the cytosol, indicating sustained Erk activation, which was
20 consistent with observation in an immunoblotting analysis (Figure S3).

21 The cells were incubated in the presence of the cApt (50 nM) and irradiated with a 365-nm peak UV
22 light. When a short pulse (1 sec) of UV irradiation was applied to the cells in 1 min interval for 6 times
23 (input A), translocation of the reporter to the cytosol started to increase within a few minutes (Figures
24 4b, 4e, and Supplementary video 1). The translocation of reporter reached its maximum after 15 min
25 from initial irradiation, and decayed to the basal level after another 10 min. This pulse-like Erk activa-
26 tion occurred several minutes after the UV-irradiation and was similar to the observation in the cells,
27 stimulated with a growth factor pulse^{2c}. The translocation of reporter was negligible by the irradiation of
28 the cells in the absence of cApt, thereby discarding the possibility of UV irradiation-dependent activa-
29 tion of the signaling under these experimental conditions (Figure S4).

30 We found that the dynamics of Erk signaling can be modulated by changing temporal pattern of UV
31 irradiation. When the interval of irradiation pulse was elongated from 1 to 5 min (input B), the reporter
32 showed a prolonged translocation (Figures 4c and 4f). While the duration for the translocation reached a
33 plateau (~ 15 min) was almost comparable to that observed in the cells, stimulated with the irradiation

Figure 4. (a) Schematic representation of the experimental workflow. Erk-KTR-Clover is a fluorescence sensor that changes its localization propensity, dependent on the Erk activity. Met activation induces the activation of Erk, thereby inducing the translocation of Erk-KTR-Clover from the nucleus to cytosol. Temporal patterns of the light-irradiation applied to the cells were shown at the top. (b)–(d) Normalized ratio of the fluorescent intensity of Erk-KTR-Clover in the cytosol and nucleus. HeLa cells, expressing Erk-KTR-Clover, were incubated with cApt (50 nM) and a 365-nm peak UV light (0.2 W/cm²) was irradiated according to the temporal pattern shown in Figure 4a. The data were expressed as average ratio, calculated from 10 cells. The colored areas indicate SD. (e)–(g) Representative time-lapse imaging, showing translocation of Erk-KTR-Clover during time-lapse imaging analysis.

pattern A, it took longer time (>30 min) until the translocation level returned to the basal level (Supplementary video 2). It was also confirmed that a repetitive UV-irradiation pattern (input C) could induce a pulsatile Erk activation pattern (Figures 4d, 4g and Supplementary video 3).

We also elucidated spatial resolution of the current system. The irradiation pattern A (Figure 4a) was applied to a selected area and the translocation of reporter in the cells, either inside or outside of the irradiated area, was monitored (Figure S5 and Supplementary video 4). In the cells located in the irradiated area, Erk-reporter showed a pulse-like translocation pattern when the cells were stimulated with the irradiation scheme A. On the contrary, the translocation of reporter was negligible in the cells outside of

the area. These results indicate that activation of Met receptor occurred only in the light-irradiated area and suggest that the influence of diffusion of uncaged aptamers was negligible. This excellent spatial resolution is presumably due to the excess amount of uncaged aptamers, existing in the non-irradiated area, which can compete for the receptor binding with the active aptamer produced in the irradiated area.

To summarize, we demonstrated for the first time, nongenetic temporal patterning of GF signaling using a non-proteinaceous growth factor surrogate. The caged DNA aptamer to RTK could control the dynamics of GF signaling with a temporal precision on the minute timescale. Unlike precedent optochemical tools, based on a combination of chemically-inducible dimerization protein, and an appropriate photo-caged dimerizer¹², this nongenetic approach applies solely a photocaged-aptamer that can directly control the protein dimerization on the living cells. Although other excellent DNA aptamer-based tools for optical control of receptor dimerization have been recently reported^{7b,c}, the current system has significant advantage. The aptamer was designed to turn on its receptor-activating potential, depending on a deprotection event of just one photo-caging group. This feature enables controlling of the GF signaling with better spatio-temporal precision over the precedent system, utilizing a DNA strand exchange reaction^{7c} or release of agonist DNA from nanoparticles^{7b}. This novel class of optochemical tools represents a promising approach for studying the association between signaling dynamics and resulting biological outcomes, aiming to manipulate cellular functions and fate of living cells.

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1. Supporting figures

Figure S1. Melting curves of the linker sequence of Apt (2.5 μM , top left) or cApt (2.5 μM , top right) in DPBS. The linker sequence of cApt was irradiated with 365 nm UV light (8 mW, 60 sec) and used for the melting curve analysis (bottom, right).

Figure S2. Representative fluorescence images showing expression of Erk-KTR-Clover in HeLa cells. The cytoplasmic versus nuclear fluorescence of the Erk-KTR-Clover (Cyt/Nuc ratio) in each cell was calculated using average nuclear or cytoplasmic fluorescent intensities, determined from three randomly selected ROIs (white circles) in a background-subtracted image. To limit the effects of variability in basal Erk activity of each cell, the cells with Cyt/Nuc ratio between 0.28 and 0.81 at the beginning of time-lapse imaging ($t = -15$ min) were randomly selected and used for the analysis.

Figure S3. (a) Immunoblotting analysis of Erk phosphorylation kinetics in HeLa cells, treated with HGF (1 nM). (b) Time-lapse imaging of translocation of Erk-KTR-Clover in HeLa cells, treated with HGF (1 nM) or vehicle control (DPBS) for 60 min. The data are expressed as average ratio from eight cells. The colored areas indicate SD.

Figure S4. Normalized ratio of the fluorescent intensity of Erk-KTR-Clover in the cytosol and nucleus. HeLa cells, expressing Erk-KTR-Clover, were incubated in the absence (gray) or presence (blue) of cApt (50 nM) and a series of 365 nm UV irradiations (purple bars). The data are expressed as average ratio from 10 cells. The colored areas indicate SD.

Figure S5. (a) Representative time-lapse imaging showing translocation of Erk-KTR-Clover. The irradiation pattern A (Figure 4a) was applied to the octagonal area (white) and the translocation of reporter in the cells, inside or outside of the irradiation area, was monitored. (b) Normalized ratio of the fluorescent intensity in the cytosol and nucleus. HeLa cells, expressing Erk-KTR-Clover, were incubated in the presence of cApt (50 nM) and light-irradiated with a temporal pattern A shown in Figure 4a. The data are expressed as average ratio and the colored areas indicate SD for the irradiated (N = 7) and the non-irradiated (N = 16) areas.

2. Methods

2-1 General information

Reagents were purchased from the standard suppliers and used without further purification. All oligonucleotides were purchased from Fasmac and Gene design and used without further purification. The cApt sequence was synthesized by Gene design, using 6-nitropiperonyl- α -methyl (NPM)-caged deoxycytidine (dC) phosphoramidite. The NPM-caged dC phosphoramidite was synthesized according to the literature¹. Refolding and annealing of DNA samples were performed with a thermal cycler. A xenon light source (MAX-301, Asahi Spectra, Tokyo, Japan) was used for uncaging experiments, shown in Figures 3b and S1. Fluorescent images were obtained with an inverted microscope (IX-81, Olympus, Tokyo, Japan), equipped with a sCMOS camera (Zyla4.2, Andor technology, Belfast, Northern Ireland). The filter cube U-MWIB3 and U-MNU2 (Olympus, Tokyo, Japan) were used for the detection of Erk-KTR-Clover and uncaging of the caged-nucleotide, respectively. Immunoblotting images were obtained with a luminescence detection system (AE-9300H, ATTO, Tokyo, Japan). Absorbance and fluorescence measurements were performed with Infinite M200 pro (Tecan, Bezirk Meilen, Switzerland). UV melting measurement was performed with UV-VIS spectrometer (UV-1650PC, Shimadzu, Kyoto, Japan).

2-2 Cell culture

DU145 cells and HeLa cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. All cells were maintained at 5% CO₂ in a humidified incubator at 37 °C.

2-3 Preparation of cell lysates

The cells were seeded onto 35 mm dishes and cultured overnight at 5% CO₂ in a humidified incubator at 37 °C. The cells were then starved for 24 h in RPMI1640 without FBS. After the starvation, the medium was replaced and the cells were stimulated with an appropriate ligand for 15 min at 37 °C. The ligand solution was pre-warmed at 37°C just before the addition to the medium. The UV light was irradiated to the ligand solution that was kept at 37°C on the thermal cycler for an appropriate time. Next, the cells were washed twice with DPBS and lysed with a lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1

mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM AEBSF, 1 μ g/mL leupeptin, 1% Triton X-100, and 10% glycerol). The cell lysates were centrifuged at $10,000\text{--}11,000 \times g$ for 20 min and then the supernatants were recovered. The total protein concentration of each cell lysate was measured with the standard BCA assay after the dilution.

2-4 ELISA

The detection of phosphorylated Met was performed using the Human Phospho-HGFR/c-MET DuoSet IC ELISA (DYC2480-2, R&D systems, Minneapolis, USA). The assay was performed according to the manufacturer's instructions. The normalized Met phosphorylation level of each cell lysate was calculated by dividing the absorbance of the assay at 450 nm to the total protein concentration of the lysate. Three independent experiments were performed and the mean values were indicated with error bars (SD). The data were expressed as relative values, compared with the normalized Met phosphorylation level, induced by the treatment with HGF (1 nM) for 15 min in each experiment.

2-5 Immunoblotting assay

The cell lysates were separated by SDS-PAGE and then transferred to a PVDF membrane. The membrane was incubated with a primary antibody at 4 °C overnight, following the incubation with an appropriate secondary antibody at room temperature for 1 h. The primary antibodies for phospho-Met (3077), Met (8198), phospho-ERK1/2 (4370), ERK1/2 (4695, figure 3b) and beta-actin (4967) were obtained from Cell Signaling Technology (Danvers, USA). The primary antibody for ERK1/2 (sc-514302, figure S3) was obtained from Santa Cruz Biotechnology (Dallas, USA). The Anti-Rabbit Immunoglobulins/HRP secondary antibody was obtained from Agilent Technologies (P0488, Santa Clara, USA). The Anti-Mouse Immunoglobulins/HRP secondary antibody (W4021) was obtained from Promega (Madison, USA). The membranes were probed using ImmunoStar LD (296-69901, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) or Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500, Merck Millipore, Burlington, USA).

2-6 UV melting measurement

UV melting measurement was performed with UV-VIS spectrometer (UV-1650PC, Shimadzu, Kyoto, Japan) over a temperature of 5–90 °C (heat rate = 0.5 °C /min) using a quartz cuvette. DNA (2.5 μM) was dissolved in Dulbecco's phosphate buffered saline and refolded with a thermal cycler (95 °C for 5 min, and then cooled to 4 °C) before measurements. The UV melting curve was obtained by subtracting the background absorbance at 320 nm from the absorbance at 260 nm. Two independent experiments were performed and the mean T_m values were indicated.

2-7 Preparation of Erk-KTR-Clover expressing cells

An episomal vector encoding Erk-KTR-Clover was constructed for time-lapse imaging of Erk-KTR-Clover translocation. The Erk-KTR-Clover sequence was amplified from pENTR-ERKKTR Clover plasmid (Addgene 59138) and inserted into pEBMulti-Puro vector. The sequence of the constructed vector was confirmed by Sanger sequencing. HeLa cells were seeded at 60 mm-plates and cultured in RPMI1640 supplemented with 10% FBS overnight. On the day of transfection, the cells were washed twice with DPBS and incubated with DNA/PEI mixture in RPMI1640 supplemented with 10% FBS overnight. To enrich Erk-KTR-Clover expressing cells, drug selection (1 mg/ml puromycin in RPMI1640 supplemented with 10% FBS) was started on the next day after the transfection. The cells were used for time-lapse imaging of Erk-KTR-Clover translocation at least 2 days after the drug selection.

2-8 Time-lapse imaging of Erk-KTR-Clover translocation

The Erk-KTR-Clover-expressing HeLa cells (2×10^4 cells/well) were seeded onto a chambered coverglass (8 wells, AGC Techno Glass, Tokyo, Japan) and cultured in RPMI1640 supplemented with 10% FBS. After the cells adhered to the coverglass, they were incubated in a starving medium (RPMI1640 without phenol red) overnight. On the day of experiment, the media was replaced and the chambered coverglass was mounted on a stage top incubator (INUG2F-IX3W, Tokai Hit, Shizuoka, Japan). The cells were incubated at 5% CO₂ in a humidified atmosphere at 37 °C during the experiment. For uncaging experiments, the cells were pre-incubated in the presence or absence of cApt (50 nM) in the

starving medium at least for 30 min. Time-lapse images were captured in 1 min intervals for 75 min (Figures 4b, 4c, 4e, 4f, S3, S5), 135 min (Figures 4d and 4g). The filter cubes U-MWIB3 and U-MNU2 (Olympus, Tokyo, Japan) were used for detection of Erk-KTR-Clover and for photo-deprotection of the caged-nucleotide, respectively. The Iris diaphragm was used for local UV irradiation. The location of UV irradiation at the sample plane was checked by observing Erk-KTR-Clover-expressing HeLa cells, cultured on the chambered coverglass in a high confluency. Both global and local irradiations were performed for the indicated duration while conducting time-lapse imaging. The cytoplasmic versus nuclear fluorescence of the Erk-KTR-Clover (Cyt/Nuc ratio) in each cell was calculated using average nuclear or cytoplasmic fluorescent intensities, determined from three randomly selected ROIs in a background-subtracted image. To limit the effects of variability in basal Erk activity of each cell, the cells with Cyt/Nuc ratio between 0.28 and 0.81 at the beginning of time-lapse imaging ($t = -15$ min) were randomly selected and used for the analysis. The image analysis was conducted using Image J software (<http://rsbweb.nih.gov/ij/>).

3. Sequence data

Apt: CGA TCG ATG GAT GGT AGC TCG GTC GGG GTG GGT GGG TTG GCA ATC GAT CGG
CTAC GTA GC

cApt: CGA TCG ATG GAT GGT AGC TCG GTC GGG GTG GGT GGG TTG GCA ATC GAT CGG
CTAX GTA GC

(X = NPM-caged deoxycytidine. Hybridization linker sequences are highlighted in red)

4. Reference

1. Govan, J. M.; Uprety, R.; Hemphill, J.; Lively, M. O.; Deiters, A. *ACS Chem. Biol.* **2012**, 7, 1247.

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