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The Bioactivity and Receptor Affinity of Recombinant Tagged EGF Designed for Tissue Engineering Applications Is Defined by the Nature and Position of the Tags

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For tissue engineering applications, growth factor immobilization on cell culture scaffolds bears the potential to stimulate cell proliferation while minimizing costs associated to soluble growth factor supply. In order to evaluate the potential of a *de novo*–designed heterodimerization peptide pair, namely the E and K coils, for epidermal growth factor (EGF) grafting on various scaffolds, production of coil-tagged EGF chimeras using a mammalian cell expression system as well as their purification have been performed. The influence of the type of coil (E or K) upon EGF bioactivity, assessed in an *in vitro* cell assay, was compared to that of the fragment crystallizable (Fc) domain of immunoglobulin G by monitoring phosphorylation of EGF receptor (EGFR) upon chimeric EGF exposure. Our results demonstrate that the type and the location of the tag have a strong impact on growth factor bioactivity (EC_{50} ranging from 5.5 to 63 nM). Additional surface plasmon resonance-based biosensor experiments were conducted to test the ability of captured chimeric EGF to bind to their receptor ectodomain *in vitro*. These experiments indicated that the oriented coiled-coil–mediated immobilization of EGF was significantly more efficient than a random approach as coil-tagged EGF displayed enhanced affinities for artificially dimerized EGFR ectodomain when compared to Fc-tagged EGF (apparent K_D of 5 pM vs. 16 nM). Altogether, our results highly suggest that coil-tagged chimeras represent an attractive avenue for the oriented immobilization of growth factors for tissue engineering applications and that HEK293 cells offer a robust platform for their expression in a bioactive form.

Introduction

Bioengineered implants are becoming extremely promising with respect to the development of biocompatible scaffolds to promote and sustain tissue regeneration.[1] Based on the well-documented effect of growth factors on cell survival, proliferation, differentiation, and chemotaxis, several research groups have developed methodologies to immobilize them on solid supports with the ultimate goal of modulating cell growth and phenotype.[2–4] Indeed, immobilization of epidermal growth factor (EGF), nerve growth factor,[5] or vascular endothelial growth factor[6] onto different supports (gold, polydimethylsiloxane, glass, and polystyrene)[7–12] has already been reported. Two different approaches are commonly applied for protein grafting, that is, nonoriented or oriented immobilization. The nonoriented immobilization strategies are based on (i) the interactions between a charged scaffold and the protein[13] or (ii) the formation of covalent linkage between the growth factor and target scaffold using bifunctional reactive chemicals as well as by photoimmobilization.[2,7,10,12,14] Although unambiguous effects on cell growth and phenotype have been reported with grafted ligands, it is likely that these immobilization methods are not optimal. That is, the surface grafting is often mediated in a random fashion by using reactive groups present on ligand amino acid side chains. The multiplicity of reactive groups results in heterogeneous ligand populations whose interactions with cell receptors may be affected by the tethering procedure and therefore this approach is poorly reproducible and inefficient. Up to now, only few methods have described the oriented immobilization of growth factors. These methods are based on the use of different tags (such as Fc or polyhistidine) that offer the potential to be applied to both purification and tethering steps.[6,9,11] More recently, based on naturally occurring coiled-coil structures, a *de novo* heterodimeric coiled-coil system formed

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by two peptides (the E and K coils) was designed and characterized.[15,16] The E/K coiled-coil system has been demonstrated to be an excellent tool for various biotechnology and biomedical applications, such as protein capture and delivery[17]; each peptide composing the E/K complex being defined by a seven-amino-acid sequence comprising both hydrophobic and charged residues that are repeated five times.[15]

EGF plays various important roles in the organism and is indeed a growth factor commonly used in tissue engineering applications.[18] EGF is synthesized as a 130 kDa precursor that is then proteolytically processed to give a mature 53-amino-acid-long polypeptide of 6 kDa[19,20] that contains three disulfide bridges. EGF mediates its biological function by promoting the oligomerization of EGF receptors (EGFRs) present at the cell surface.[21,22] Ligand-mediated receptor oligomerization initiates complex cascades of intracellular events resulting in a broad spectrum of phenotypic responses such as the proliferation and differentiation of epithelial or mesenchymal cells. Its role in neovascularization and chemotaxis in wound healing processes has also been demonstrated.[23]

In order to evaluate the potential of the E/K coiled-coil system for EGF grafting on various scaffolds and surfaces used in tissue engineering applications, production of coil-tagged EGF chimeras using a mammalian cell expression system as well as their purification and characterization have been performed. The influence of the type of coil (E or K) on EGF bioactivity was compared to that of the Fc domain of immunoglobulin G (IgG). We showed that the type and the location of the tag have a strong impact on growth factor bioactivity. Additional surface plasmon resonance (SPR)–based biosensor experiments indicated that the oriented coiled-coil–mediated capture of EGF was significantly more efficient than a random approach. Altogether, our results highly suggest that coil-tagged chimeras represent an attractive avenue for the oriented immobilization of growth factors for tissue engineering applications and that human embryonic kidney cells (HEK293) offer a robust platform for their expression in a bioactive form.

Materials and Methods

Chemicals and reagents

Chemicals and reagent used are linear 25 kDa polyethylenimine (PEI; Polysciences, Warrington, PA), prepared as previously described[24]; Salmon testes DNA (stDNA) and pluronic acid F-68 (F68) (Sigma-Aldrich, Oakville, ON); TN1 peptone (OrganoTechnie SA, Teknisciences, Canada); geneticin (Gibco BRL, Burlington, ON).

All the SPR experiments were performed using a Biacore 3000 and Biacore T100 biosensors. The thiol coupling kit containing N-hydroxysuccinimide (NHS), ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-(2-pyridyldithio) ethaneamine (PDEA), and CM4 sensor chips was purchased from Biacore (Piscataway, NJ).

Cells

A human embryonic kidney 293 cell line stably expressing EBNA1 (293-6E) was maintained in suspension culture in shake flasks (110 rpm) in F17 medium (Invitrogen, Burlington, ON) supplemented with geneticin (25 μg/mL), glutamine (4 mM), and pluronic acid (0.1%) in a humidified incubator at 37°C with 5% CO_2. The A-431 cells were maintained in 175 cm^2 flasks in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Invitrogen).

Plasmids

All the vectors were derived from the pTT plasmid previously described.[25] Four constructs (Fig. 1) were used to express the EGF cDNA and tags (Ecoil, Kcoil, and Fc). A codon-optimized human EGF cDNA containing an N-terminal interleukin 2 signal peptide (IL2SP) was synthesized by GeneArt (Regensburg, Germany) and cloned in pTT vector as a *NotI–BamHI* fragments.

Plasmid encoding Fc tag. pTT5-EGF was digested with *NheI* and *BamHI*; EGF cDNA was inserted into pYD5 that encoded the human IgG signal peptide (IgGSP) and Fc (Fig. 1). pYD11-EGF-Fc was constructed by PCR amplification of

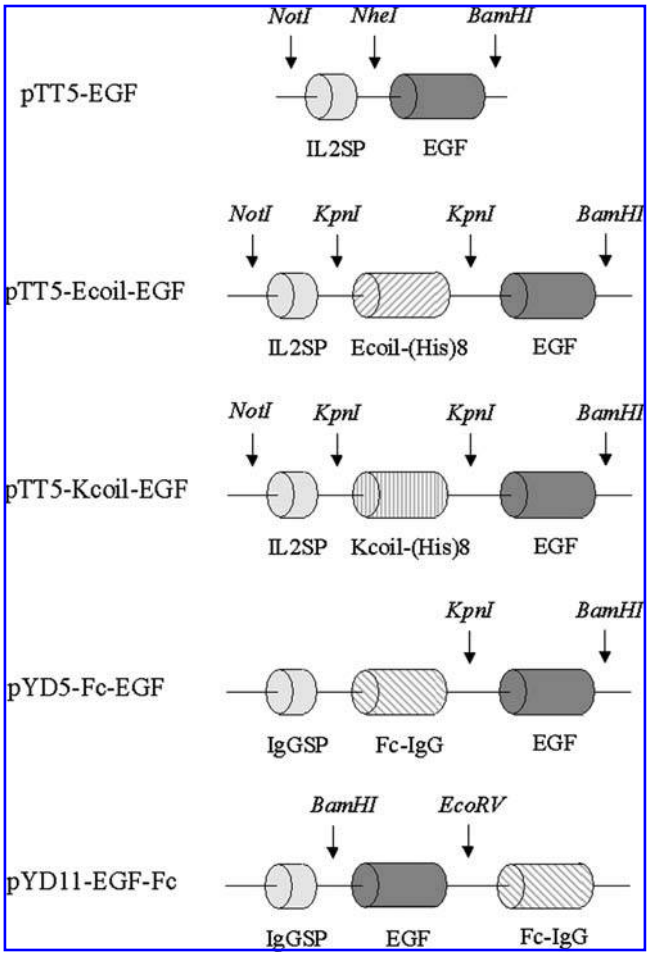

FIG. 1. Expression plasmids for the production of the various tagged EGFs. Each plasmid encodes human EGF cDNA tagged with Ecoil and His-tag (pTT5-Ecoil-EGF plasmid), or with Kcoil and His-tag (pTT5-Kcoil-EGF plasmid) or with the Fc portion of an IgG (pYD5-Fc-EGF and pYD11-EGF-Fc plasmids).

EGF cDNA with forward (5′-AAAGGATCCATGAATTCT GACAGCGAGTGCCC-3′) and reverse (5′-AAATCTAGAT CTCAGCTCCCACCACTTCAG-3′) primers. The PCR product was digested by *BamHI* and cloned in frame with pYD11 digested with *BamHI* and *EcoRV* (Fig. 1).

Plasmids encoding Ecoil or Kcoil tag. pTT5-E/Kcoil-EGF were constructed by PCR amplification of the Ecoil or Kcoil sequences with forward (5′-AAAGGTACCATGGAGGTA TCCGCTTTAGAGAAAG-3′ for Ecoil sequence and 5′-AAA GGTACCAAGGTATCCGCTTTAAAGGAG-3′ for Kcoil sequence) and reverse (5′-AAAGGTACCATGGTGATGGTGA TGGTGATGATGCAATTCAGAGCCACCGCCACCGCTGC C-3′) primers. The PCR products were digested by *KpnI* and cloned in frame with pTT5-EGF digested with the same enzyme (Fig. 1).

Plasmids were amplified in DH5α *Escherichia coli* grown in Circlegrow medium (Q-Biogene, Carlsbad, ON) and purified using Maxi-Prep kits (Qiagen, Mississauga, ON). Plasmids were quantified following dilution in 50 mM Tris-HCl, pH 8.0, by measuring absorbance at 260 nm. The *A*260/*A*280 ratio was between 1.80 and 1.95 for all plasmids used for transient transfection.

Transfection

To express the various tagged EGF, 293-6E cells were first distributed in 6-well plates at 1.67×10^6 cells/mL in 1.8 mL medium. The transfection mixture was then prepared as follows: 2 μg plasmids (95% pTT vector and 5% pTT-GFPq) were diluted in 100 μL F17 medium, and 100 μL F17 medium containing 4 μg of 25 kDa linear PEI was added. The mixture was immediately vortexed and incubated for 15 min at room temperature prior to addition to the cells. Cells were fed 24 hours posttransfection (hpt) with 0.5% TN1 peptone (w/v, final).[24] The supernatants were harvested 5 days posttransfection.

Purification

Production was performed in shake flasks. Each cell culture supernatant (500 mL) was harvested 120 hpt and centrifuged to eliminate cells and debris. The supernatants were then filtered on 0.45-μm filters (Millipore, Mississauga, ON). Ecoil- and Kcoil-tagged EGF were purified by immobilized metal ion affinity chromatography (IMAC) based on the interaction existing between metallic ion and the $(His)_8$ tag (Fig. 1), whereas N- and C-terminally Fc-tagged EGF were purified by affinity chromatography based on the interaction existing between the Fc portion from IgG and protein A.

E/Kcoil-tagged EGF. Fractogel EMD Chelate gravity flow columns (5 mL bed size) were charged successively with four column volumes (CVs) of 200 mM $CoCl_2$, five CVs of water, and five CVs of PBS. After E/Kcoil-EGF loading, the columns were washed with 10 CVs of 50 mM NaH_2PO_4, pH 7.0; 300 mM NaCl; and 25 mM imidazole, pH 7.0. E/Kcoil-EGF were eluted in 1 mL fractions using the same buffer but containing 300 mM imidazole.

Fc-tagged EGF. The filtered supernatants were loaded onto a 2 mL protein A column (GE Healthcare, Piscataway, NJ). The columns were washed with 10 CVs of PBS. The Fc-tagged EGF was then eluted with 0.1 M citrate of pH 3.6 in 1 mL fractions.

The protein-containing fractions were detected with Bradford reagent, pooled, and desalted on Econo-Pac® 10 columns (Bio-Rad Laboratories, Mississauga, ON) previously equilibrated with PBS according to the manufacturer's specifications and quantified by Bradford. To assess the purity level, proteins were analyzed by SDS-PAGE (4–12% NuPAGE Bis-Tris gradient gel) followed by Coomassie staining.

In vitro *cell assay*

Bioactivity of the purified constructs was investigated by testing EGF-induced autophosphorylation of EGFR in A-431 cells. Consequently, A-431 cells were distributed in 6-well plates at 0.5×10^6 cells/mL, 2 mL/well. Eighteen hours later, the cells (80–90% confluence) were treated with tagged EGF (final concentrations ranging from 0 to 1 μM) for 5 min in a humidified incubator at 37°C with 5% CO_2. Cells were then washed twice with PBS supplemented with sodium-orthovanadate (1 mM; Sigma) and lysed (lysis buffer supplemented with 0.1 mM Na_3VO_4, 20 mM β-glycerophosphate, 10 mM $Na_4P_2O_7\cdot10\ H_2O$, and 0.5 μM microcystin). Commercially available untagged EGF (R&D Systems, Minneapolis, MN) was used as a positive control.

SDS–PAGE and Western blot analyses

Cells from one well of a 6-well plate were lysed in 300 μL of lysis buffer (50 mM HEPES of pH 7.4, 150 mM NaCl, 1% Thesit, and 0.5% sodium deoxycholate), and insoluble material was removed by centrifugation at $10{,}000\times g$ for 5 min at 4°C. Samples (cell lysates or culture medium) were mixed 3:1 (v:v) with 4× NuPage sample buffer (Invitrogen) containing 50 mM DTT (for EGFR1) and heated to 70°C for 10 min. Electrophoresis was performed using 4–12% Bis-Tris NuPage gels (Invitrogen) at 200 V in MES SDS (tagged EGF) or MOPS SDS buffer (EGFR1). Mark12 MW standard (Invitrogen) was applied to each gel to determine molecular weights. Protein purity was determined by staining with Coomassie blue R250. For Western blots, proteins were transferred to a 0.2 μm Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using Tris-glycine buffer for 1 h at 300 mA. The membrane was incubated in blocking reagent (Roche Diagnostics, Indianapolis, IN), and then probed with appropriate antibodies against EGF (Z-12) or phosphotyrosine (PY99) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Detection was performed using BM Chemiluminescence Blotting Substrate (Roche Diagnostics) with a Kodak Digital Science Image Station 440cf equipped with Kodak Digital Science 1D image analysis software version 3.0 (Eastman Kodak, New York, NY).

SPR assay

The affinity of the various tagged EGF for the extracellular domain of EGFR artificially dimerized through the Fc portion of an IgG (EGFRED-Fc; R&D Systems) was studied by SPR.

SPR experiments were conducted at 25°C using HBS-T (10 mM HEPES of pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.05% Tween 20) for sample dilution and as running buffer. Cysteine-tagged Kcoil and Ecoil peptides were

independently and covalently immobilized onto CM4 sensor chip surfaces using standard ligand thiol coupling procedure as previously described.[17] In addition to coil surfaces, mock surfaces were prepared using the same protocol by replacing coil injections with running buffer injections.

N- and C-terminally Fc-tagged EGF were covalently immobilized onto CM5 sensor chip surfaces adapting standard amine coupling procedure as previously described,[26] and mock surfaces were prepared using the same protocol by replacing Fc-tagged EGF injections by running buffer injections.

EGFRED-Fc binding to covalently immobilized Fc-tagged EGF. Experiments were carried out at a flow rate of 50 μL/min. Artificially dimerized EGFR ectodomain (EGFRED-Fc) was then injected at concentrations ranging from 5 to 80 nM (in addition to HBS-EP buffer injections), in duplicate over EGF and control surfaces. Regeneration of the sensor chip surfaces was accomplished by HBS-EP (10 mM HEPES, pH 6.0; 150 mM NaCl; 3.0 mM EDTA; and 0.05% surfactant P20) buffer injection until original baseline levels were reached.

EGFRED-Fc binding to captured coil-tagged EGF. Experiments were carried out at a flow rate of 50 μL/min. Coil-tagged EGF was captured (100 Resonance Units, RUs) on surfaces on which their coil partner had been previously immobilized. After EGF capture via coiled-coil interactions, the artificially dimerized EGFRED-Fc (R&D Systems) was then injected over coil-tagged EGF and control surfaces (60 s). Regeneration of the sensor chip (i.e., removal of both EGFRED-Fc and coil-tagged EGF) was accomplished by two pulses of guanidium-HCl (25 μL at 100 μL/min). This cycle was repeated by capturing the same amount of coil-tagged EGF, followed by injection of the artificially dimerized EGFR ectodomain at concentrations ranging from 1.25 to 50 nM (duplicate), in addition to buffer. For coil-tagged EGF interactions with the artificially dimerized EGFRED-Fc, sensorgrams were globally analyzed using a simple binding model including a mass transport limitation step, available in the Biaevaluation 4.1 software package of the biosensors.

Results

Cloning strategies and production of tagged EGF in HEK293 cells

Plasmids corresponding to EGF tagged with (i) the Ecoil or Kcoil sequences added to the N-terminus of EGF (denoted Ecoil- and Kcoil-EGF, respectively) or with (ii) the Fc portion from IgG added to the N- or C-terminus of EGF (denoted Fc-EGF and EGF-Fc, respectively) were generated (Fig. 1). In order to ease subsequent purification, constructs were designed to favor chimeric protein secretion by adding sequences corresponding to signal peptide of the interleukin-2 (IL2SP, Fig. 1) or of IgG (IgGSP, Fig. 1).

293-6E cells were then transfected in 6-well plates (2 mL) with each expression vector as described in "Materials and Methods" section. One day posttransfection, TN1 tryptone was added to the cell medium in order to increase protein productivity.[24] Western blot analyses were performed to verify expression. EGF chimeras were found to be efficiently secreted (Fig. 2, compare signal intensity for cell supernatants vs. related cell lysates).

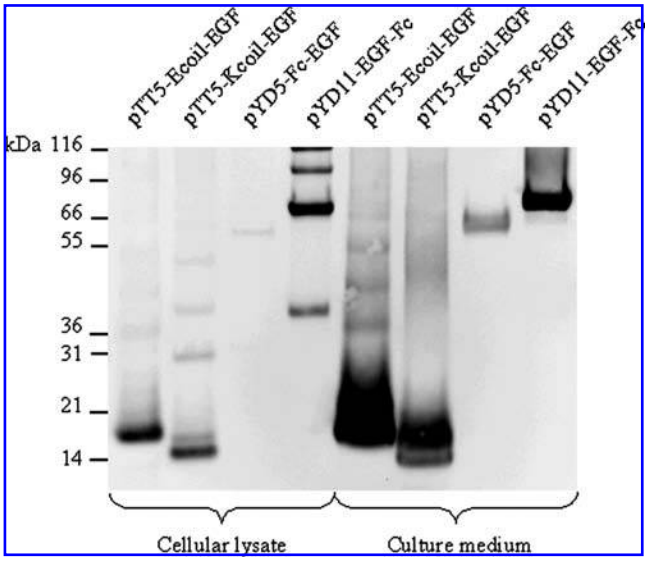

FIG. 2. Small-scale expression of tagged EGF in 293-6E cells. Presence of the various EGF fusion proteins was tested in the culture medium and cell lysate 5 days posttransfection by Western blotting. The same volume (20 μL) of cell extract (total 300 μL) and culture medium (total 2 mL) was loaded in each lane.

Purification of various tagged EGF

For each construct, production was then performed in 500-mL flasks in serum-free medium to facilitate subsequent purification. Due to the presence of the $(His)_8$ tag, Ecoil-EGF and Kcoil-EGF were purified by immobilized metal affinity chromatography (IMAC). Fc-EGF and EGF-Fc were purified by protein A affinity chromatography. Results for the purification of the different tagged EGF are shown in Figure 3. The purification was efficient for each construct with a yield of 4.0, 0.6, 7.2, and 4.3 mg per liter of medium for Ecoil-EGF, Kcoil-EGF, Fc-EGF, and EGF-Fc, respectively.

In vitro *cell assays*

To test the bioactivity of the different fusion proteins corresponding to tagged EGF, we evaluated their ability to induce EGFR phosphorylation upon EGF binding in an *in vitro* cell assay. Increasing quantities of purified EGF constructs were added to A-431 cells that are known to express high levels of EGFR.[27] Cells were lysed 5 min after EGF addition, and the phosphorylation level of EGFR was quantified by Western blot using antiphosphotyrosine antibody (Fig. 4A). Ecoil-EGF, Kcoil-EGF, and EGF-Fc efficiently induced EGFR phosphorylation, whereas no phosphorylation was observed for Fc-EGF (Fig. 4B). Ecoil-EGF was found to be as active as untagged EGF (EC_{50} of 5.5 and 4.3 nM, respectively), while EGF-Fc and Kcoil-EGF had EC_{50} of 17 and 63 nM, respectively (Fig. 4B).

Binding of the artificially dimerized EGFRED-Fc to immobilized tagged EGF

In order to complement our *in vitro* cell assay results, the interactions of both N- and C-terminally Fc-tagged EGF

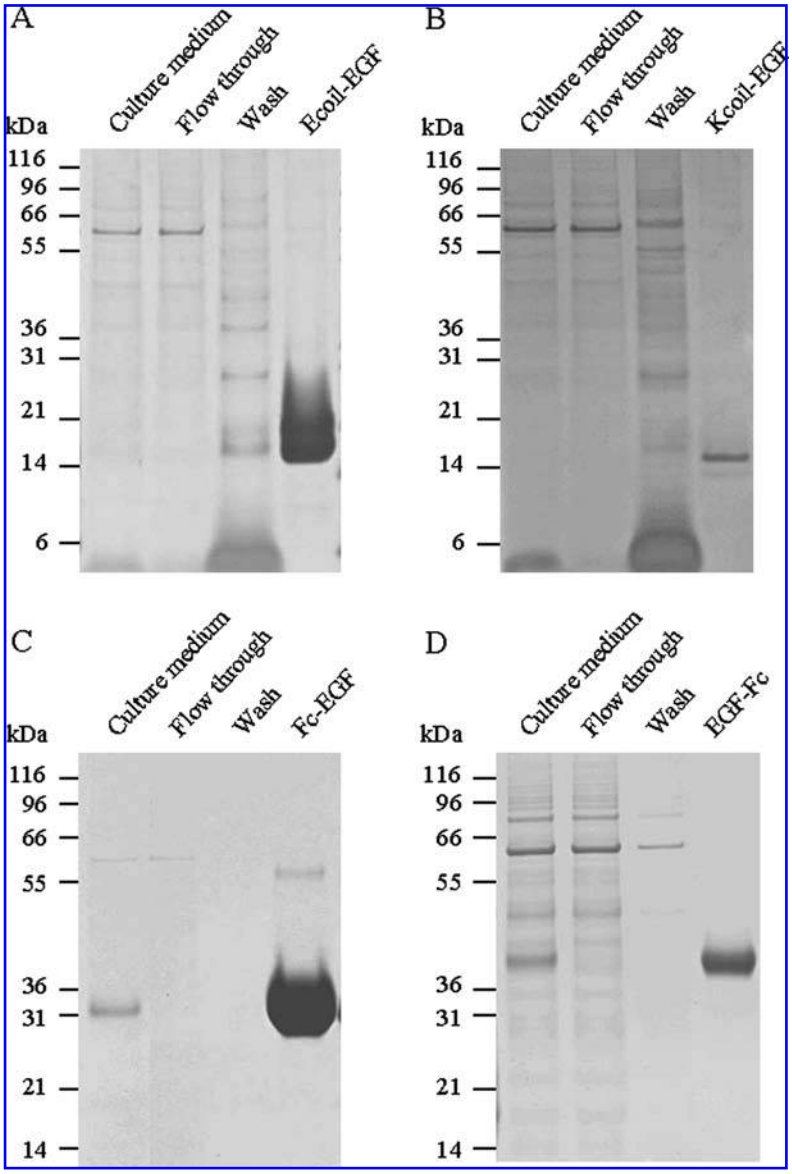

FIG. 3. Production and purification of tagged EGFs. SDS-PAGE analysis of Ecoil-EGF (**A**), Kcoil-EGF (**B**), Fc-EGF (**C**), and EGF-Fc (**D**) purified by affinity chromatography as described in the "Materials and Methods" section. The gel was stained with Coomassie blue R250.

with an artificially dimerized version of the extracellular domain of EGFR (EGFRED-Fc) were then studied in real time by SPR. EGF-Fc and Fc-EGF were covalently immobilized (2800 and 3900 RUs, respectively) onto different biosensor surfaces through their amine groups in a non-oriented fashion. Several concentrations of the artificially dimerized EGFRED-Fc ranging from 5 to 80 nM were then injected in duplicate over both C- and N-terminally tagged EGFs as well as over related control surfaces. Control-corrected sensorgrams related to these series of experiments are shown in Figure 5A and B, respectively. In the case of EGF-Fc (Fig. 5A), the receptor ectodomain binding was detected at concentrations as low as 5 nM. Also, at the biosensor surface, the receptor ectodomain/EGF-Fc complex was found to be stable. That is, a global analysis of the corrected sensorgrams shown in Figure 5A indicated that the interaction was characterized by an apparent kinetic dissociation constant of $1.11 \pm 0.01 \times 10^{-4}\,s^{-1}$. The apparent thermodynamic dissociation constant, K_D, for EGFRED-Fc/EGF-Fc interactions was in the low nanomolar range (16 ± 0.5 nM).

In contrast, when EGFRED-Fc injections were performed over N-terminally immobilized Fc-tagged EGF, a weaker signal was recorded with Fc-EGF as compared to EGF-Fc (3 RUs vs. 28 RUs with 80 nM EGFRED-Fc, compare Fig. 5A and B). Numerical analysis of this set of sensorgrams was not possible as a visual inspection of the EGFRED-Fc/Fc-EGF binding clearly indicated that the interactions were transient, that is, characterized by extremely fast dissociation rates and very low affinity ($K_D >> 100$ nM).

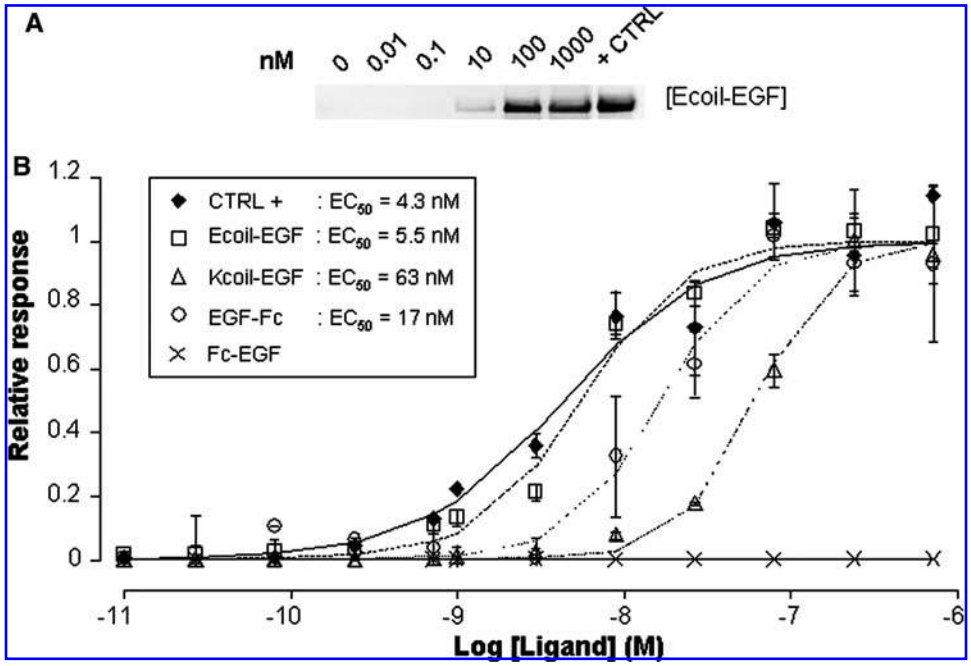

FIG. 4. *In vitro* cell assays. A-431 cells were treated with various concentrations of untagged (control) or tagged EGF ranging from 0 to 1 μM for 5 min. Cells were then lysed and autophosphorylation of EGFR was monitored by Western blot using a chemiluminescent substrate (**A**). The 180 kDa band immunoreactive to anti-pTyr antibody (corresponding to EGFR) was quantified using a Kodak 440cf imager ($n = 3$ for commercial EGF and Ecoil-EGF and $n = 2$ for Kcoil-EGF and Fc-tagged EGF) (**B**).

Binding of the artificially dimerized extracellular domain of EGFR, EGFRED-Fc, to immobilized coil-tagged EGF

Our SPR kinetic study was complemented by monitoring the interactions of the artificially dimerized EGFRED-Fc with surface-immobilized coil-tagged EGF. As opposed to the coupling strategy we used in the case of Fc-tagged EGF, coil-tagged EGFs were noncovalently captured at the biosensor surface in an oriented fashion. This was achieved through coiled-coil interactions. In a first step, E and K coil peptides harboring an engineered cysteine at their N-termini were covalently immobilized using thiol chemistry over different biosensor surfaces (400 RUs of each coil). Injections of coil-tagged EGF over surfaces on which the appropriate coil partner had been immobilized resulted in its noncovalent but stable capture (Fig. 6). Interactions of captured EGF with the artificially dimerized EGFRED-Fc were then monitored by injecting the latter over coil-tagged EGF and related mock surfaces.

The control-corrected sensorgrams corresponding to ectodomain injections (concentrations ranging from 0.25 to 10 nM) over 100 RUs of captured Ecoil-EGF and Kcoil-EGF were similar (Fig. 6). However, the amplitude of the signal corresponding to ectodomain net accumulation at the end of the injection for the highest concentration differed by 20% (410 vs. 360 RUs of accumulated material over captured Ecoil-EGF and Kcoil-EGF, respectively). Of interest, both interactions were characterized by similar fast on-rates and extremely low off-rates (Table 1), resulting in almost equal apparent K_D (44 ± 5 and 55 ± 7 pM for Ecoil-EGF and Kcoil-EGF, respectively, see Table 1).

Discussion

Production of EGF in *E. coli* had been previously reported to lead to high final concentration in EGF but mainly present in inclusion bodies (87 mg/L of culture medium[28]). The restoration of EGF bioactivity was, however, reported to require labor-intensive and time-consuming refolding steps.[28] EGF expression in mammalian systems has also been reported by Mroczkowski *et al.* in NIH 3T3 cells.[20,29] In that

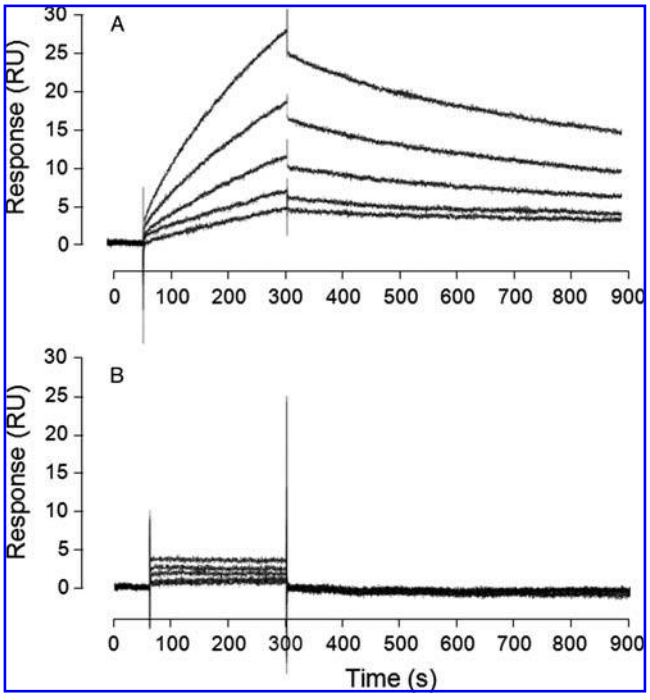

FIG. 5. Control-corrected sensorgrams corresponding to Fc-tagged EGF interactions with EGFRED-Fc. Increasing concentrations of EGFRED-Fc ranging from 5 to 80 nM were injected in duplicate over biosensor surfaces coated with EGF-Fc (**A**) or Fc-EGF (**B**).

Table 1. Kinetic and Thermodynamic Parameters Corresponding to the Interactions of EGFRED-Fc with Captured Ecoil-EGF and Kcoil-EGF Shown in Figure 6

Parameters	*Ecoil-EGF*	*Kcoil-EGF*
k_{on} (M^{-1} s^{-1})	$(0.745 \pm 0.003) \times 10^6$	$(1.19 \pm 0.05) \times 10^6$
k_{off} (s^{-1})	$(3.3 \pm 0.3) \times 10^{-5}$	$(6.5 \pm 0.4) \times 10^{-5}$
K_D (pM)	44 ± 5	55 ± 7

Values correspond to average value ± the standard deviation of two independent experiments.

specific case, however, EGF was expressed in its precursor form and was found as membrane-associated as well as in the conditioned medium.

In order to obtain fully bioactive tagged EGF (for subsequent immobilization on scaffolds) as well as to avoid downstream processing steps, such as refolding and precursor cleavage, we thus set up an experimental strategy aiming at secreting high levels of bioactive tagged EGF. The latter is based on the use of HEK293 cells, combined with PEI-mediated transfection, as we previously reported excellent yields for the expression of various bioactive proteins using this approach.[24]

Expression vectors coding for various versions of tagged EGF were thus generated using mature EGF cDNA. Two types of tags were also selected, based on their potential use for peptide or protein immobilization in the field of tissue engineering. Those correspond to (i) *de novo*–designed coils (Ecoil or Kcoil)[15] since we already demonstrated that the E/K coiled-coil system is efficient for protein capture on three-dimensional surfaces[17] and since functionalization of hydrogels with a similar peptide-based heterodimeric system had been recently reported[30] and (ii) the Fc portion of IgG.[11] For each version of tagged EGF, expression vectors were designed in order to allow efficient secretion. This was achieved by inserting cDNA coding for interleukin-2 or IgG signal peptides upstream of the EGF cDNA (IL2SP and IgGSP, Fig. 1). Our approach was validated as we found that both IL2SP and IgGSP efficiently promoted secretion of coil-tagged and Fc-tagged EGF chimera, respectively (Fig. 2). After production (500-mL scale), EGF chimeras present in conditioned serum-free media were purified by a single affinity purification step (Fig. 3), yielding 0.6 to 7.2 mg of highly pure tagged EGF per liter of medium. In the case of Fc-tagged EGF chimera, the capacity of Fc-tagged protein to form covalent dimer[31] was also confirmed by Western blotting and Coomassie blue staining detection performed after SDS-PAGE in nonreducing and reducing conditions, respectively (Figs. 2 and 3).

The bioactivity of each EGF fusion protein was then evaluated in an *in vitro* cell assay examining their ability, when in solution, to promote phosphorylation of EGFR present at the surface of A-431 cells. *In vitro* cell assay results unambiguously demonstrated that purified Ecoil-EGF was as active as our positive control (i.e., untagged commercially available EGF, Fig. 4), as both untagged and Ecoil-tagged EGF were characterized by similar EC_{50} in that assay (4.3 and 5.5 nM, respectively). In contrast, Fc-EGF was not able to promote phosphorylation in the same assay. This finding is in excellent agreement with our SPR results, indicating that captured Fc-EGF was not able to bind efficiently to EGFR ectodomain (Fig. 5B) while EGF-Fc was (Fig. 5A). The other tagged versions of EGF were all characterized by higher EC_{50} values than that of Ecoil-EGF (17 and 63 nM for EGF-Fc and Kcoil-EGF, respectively), indicating nonoptimal bioactivity of the EGF portion of these fusion proteins (Fig. 4). Thus, the type of tag (Ecoil, Kcoil, or Fc) as well as its location within the chimera (N- or C-terminal) has a strong influence on the bioactivity of the latter. Such a negative impact on bioactivity may be attributed to the high pI (10.36) of the Kcoil moiety that may interact nonspecifically with cell-surface heparan sulphate proteoglycans, thus reducing its effective concentration in the medium. Neutralization of the Kcoil with free Ecoil prior to its addition to the cells should be performed to confirm this hypothesis.

Finally, additional SPR experiments demonstrated the usefulness of the E/K coiled-coil system in tissue engineering for achieving optimal ligand tethering (Fig. 6) as both Ecoil- and Kcoil-EGF, when captured to biosensor surfaces via coiled-coil interactions, were demonstrated to bind to the artificially dimerized extracellular domain of EGFR in a

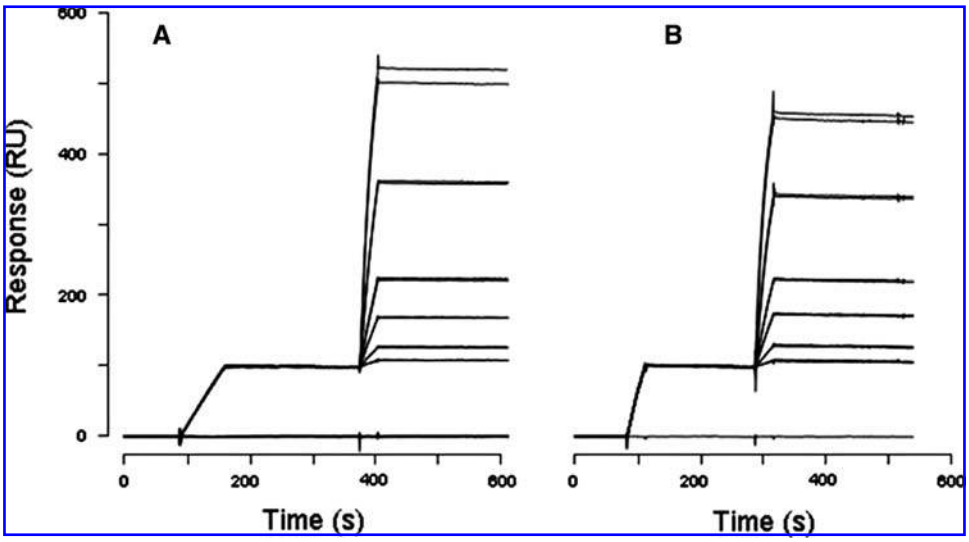

FIG. 6. Control-corrected sensorgrams corresponding to EGFRED-Fc interactions with captured E or Kcoil-EGF. Increasing concentrations of EGFRED-Fc ranging from 0.25 to 10 nM were injected in duplicate over 100 RUs of Ecoil-EGF (**A**) or Kcoil-EGF (**B**) previously captured to biosensor surfaces via coiled-coil interactions.

highly stable fashion (dissociation rates of 3.3×10^{-5} and $6.5\times10^{-5}\,s^{-1}$, Table 1) with strong apparent affinities (K_D of approx. 50 pM, Table 1). Of interest, a comparison of EGFR ectodomain interactions with oriented Kcoil-EGF (Fig. 6A) to those with EGF-Fc (Fig. 5A) further highlights the benefit of an oriented capture approach of EGF. That is, even though higher molar amounts of EGF-Fc were immobilized for SPR experiments (3900 RUs of immobilized EGF-Fc corresponds to $40\times10^{-15}\,mol/mm^2$ while 100 RUs of Kcoil-EGF corresponds to $6\times10^{-15}\,mol/mm^2$) and even though EGF-Fc was found to be more active *in vitro* (see Fig. 4), injections of the EGFR moiety resulted in a higher accumulation and in the formation of a more stable EGFR complex on the Kcoil-EGF surface (optimized capture) versus EGF-Fc surface (random coupling) (compare accumulation and stability in Figs. 5A and 6A).

In conclusion, the present study highly suggests that combining the Ecoil tag to an efficient mammalian expression system (transient gene expression in HEK293 cells cultured in serum-free medium) allows for the rapid production, purification, and efficient capture of bioactive EGF that can be immobilized onto various Kcoil supports. Further, our strategy can be readily applied to other growth factors. In that respect, the use of the Ecoil tag would significantly ease the grafting of any growth factor since only one type of support preparation (e.g., Kcoil functionalization) would be compatible with any Ecoil-tagged protein. This concept sharply contrasts with current approaches relying on direct chemical coupling, which is a trial-and-error process dependent on the physiochemical properties of each individual protein.

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