Artificial Receptors

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Signal Transduction Using an Artificial Receptor System that Undergoes Dimerization Upon Addition of a Bivalent Leucine-Zipper Ligand**

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Dimerization and clustering of biological receptors on the plasma membrane lead to activation and subsequent signal transduction. If this dimerization event could be artificially controlled, it would be a valuable tool for studying signal transduction and modulating cellular functions. Previous studies have applied antibodies that recognize pre-incorporated tags on cell-surface receptors to stimulate receptor dimerization.[1–3] Dimerization of receptors expressing a single-chain antibody against fluorescein by adding fluorescein-modified albumins were also reported.[3,4] Although antibodies are specific and have high ligand-binding affinities, their bulky structures may limit the design of ligand-receptor recognition systems. Other approaches caused intracellular cross-linking using FK506- and FKBP-tagged intracellular signaling domains of receptors to form dimers when FKBP binds FK506.[5] These approaches often employed multiple intracellular FKBP domains, which could lead to the assembly of more than two intracellular domains upon the addition of dimeric FK506. Possible steric interference of FKBP multimers might induce interaction of intracellular domains different from those of the wild-type receptors.

Herein, we propose a novel approach to controlled receptor activation that employs a leucine-zipper coiled-coil as a recognition element to stimulate dimerization (Figure 1). This approach provides flexibility in the selection of the different combinations of leucine zippers, as recognition elements, and the linkers tethering them to the receptors being studied.

To demonstrate the feasibility of this approach, we used a heterodimeric coiled-coil developed by Hodges and coworkers (E/K coil).[6] This recognition motif has already been used to fluorescently label cell-surface proteins.[7,8] Peptide probes K3 (=$(KIAALKE)_3$) and K4 (=$(KIAALKE)_4$), labeled with a fluorophore, specifically stained the surface-exposed tag sequence E3 (=$(EIAALEK)_3$) attached to the N-terminus of the proteins. Recognition of these peptides is quick (<1 min) with a high affinity (K_d = 64 nM for K3 and 6 nM for K4),[7] which should also allow for efficient dimerization of receptor proteins. Considering that the K4 peptide showed a higher affinity to E3 than K3, we designed a system using bivalent K4 ligands to bring together cell-surface receptors bearing the E3 tag (Figure 1). One recent study employed an extracellular leucine-zipper dimerization domain of epidermal growth factor receptor (EGFR).[20] This previous work used a constitutively dimerized leucine-zipper–EGFR fusion to analyze the mechanism of an intracellular EGFR activating factor. On the other hand, our approach could increase the flexibility in selection of combinations of leucine zippers as recognition elements and in the linkers tethering them to the receptors. In addition, this method should provide an additional level of control by allowing a triggered dimerization event in biochemical and cell-biological experiments.

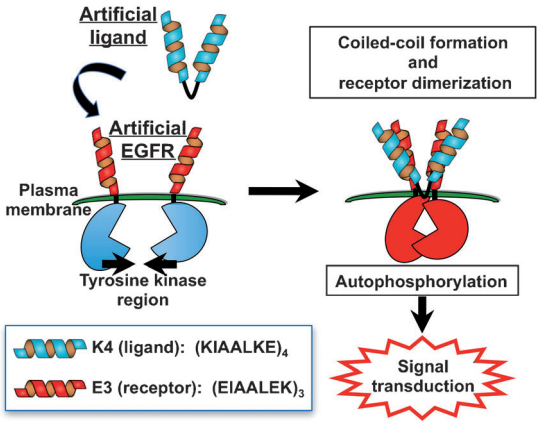

Figure 1. Scheme of artificial EGFR activation by a helical peptide through coiled-coil formation.

EGFR is a representative receptor tyrosine kinase (RTK) that regulates proliferation, motility, and survival in normal cells, and is also implicated in many human cancers.[9–11] Binding of EGF promotes dimerization of the EGFR, which causes phosphorylation of the cytoplasmic domain and activation of downstream signaling pathways.[9,10,12,13] The extracellular region of EGFR contains four subdomains (domains I–IV).[9,12] We designed an EGFR receptor lacking domains I–III and a part of domain IV (which are responsible for binding to EGF and dimerization) but included hemagglutinin A (HA) and E3 tags (E3-EGFR; tags are for the

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detection and dimerization of the receptor, respectively; see Supporting Information, Figure S1 for a schematic depiction). As a control, we also prepared wild-type EGFR (wtEGFR) fused to an HA tag on the N-terminus. The pDisplay vector was used to express E3-EGFR and wtEGFR on plasma membranes. CHO-K1 cells were used for transfection because of their low intrinsic expression of wtEGFR[14] (see Supporting Information).

Bivalent K4-ligands were prepared to induce dimerization of E3-EGFR on plasma membranes and activate the receptor. EGFR dimerization is mediated by receptor contacts within domain IV.[12] Because K4 forms a parallel coiled-coil with E3 and the C-terminal residue of E3 is located near the membrane in the E3-EGFR sequence, we placed a cysteine at the C-terminus of K4 (connected with a Gly-Gly linker) to cross-link two K4 peptides using bis(maleimido)ethane (BMOE-C; Figure 2a). This bivalent K4 ligand was predicted to bind and hold two E3-EGFR molecules in close proximity. To evaluate the effect of this type of cross-link, we prepared another ligand where a pair of K4 segments were connected at their N-termini (BMOE-N). We also prepared an analogue of BMOE-N with a longer spacer (BMH-N; Figure 2b).

We confirmed that BMOE-C recognized E3-EGFR on plasma membranes using confocal microscopy after treating

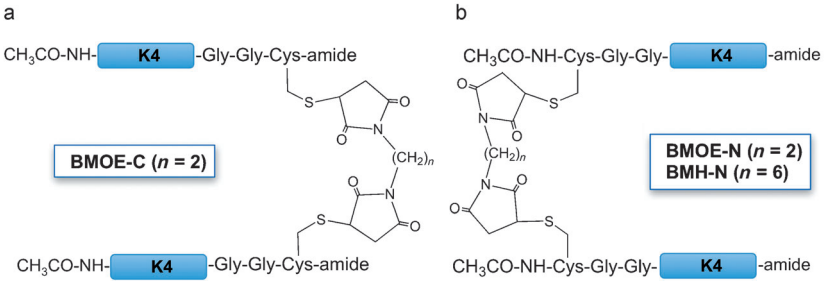

Figure 2. Structure of bivalent K4 ligands. a) BMOE-C, b) BMOE-N and BMH-N.

E3-EGFR-expressing CHO cells with fluorescently labeled BMOE-C peptide (Supporting Information, Figure S2) for 15 min at 4 °C. Performing this experiment at 4 °C prevents internalization of E3-EGFR by endocytosis (Figure 3 and Supporting Information, Figure S3). Localization of E3-EGFR on the plasma membrane was simultaneously analyzed using an anti-HA antibody, because E3-EGFR has an HA tag in the extracellular domain (Supporting Information, Figure S1). In contrast to the colocalization of BMOE-C with HA-antibody on plasma membranes in cells expressing E3-EGFR (Figure 3, upper panel), no BMOE-C was observed in wtEGFR-expressing cells (Figure 3, lower panel). Additionally, there appeared to be less labeling of E3-EGFR by the anti-HA antibody on cell surfaces than fluorescein-labeled BMOE-C, which could be due to steric hindrance between antibodies. This difference illustrates the advantage of the less hindered leucine-zipper linkers for receptor dimerization. We further confirmed that BMOE-C recognized E3-EGFR using fluorescence resonance transfer (FRET)[15] between a donor (fluorescein-BMOE-C) and an acceptor (Alexa Fluor 568 labeled anti-HA antibody; Figure 4).

Dimerization of EGFR leads to autophosphorylation, which activates downstream signaling pathways including the Ras/Raf/mitogen-activated protein kinase pathway (extracellular signal-regulated kinase (ERK) 1 and 2), the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and the signal transduction and activator of transcription (STAT).[16] We next examined whether BMOE-C binding results in activation of the receptor. CHO-K1 cells expressing E3-EGFR or wtEGFR were treated with K4 ligands (10 μM) for 1, 2, 5, 10, or 20 min at 37 °C. Note, although E3-EGFR was also activated by 1 μM BMOE-C, we used 10 μM BMOE-C to maintain high reproducibility in the following studies. We analyzed phosphorylation of EGFR tyrosine 1173 (Y1173), as a measure of autophosphorylation,[17] by Western blot using a phosphorylated Y1173-specific antibody (pY1173; Supporting Information, Figure S4; note that the number of the corresponding tyrosine in E3-EGFR is different from 1173, but for simplicity Y1173 was used to describe the tyrosines where phos-

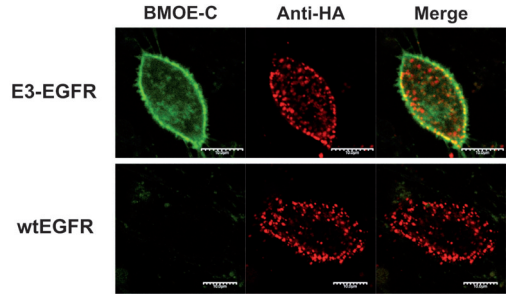

Figure 3. Confocal microscopic images of CHO-K1 cells expressing E3-EGFR or wtEGFR and treated with fluorescently labeled BMOE-C and anti-HA antibody, as described in the Supporting Information. Scale bar = 10 μm.

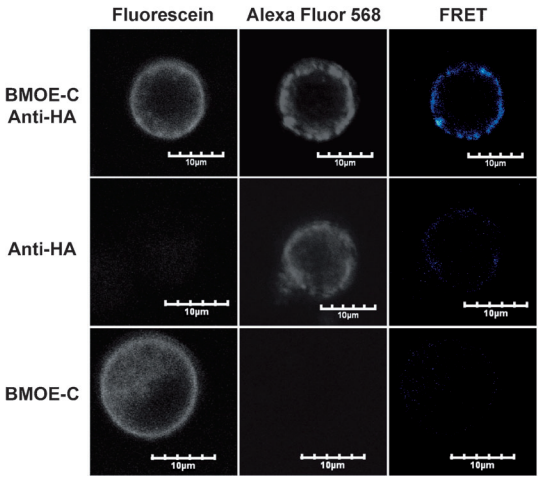

Figure 4. Fluorescent and FRET images of CHO-K1 cells expressing E3-EGFR and treated with fluorescein-BMOE-C (BMOE-C) and/or Alexa Fluor 568 labeled anti-HA antibody (Anti-HA). Fluorescein-BMOE-C (excitation: 488 nm, emission: 500–545 nm), Alexa Fluor 568 labeled anti-HA antibody (excitation: 559 nm, emission: 570–670 nm), and FRET (excitation: 488 nm, emission: 570–670). Scale bar = 10 μm.

phorylation occurs in both wtEGFR and E3-EGFR). Stimulation of cells with BMOE-N, BMOE-C, and BMH-N resulted in increased phosphorylation of Y1173 in E3-EGFR at 5 min (Figure 5 and Supporting Information, Figure S4), which was similar to stimulation of wtEGFR by EGF (Figure S4). In contrast, there was no increase in Y1173

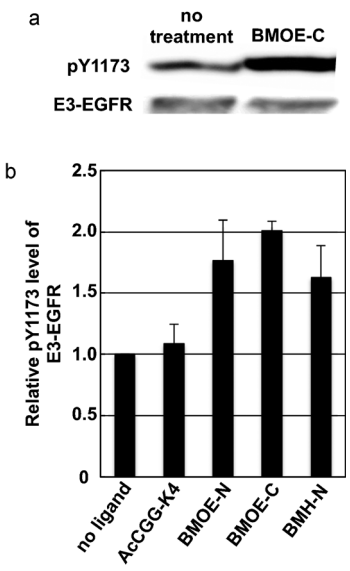

Figure 5. a) Western blot of phosphorylation of E3-EGFR Y1173 stimulated with BMOE-C (10 μM) for 5 min. b) Relative phosphorylation level of E3-EGFR Y1173 stimulated with artificial ligands (10 μM) for 5 min at 37 °C. Data represent the average (± standard deviation, SD) of three experiments.

phosphorylation in E3-EGFR when the cells were treated with monomeric K4 peptide (AcCGG-K4), and wtEGFR was not activated by BMOE-N (Figure 5b and Figure S4). Densitometric analysis of the Western blot suggested a twofold increase in Y1173 phosphorylation after treatment with BMOE-C in E3-EGFR-expressing cells. A comparable increase in phosphorylation was detected after treatment with BMOE-N. Treatment with BMH-N had a slightly less-significant effect (Figure 5b).

Stimulation of EGFR by EGF leads to actin reorganization and the formation of lamellipodia, sheet-like plasma-membrane protrusions formed by branched actin networks.[18] Lamellipodia also formed after treatment of E3-EGFR-expressing CHO-K1 cells with bivalent K4 ligands. The cells were treated with the K4 ligands for 10 min at 37 °C, followed by actin staining with phalloidin-TRITC. BMOE-C and BMOE-N efficiently induced lamellipodia formation (Supporting Information, Figure S5). Less lamellipodia formation was observed after BMH-N treatment (Supporting Information, Figure S5), and no significant lamellipodia formation was observed with monomeric K4 peptide in E3-EGFR-expressing cells (Supporting Information, Figure S5) or with bivalent K4 ligands in wtEGFR-expressing CHO-K1 cells (data not shown), further confirming the specific activation of E3-EGFR by bivalent K4 linkers.

Activation of EGFR also stimulates cell migration through the PI3K/Akt and STAT pathways.[2,19] This was also observed after stimulating E3-EGFR-expressing cells with bivalent K4 ligands (Figure 6). The E3-EGFR-expressing CHO-K1 cells were partially scraped (as described in Supporting Information) and treated with the bivalent K4

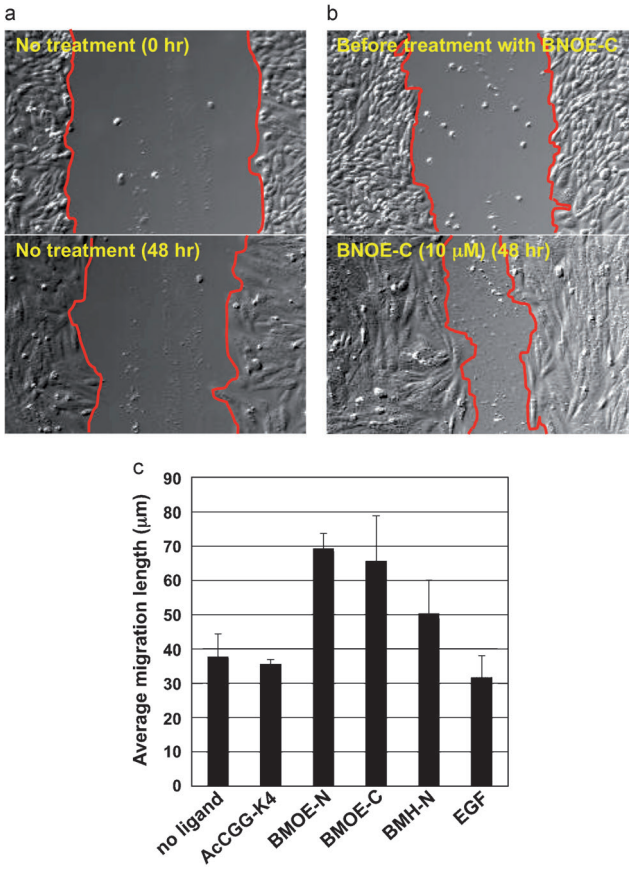

Figure 6. a, b) Migration of E3-EGFR expressing CHO cells treated with 10 μM BMOE-C (b) and without any ligand (a) for 48 h at 37 °C, observed using a microscope. c) Average migration length of E3-EGFR expressing CHO cells treated with AcCGG-K4, BMOE-N, BMOE-C, BMH-N (each 10 μM), or EGF (100 nM) in F-12(−) medium for 48 h at 37 °C under 5% CO_2 as described in the Supporting Information. Average migration length during ligand stimulation was calculated using software from an FV1000 confocal microscope (FV10-ASW). Data represent the average (± SD) of 150–200 cells.

ligand for 48 h. Then the distance the cells moved was analyzed using a scratch assay. After treatment with BMOE-C and BMOE-N, we determined an approximately twofold increase in migration length compared to that of nontreated control cells (Figure 6). BMH-N also yielded a shorter migration length compared to cells treated with BMOE-C or BMOE-N. Based on these studies, BMH-N has less of an effect than BMOE-C and BMOE-N. The longer linker in BMH-N may not tether the E3-EGFR molecules as tightly as in BMOE-C and BMOE-N, which could reduce the biological responses evoked by dimerization of the receptor molecules. The monomeric K4 peptide (AcCGG-K4) and EGF did not increase the migration of E3-EGFR-expressing CHO cells,

suggesting that bivalent K4 ligands specifically stimulate the migration of such cells. No stimulation of migration was observed after the treatment of CHO-K1 cells (having low intrinsic expression of wtEGFR) with EGF, and this is another benefit of our approach, to properly analyze the effects of dimerization under the control of bivalent K4 ligands.

In conclusion, we demonstrated that dimerization and activation of receptors on plasma membranes through coiled-coil formation could be induced with artificial bivalent ligands. This system provides greater flexibility in design, compared to current methods, such as using antibodies. More detailed studies are necessary with regard to the positioning of leucine-zipper segments in the receptors and the downstream responses produced by the addition of bivalent ligands, as well as the effect of the linkers. The bivalent ligand is easily prepared, compact, resistant to denaturation, and easily handled under cell culture conditions. The availability of different combinations of leucine-zipper recognition peptides and cross-linkers is another advantage of this approach. Therefore, this approach could be used to study receptor recognition, activation, and signal transduction.

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