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Inhibitory effect of a dimerization-arm-mimetic peptide on EGF receptor activation

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ABSTRACT

A cyclic decapeptide was chemically synthesized that mimics the loop structure of a β-hairpin arm of the EGF receptor, which is highly involved in receptor dimerization upon activation by ligand binding. This peptide was revealed to reduce dimer formation of the receptor in a detergent-solubilized extract of epidermoid carcinoma A431 cells and to inhibit receptor autophosphorylation at less than 10 μM in the intact cells.

In response to ligand binding, epidermal growth factor (EGF) receptor on the cellular membrane is activated, causing proliferation and differentiation of various cells. The overexpression and unregulated activation of the receptor are known to initiate cancers in cells, as often observed in lung carcinoma.[1–4] Therefore, the receptor has recently become a good medicinal target for designing anti-cancer drugs. For example, *gefitinib* (Iressa®) has been developed as a molecular-targeted agent to directly inhibit the intracellular tyrosine kinase of the receptor.[5,6] In addition, antibody drugs against the receptor have been developed, such as *cetuximab* (Erbitux®), which is a monoclonal antibody recognizing the extracellular ligand-binding region of the receptor.[7–9] However, the use of such agents is hindered by some serious problems, such as side effects[10] and the exorbitant medication cost,[11,12] although they have shown certain efficacy in cancer therapeutics. A new type of inhibitor against the EGF receptor is now eagerly anticipated, to further improve the quality of life of cancer patients.

The EGF receptor is a single-spanning membrane protein on the cell surface. Ligand binding to the receptor extracellular region dramatically changes its conformation, and consequently drives the receptor molecules into dimers. The receptor dimerization successively causes intracellular autophosphorylation, in which the kinase transphosphorylates tyrosine residues in the C-terminal tail of the dimerization partner.[13–15] Since dimerization may be one of the most crucial steps in receptor activation, in this study, we designed and synthesized a peptide that would prevent the dimerization and inhibit the autophosphorylation of the EGF receptor.

The structure of the EGF receptor extracellular region, dimerized by binding the EGF ligand, was previously elucidated by X-ray crystallography (PDB ID: 1IVO) (Fig. 1).[16] The extracellular region of the receptor consists of four domains (I–IV), and in the dimerized structure, the interface between the receptor monomers is mostly occupied by domain II. Especially, a β-hairpin arm (amino-acid residues 242–259) protruding from this domain seems to be most responsible for the dimerization, since a considerable number of hydrogen-bonds are observed between the residues at a turn head of the arm and those of the partner receptor molecule (Fig. 1, inset). Therefore, the β-hairpin structure is called a 'dimerization arm'.[16]

To develop dimerization inhibitors against the EGF receptor, a decapeptide mimicking the loop structure of the arm head, CYNPTTYQMC (**1**), was designed. Peptide **1** has the amino-acid sequence of the turn head and two additional cysteine residues at both the N- and C-termini, for cyclization with a disulfide bond between them, and it was chemically synthesized by conventional Fmoc-based solid-phase methodology. Namely, the first C-terminal

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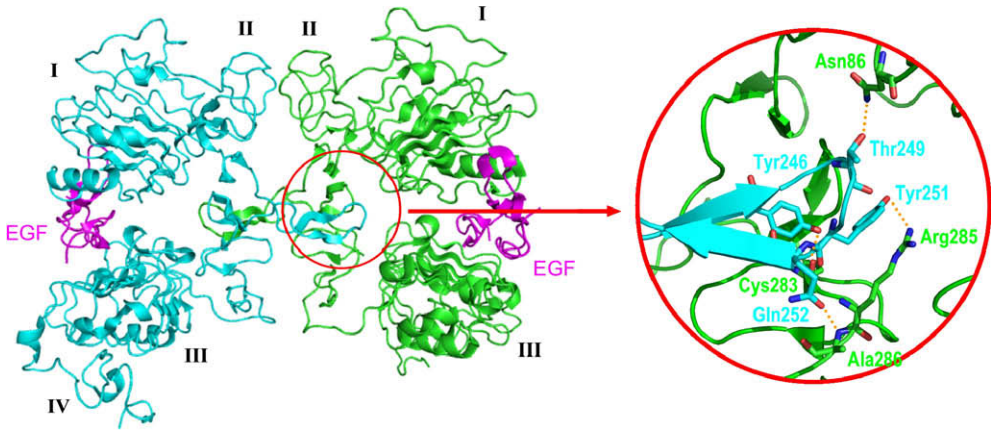

Figure 1. Crystal structure of dimerized EGF receptor ectodomains (left) and the intermolecular hydrogen-bond network at the turn head of the dimerization arm (right inset) (PDB ID: 1IVO). The dimer interface is mostly occupied by domain II, and several hydrogen-bonds are observed between the residues at the turn head of the protruding dimerization arm and those of the partner receptor molecule. Four representative hydrogen-bonds are drawn in the inset: between Tyr246^{A} and Cys283^{B}, Thr249^{A} and Asn86^{B}, Tyr251^{A} and Arg285^{B}, and Gln252^{A} and Ala286^{B} (superscript suffixes, A and B, on residue numbers indicate each of the receptor molecules in the dimer, with monomer structures drawn in blue and green, respectively).

residue, Fmoc-L-Cys(Trt)-OH, was attached to 2-chlorotrityl chloride resin using diisopropylethylamine in 1,2-dichloroethane. Fmoc-protected L-amino acids (2.5 equiv) were successively coupled with the aid of 1-hydroxybenzotriazole (2.5 equiv) and diisopropylcarbodiimide (2.5 equiv) in DMF, and completion of the coupling reactions was monitored by Kaiser's ninhydrin test. A treatment with 20% (v/v) piperidine was utilized for the removal of the Fmoc group. Finally, the desired peptide was cleaved and released from the completed resin using a solution of trifluoroacetic acid (TFA)–ethanedithiol–triisopropylsilane–H_2O (94.5:2.5:1:2.5, v/v) at room temperature for 90 min. After removal of the resin with a glass filter, an excess volume of cold diethyl ether was added to precipitate the cleaved peptide, which was then redissolved in H_2O and lyophilized. A solution (0.1 mg/mL) of the lyophilized peptide in 100 mM NH_4HCO_3 (pH ~8) was stirred in air at room temperature, and oxidative cyclization of the peptide to yield **1** was monitored by analytical reversed-phase HPLC (Fig. 2). Even immediately after the dissolution at 0 h, the cyclized peptide **1** (peak b) was detected as well as the starting material without a disulfide bond (peak a). The amount of the resultant peptide **1** gradually increased, and the reaction was mostly completed within 3 h. The peptide sequence was quite readily cyclized to yield the product **1**. After purification by preparative HPLC, formation of the disulfide bond was confirmed with MALDI-TOF-MS (monoisotopic mass: found, 1243.50; calcd for $[M+Na]^+$, 1243.42). As a reference compound, a linear octapeptide lacking the cysteine residues, YNPTTYQM (**2**), was similarly prepared by the Fmoc-based solid-phase synthesis. After cleavage from the resin and precipitation with cold ether, the peptide was directly subjected to preparative HPLC purification, without the oxidative cyclization step. The purified product of **2** showed a quite acceptable mass value (monoisotopic mass: found, 1017.36; calcd for $[M+H]^+$, 1017.43).

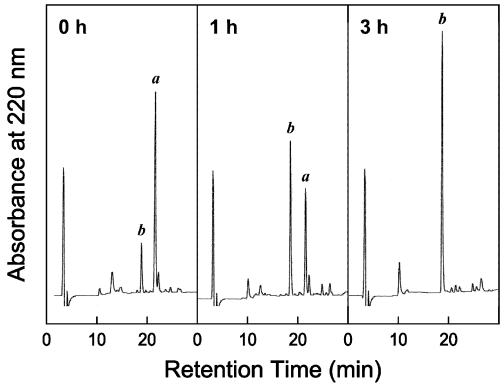

Figure 2. Oxidative disulfide bond formation in peptide **1**. The parent linear peptide CYNPTTYQMC was dissolved in 100 mM NH_4HCO_3, and the solution (0.1 mg/mL) was stirred in air at room temperature. The cyclization reaction was monitored by analytical HPLC [Column, YMC-Pack ODS-A A-312 (6.0 × 150 mm); solvent, 0.05% TFA/CH_3CN–H_2O (gradient from 15:85 to 35:65 over a period of 40 min) at a flow rate of 1.0 mL/min]. Even immediately after the dissolution of the starting material at 0 h, both the parent peptide (peak a; *m/z* 1245.41) and the resultant cyclic one (peak b; *m/z* 1243.50) were detected. The peak intensity of the product peptide **1** (b) gradually increased at 1 h, and, finally, the peak of the parent peptide (a) had mostly disappeared by 3 h. Calculated monoisotopic *m/z* values for $[M+Na]^+$ ion of the parent linear and product cyclic peptides are 1245.43 and 1243.42, respectively.

First of all, the inhibitory activities of the synthetic peptides against the EGF receptor dimerization were assessed. Formation of the dimer was monitored by cross-linking of the receptor molecules in the ligand-induced dimer state. In this study, as fully described in Supplementary data, a hydrophilic, membrane-impermeant cross-linker, bis(sulfosuccinimidyl) suberate (BS^3),[17] was employed, since Staros and his co-workers had successfully trapped the EGF receptor dimer in extracts solubilized from human epidermal carcinoma A431 cells by the use of this cross-linker.[18] The carcinoma cells abundantly express the EGF receptor on their cellular surface, and the receptor, in a Triton X-100-solubilized membrane fraction of the cells, was easily detected as a protein band at ~170 kDa on SDS–PAGE gels, by immunoblotting with an anti-EGFR antibody (Fig. 3A). In response to stimulation by the ligand EGF, an additional band obviously appeared at ~350 kDa, corresponding to the molecular mass of the BS^3-trapped receptor dimer. The dimer band became slightly thinner, as larger amounts of peptide **1** were co-incubated during the ligand stimulation, indicating that the dimerization-arm-mimetic peptide reduced the EGF-induced dimerization of the receptor. The degrees of reduction in the dimer formation were quantified by densitometry of the bands, and 1 μM peptide **1** was revealed to decrease the dimer formation to less than 80% of that without the peptide (Fig. 3B). Unfortunately, amounts larger than 1 μM of peptide **1** generated only poorly-reproducible data with large deviations, perhaps due to perturbation of the solubilized state of the receptor itself, and thus complete quenching of the dimer formation could not be accomplished by peptide **1**. On the other hand, the linear peptide **2** hardly inhibited the receptor dimerization in the concentration range studied here (Fig. 3B). Since the inhibitory activity of

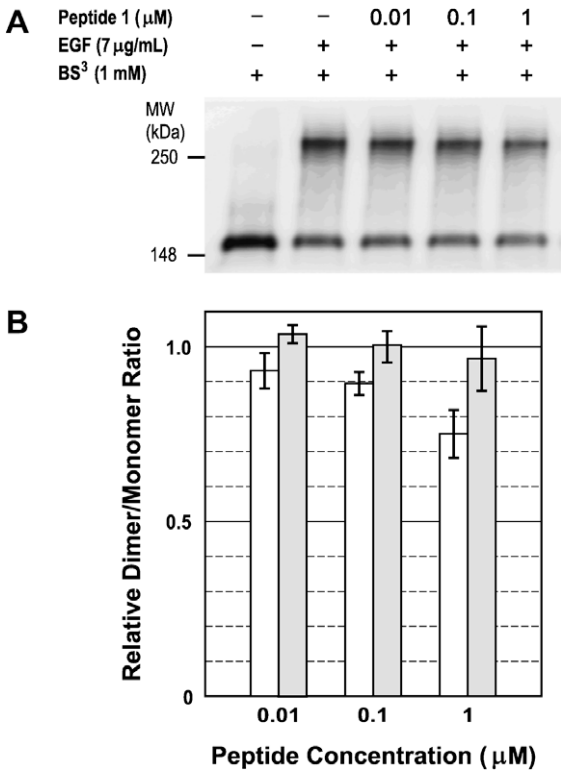

Figure 3. Inhibitory effects of the cyclic (**1**) and linear (**2**) peptides on dimerization of the EGF receptor in a solubilized membrane fraction prepared from human epidermal carcinoma A431 cells. Solubilized extracts containing the EGF receptor were incubated with or without 7 μg/mL EGF for 10 min in the presence of 0, 0.01, 0.1, or 1 μM peptide **1** or **2**. Then, the extracts were treated with 1 mM BS^3 for 10 min to covalently cross-link the EGF receptor molecules in the dimer state. After termination of the cross-linking reaction with 50 mM glycine, the complexes were separated by SDS–PAGE, transferred to a PVDF membrane, and visualized by immunoblotting with an anti-EGFR antibody. (A) Typical immunoblots for inhibition assays of the EGF receptor dimerization by peptide **1**. (B) Inhibitory activities of peptides **1** (open bars) and **2** (gray bars) against the receptor dimerization. Relative ratios of apparent band densities between receptor dimer and monomer are shown as the mean ± standard deviation values of at least three independent experiments, and were normalized such that the positive control (EGF-stimulated but in the absence of the peptides) represents a ratio = 1.0.

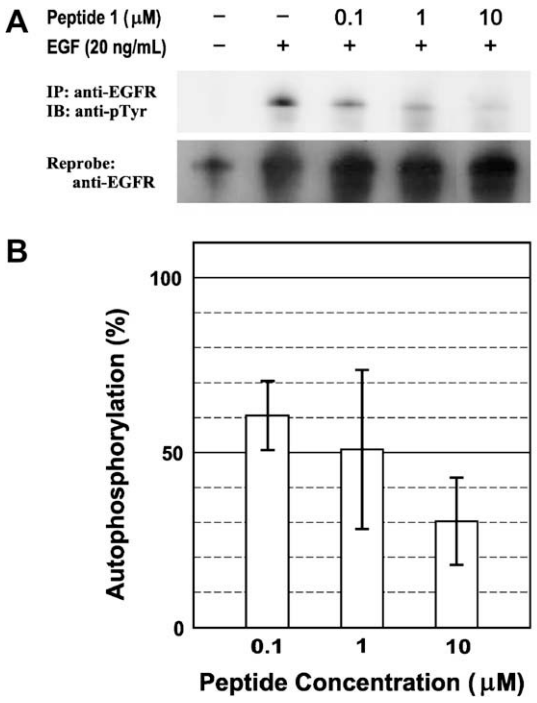

Figure 4. Inhibitory effect of peptide **1** on EGF receptor autophosphorylation in intact A431 cells. The cells were serum-starved for 24 h in serum-free medium containing 1 mg/mL BSA. After the starvation, they were stimulated with 20 ng/mL EGF for 5 min in the presence of 0, 0.1, 1, or 10 μM peptide **1**. Cell lysates were subjected to immunoprecipitation (IP) with an anti-EGFR antibody. Precipitates were resolved by SDS–PAGE, transferred to a PVDF membrane, and visualized by immunoblotting (IB) with an anti-phosphotyrosine antibody. Membranes were reprobed with an anti-EGFR antibody to control for protein loading. (A) Typical immunoblots for autophosphorylation assays of the EGF receptor. (B) Inhibitory activities of peptide **1** against EGF receptor autophosphorylation. Data represent the mean ± standard deviation values of at least three independent experiments, and were normalized such that the positive control (EGF-stimulated but in the absence of the peptide) represents 100% receptor autophosphorylation.

peptide **2** was considerably lower than that of peptide **1**, the loop structure of the arm-mimetic peptide, maintained by the disulfide bond, may play an important role in retaining the affinity for the EGF receptor molecule to competitively inhibit the receptor dimerization.

Next, the cyclic peptide **1**, which showed weak but certain inhibitory activity against the receptor dimerization, was subjected to another assay of receptor autophosphorylation, using intact A431 cells. The cell-based assay was performed as described previously[19] with minor modifications (for details of the assay procedure, see Supplementary data). After the cells were stimulated with EGF in the presence of an appropriate amount of the peptide, the receptor was immunoprecipitated with an anti-EGFR antibody, and autophosphorylation of the receptor was observed by immunoblotting with an anti-phosphotyrosine antibody (Fig. 4A). The cyclic peptide **1** showed obvious inhibition of the receptor autophosphorylation. Quantitative densitometric analyses of the immunostained bands revealed that the residual autophosphorylation level of the EGF receptor was suppressed to 61%, 51%, and 30%, by the addition of 0.1, 1, and 10 μM peptide **1**, respectively, to the culture medium (Fig. 4B). The dimerization-inhibiting peptide absolutely reduced the EGF-stimulated receptor activation, in terms of the intracellular autophosphorylation in living cells.

Our simple strategy for the inhibition of EGF receptor activation via that of the dimerization by the arm-mimetic cyclic peptide **1** seems quite successful, although the peptide-binding site on the receptor should be confirmed in the future. The cyclic peptide can bind to the extracellular region of the receptor, at least, since it might not penetrate the hydrophobic cellular membrane to the cytoplasm of A431 cells, but it obviously inhibited the receptor autophosphorylation in the intact cells. On the other hand, 25–40-residue fragments of a negative regulatory protein of the EGF receptor, MIG6, were reported to directly inhibit the receptor autophosphorylation by preventing the interaction between a phosphorylating kinase domain and a phosphorylated substrate domain; that is, the asymmetric dimer formation of the intracellular kinase domain.[20] However, it is still unknown whether the fragments can inhibit the dimerization of not only the kinase domain but also a 'whole' receptor molecule.

Another cyclic peptide, $\overline{\text{FCDGFYAC}}\text{YMDV}$, was designed and found to inhibit the activity of ErbB-2 (HER2), a homologue of the EGF receptor, by Murali and his co-workers.[21,22] However, in contrast to this study, the sequence of their peptide was derived from the structure of the CDR-H3 loop of the anti-$p185^{HER2/neu}$ monoclonal antibody, 4D5 (*herceptin*; Trastuzumab®), and thus the peptide is referred to as an anti-HER2/neu peptide (AHNP). AHNP may bind domain IV, not domain II, because its parent antibody binds domain IV of ErbB-2. When inhibiting the ErbB-2 activity, it does not compete with the dimerization arm of the receptor, while our cyclic peptide might do so with that of the

EGF receptor. Therefore, peptide **1** is the first example of a peptide that was designed based on the loop structure of the dimerization arm and exhibits inhibitory activity against the EGF receptor.

In conclusion, the cyclic peptide **1** that mimics the loop of the dimerization arm in the crystal structure was revealed to inhibit the EGF receptor activity at less than 10 μM. Although the peptide should be optimized for higher inhibitory activity, it represents a promising lead for developing a new type of inhibitor against cancers arising from uncontrolled activation of the EGF receptor.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.080.

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