YAC tripeptide of epidermal growth factor promotes the proliferation of HaCaT keratinocytes through activation of EGFR

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Epidermal growth factor (EGF) is known to play key roles in skin regeneration and wound-healing. Here, we demonstrate that Pep2-YAC, a tripeptide covering residues 29-31 in the B loop of EGF, promotes the proliferation of HaCaT keratinocytes with activity comparable to EGF. The treatment of HaCaT cells with Pep2-YAC induced phosphorylation, internalization, and degradation of EGFR and organization of signaling complexes, which consist of Grb2, Gab1, SHP2, and PI3K. In addition, it stimulated the phosphorylation of ERK1/2 at Thr 202/Tyr 204 and of Akt1 at Ser 473 and the nuclear translocation of EGFR, STAT3, c-Jun, and c-Fos. These results suggest that Pep2-YAC may be useful as a therapeutic agent for skin regeneration and wound-healing as an EGFR agonist. [BMB Reports 2014; 47(10): 581-586]

INTRODUCTION

EGFR signaling plays key roles in skin regeneration and wound healing by stimulating epidermal keratinocyte proliferation (1). Additionally, EGFR is essential for epidermal and hair follicle homeostasis, and is important for the massive ex vivo expansion of human keratinocyte stem cells in patients with extensive burns and genetic disorders (2). EGFR is a 170-kDa transmembrane protein that belongs to ErbB family receptors, which includes three other members (ErbB2/HER-2, ErbB3/HER-3, and ErbB4/HER-4). Ligand binding to EGFR results in its dimerization and tyrosine autophosphorylation, and subsequent activation of several downstream pathways, such as the RAS-ERK, PI3K-Akt, and STAT3 pathways, which stimulate cell proliferation, survival, and migration (3, 4). Epidermal growth factor, a major ligand for the ErbB family, is a 53-residue polypeptide expressed in many tissues and body fluids of mammals. It has been shown to improve wound healing by increasing keratinocyte proliferation and the tensile strength of the healed dermis (5). All EGF family members contain one or more repeats of a conserved six-cysteine-containing motif (6). These six cysteine residues are contained within a sequence of 35-40 amino acids and have the potential to form three intra-molecular disulfide bond pairings to produce three loops that are essential for high-affinity binding to the EGFR receptor (7). The A loop contains a short alpha helix-like segment, while the B and C loops each contain a short antiparallel beta sheet structures. Crystal structure analysis showed that the B loop of EGF interacts hydrophobically with domain I of EGFR (8). The B loop fragment of EGF produced mitogenic and angiogenic effects like its parent molecule (9).

Due to its mitogenic activity, EGF has been used as a therapeutic reagent for skin wound regeneration (10). However, poor skin penetration of macromolecules with a molecular weight of more than 500 Da limits the use of EGF in topical treatments for enhancing wound healing (11). Here, we demonstrate that a tripeptide derived from the EGF B-loop enhances keratinocyte proliferation through activation of EGFR. Using analyses of intracellular signal protein interaction, EGFR activation studies, and in vitro proliferation assays, we show that the EGF-derived peptide can activate EGFR signaling pathway in keratinocytes with activity comparable to its parent EGF protein.

RESULTS

Identification of an EGF-derived agonist peptide Pep2-YAC

Because previous studies showed that the B loop region of EGF plays an important role in the activation of the EGFR signaling pathway (6), we performed homologous sequence analysis of the B loop region to identify EGFR agonist peptides. Based on a protein sequence homology search, we synthesized three EGFR agonist peptide candidates with molecular weights below 500 Da to examine their agonistic effects on EGFR activation (Fig. 1A). First, we investigated whether the three peptide candidates induced a physical interaction between EGFR and Grb2 or Gab1 and SHP2. HaCaT keratinocytes...
cytes were treated with three EGF-derived peptide candidates for 15 min and then the physical interaction of the signaling molecules was examined by an *in situ* proximity ligation assay (PLA). EGF itself was used as a positive control. Among peptide candidates, Pep2-YAC, a tripeptide covering residues 29-31 of EGF, enhanced the association of EGFR with Grb2 or Gab1 with SHP2 (Fig. 1B). Consistently, it stimulated keratinocyte proliferation with mitogenic activity comparable to EGF (Fig. 1C). From these data, we identified Pep2-YAC as an effective EGF agonist peptide.

**Pep2-YAC activated ERK and Akt signaling pathway and promoted cell proliferation through EGF**

EGF is known to promote the activation of ERK and Akt signaling pathway (12), which prompted us to examine whether Pep2-YAC induced phosphorylation of ERK1/2 and Akt1. As shown in Fig. 2A, treatment of HaCaT cells with Pep2-YAC showed phosphorylation of ERK1/2 and Akt1, dose-dependently. To confirm whether the effect of Pep2-YAC on phos-
An EGF-derived tripeptide activates EGFR
Yeon Ho Yoo, et al.

583
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Fig. 3. Pep2-YAC induces not only interaction of EGFR signaling molecules but nuclear translocation of transcription factors via EGFR.

(A) HaCaT cells were transfected with control siRNA (30 nM), or EGFR siRNA (30 nM) for 48 h and then treated with EGF (10 ng/ml) or Pep2-YAC (10 μM) for 15 min. After fixation, in situ PLA for Gab1-SHP2, Gab1-PI3K, and c-Jun-c-Fos were performed with specific antibodies. Nuclei were stained with DAPI (blue). Red spots represent physical interaction between the two indicated molecules. (C) HaCaT cells treated with Pep2-YAC, as with EGF, which was abolished, to control levels, in EGFR knockdown cells (Fig. 3B). Previous studies showed that EGFR activation results in the nuclear translocation of c-Jun, c-Fos, and STAT3 and transcription of target genes in cells with a high proliferative index (13). Notably, the nuclear interaction of c-Jun and c-Fos and nuclear translocation of STAT3 was observed in the HaCaT cells stimulated with Pep2-YAC, but not in EGFR knockdown cells (Fig. 3B, C).

Finally, we investigated whether Pep2-YAC induced the nuclear translocation of EGFR. Previous studies have shown that EGFR activation leads to its internalization and subsequent nuclear translocation, which regulates gene expression required for cell proliferation by interacting with STAT3 (14). Like EGF, Pep2-YAC induced the nuclear translocation of EGFR, suggesting that Pep2-YAC may promote cell proliferation through EGFR nuclear translocation (Fig. 3D). Collectively, our results demonstrated that Pep2-YAC has the ability to promote cell proliferation by activating the EGFR signaling pathway.

DISCUSSION

Here, we demonstrated that Pep2-YAC, an EGF-derived tripeptide, has a similar ability to EGF to activate EGFR. The treatment of HaCaT cells with Pep2-YAC induced phosphorylation of EGFR and organization of signaling complexes, which consist of Grb2, Gab1, SHP2, and PI3K. Furthermore, it enhanced phosphorylation of ERK1/2 and Akt1 and subsequent nuclear translocation of EGFR, STAT3, c-Jun, and c-Fos, which would promote keratinocyte proliferation.

Pep2-YAC was identified based on a sequence homology search. To search for EGFR agonist peptides, we focused on amino acid sequences in the EGF B loop region, which have

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been shown to play a key role in the activation of EGFR (6). Previous structural analyses showed that Met21, Ile23, and Leu26 in the B loop of EGF interact with EGFR (8). However, peptide mimics containing these residues inhibited EGF from interacting with its receptor (15). Additionally, these residues are not evolutionarily conserved, suggesting that these amino acid sequences may not be directly involved in the activation of EGFR. These observations caused us to focus on the B-sheet sequence (residues 19-23 and 28-32) in the B loop of EGF that contains relatively highly evolutionarily conserved regions (16). The evolutionary information in a multiple sequence alignment showed the conserved amino acid sequences covering the 19-21 and 29-32 region of EGF. Thus, we synthesized them as three EGFR agonist peptide candidates. Our data revealed that one of them, Pep2-YAC, was functionally significant in the activation of EGFR.

In the present report, we demonstrated that Pep2-YAC promoted keratinocyte proliferation by EGFR activation. Our in situ PLA analysis showed that Pep2-YAC increased the physical interaction between EGFR and Grb2, Gab1 and SHP2 or PI3K through EGFR. Binding of EGF to its receptor leads to receptor dimerization and the activation of an intrinsic tyrosine kinase (17). Autophosphorylated EGFR then recruits Gab1 through Grb2. Subsequently, Gab1 activates SHP2 (18) and the p85/p110 subtype of phosphoinositide 3-kinase (PI3K) on juxtamembrane EGFR (19, 20), which are important for EGFR-mediated biological responses. In an immunoblotting assay, Pep2-YAC phosphorylated EGFR, ERK1/2, and Akt1 through EGFR, suggesting that it induces the activation of major EGF/EGFR signaling pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway and phosphoinositide 3-kinase (PI3K)-Akt pathways (21, 22). Furthermore, Pep2-YAC, like its parent EGF, induced EGFR degradation, which plays an important role in the regulation of the EGFR-mediated ERK and Akt signaling pathway in pathological and physiological conditions (23).

Here, we showed that Pep2-YAC induced the nuclear translocation of EGFR and STAT3. EGFR signaling can be transmitted, via EGFR nuclear translocation, directly from the cytoplasmic membrane to the transcriptional targets in the nucleus. EGFR has transcriptional activity and is able to translocate into the nucleus, but requires a DNA-binding transcription cofactor for its transcriptional function, such as STAT3, due to its lack of a DNA-binding domain (24). STAT3 represents an essential effector pathway of Rho GTPases in regulating multiple cellular functions, including actin cytoskeleton reorganization, cell migration, gene activation, and proliferation (25), and transit from the cell membrane to the nuclear region in response to growth factor stimulation (26). Additionally, nuclear translocation of c-Jun/c-Fos and their interaction were increased by Pep2-YAC or EGF treatment. EGF activates different mitogen-activated protein kinases including ERK, JNK, and p38, which, in turn, activate c-Jun and c-Fos, leading to cell growth and differentiation (13). Consistent with these results, our proliferation assay showed Pep2-YAC significantly enhanced keratinocyte proliferation. Overall, our results demonstrated that Pep2-YAC can function as an EGFR agonist, and, therefore, may be useful as a therapeutic agent in skin regeneration and wound-healing.

**MATERIALS AND METHODS**

**Cell culture**

HaCaT keratinocytes were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, 5% penicillin/streptomycin and maintained at 37°C, 5% CO2.

**RNA interference**

Small-interfering RNAs (siRNAs) against human EGFR and a non-specific siRNA control were purchased from Santa Cruz Biotechnology. HaCaT cells were cultured to 50-60% confluence, and then transfected with the siRNA duplex using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s protocol. Non-specific siRNA was also transfected as a control.

**In situ PLA**

To determine changes in protein-protein interactions by EGFR signaling, we performed an in situ PLA assay. The in situ PLA studies on fixed HaCaT keratinocytes were performed as follows (O-LINK Bioscience, Upsalla, Sweden). EGFR or control siRNA transfected cells were grown on slide glasses for at least 16 h, and treated with EGF or EGF-pep for 15 min in 5% CO2 at 37°C and washed with PBS twice. In situ PLA detection was carried out using the appropriate DUOLINK II in situ kit components according to the manufacturer’s protocol. Briefly, cells were washed once with PBS, and then subjected to blocking using the DUOLINK blocking solution (1 drop) at 37°C in a wet chamber for 30 min. After tapping off the blocking solution from the slides, antibodies were added at a dilution of 1: 100 in 40 μl DUOLINK antibody diluent and incubated in a wet chamber at 37°C for 30 min. The slides were washed twice with wash buffer A for 5 min each, and then secondary antibodies (DUOLINK anti-rabbit PLA-plus probe, DUOLINK anti-goat PLA minus probe) were added and incubated at 37°C for 1 h. Two washes with wash buffer A were then followed by addition of the ligation mix and incubation at 37°C for 30 min, followed by another two washes. Then, the amplification reaction was carried out at 37°C for 100 min. Subsequently, the slides were washed twice with wash buffer B, and once with 0.1×wash buffer B. Mounting was done with mounting solution containing DAPI. Antibodies used for PLA were as follows: rabbit anti-EGFR Ab (Santa Cruz Biotechnology), mouse anti-Grb2 Ab (Santa Cruz Biotechnology), rabbit anti-Gab1 Ab (Cell signaling technology), mouse anti-SHP2 Ab (Santa Cruz Biotechnology), rabbit anti-Gab1 Ab (Cell Signaling Technology), mouse anti-PI3K p85 Ab (Santa Cruz Biotechnology), rab-
Independent experiments were repeated in triplicate. Data are expressed as the average of mean values obtained ± t-test. The statistical software GraphPad Prism (ver. 4.0). All experiments were conducted three times or more to ensure reproducible results. Representative data are shown in the figures.

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