Escherichia coli expression and refolding of E/K-coil-tagged EGF generates fully bioactive EGF for diverse applications

Le, Phuong U.; Lenferink, Anne E. G.; Pinard, Maxime; Baardsnes, Jason; Massie, Bernard; O'Connor-McCourt, Maureen D.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.pep.2008.11.005
Protein Expression and Purification, 64, 2, pp. 108-117, 2008-11-25

Protein Expression and Purification 64 (2009) 108–117

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Escherichia coli expression and refolding of E/K-coil-tagged EGF generates fully bioactive EGF for diverse applications

Phuong U. Le [a], Anne E.G. Lenferink [a,*], Maxime Pinard [a,c], Jason Baardsnes [a], Bernard Massie [a,c], Maureen D. O'Connor-McCourt [a,b]

[a] Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montréal, Québec, Canada H4P 2R2
[b] Department of Biochemistry, McGill University, Montréal, Québec, Canada H3G 1Y6
[c] Department of Microbiology and Immunology, Université de Montréal, Québec, Canada H3T 1J4

ARTICLE INFO

Article history:
Received 13 May 2008
and in revised form 3 November 2008
Available online 25 November 2008

Keywords:
EGF
E/K-coil
Refolding
Inclusion bodies
Tissue engineering

ABSTRACT

Heterodimerizing peptides, such as the *de novo* designed E5/K5 peptide pair, have several applications including as tags for protein purification or immobilization. Recently, we demonstrated that E5-tagged epidermal growth factor (EGF), when bound to a K4 expressing adenovirus, promotes retargeting of the adenovirus to EGFR expressing target cells. In this study, we present the *Escherichia coli* expression, refolding and purification of human EGF fused with the E5-coil (E5-coil-EGF) or with the K5-coil (K5-coil-EGF). EGF receptor phosphorylation and cell proliferation assays demonstrated that the biological activity of the coil-tagged EGF versions was comparable to that of non-tagged EGF. Additionally, analysis of the binding of E5/K5-coil-EGF to cell surface EGFR or to soluble EGFR ectodomain, as measured by cell-based binding competition assays and by SPR-based biosensor experiments, indicated that the coil-tagged EGF versions bound to EGFR with affinities similar to that of non-tagged EGF. Finally, we show that E-coil-tagged EGF, but not non-tagged EGF, can retarget a K-coil containing adenovirus to EGF receptor expressing glioblastoma tumor cells. Overall these results indicate that *E. coli* expression offers a practical platform for the reproducible production of fully biologically active E5/K5-coil-tagged EGF, and support applications of heterodimerizing coil-tagged ligands, e.g. the targeting of viruses or other entities such as nanoparticles to tumor cells, or growth factor immobilization on cell culture scaffolds for tissue engineering.

Epidermal growth factor (EGF)[1] is a 53 amino acid long polypeptide growth factor, which contains a conserved six-cysteine residue motif that is characteristic of the EGF-domain. Many reports have shown that EGF plays a key role in the regulation of cell proliferation, differentiation and migration [1]. EGF binds and activates the epidermal growth factor receptor (EGFR), a single transmembrane domain glycoprotein, which has frequently been implicated in various types of cancer. There are various mechanisms by which the EGFR can become oncogenic, e.g. by the presence of auto/paracrine ligand loops, mutations that render the receptor active, or the failure to attenuate signaling through receptor down regulation (e.g. through heterodimerization of EGFR with the orphan ErbB2 receptor). In most cancer types, it is the overexpression and/or amplification of the EGFR gene that prevails.

Because of its role in tumor development, the EGFR has been studied intensively as a therapeutic target. Currently, the two main types of therapeutics that are being applied are antibodies that bind to the EGFR extracellular domain, and small-molecule inhibitors that target its intracellular tyrosine kinase domain [2]. While efficient inhibition of EGFR with these drugs can be observed, evidence of resistance to these drugs has been described [3]. Therefore, the development of other EGFR targeting strategies is required. Targeted adenovirus-based gene therapy can potentially be used as an effective treatment for cancer. The application of adenovirus in cancer treatment has however been limited due to the wide tropism of the adenovirus. This is caused by the ubiquitous cell surface expression pattern of the CAR and integrin receptors, which then leads to undesired virus uptake and gene expression in non-targeted tissues. To address this viral tropism problem, several research groups have introduced modifications and adaptor targeting-molecules to the virus fiber, which allows for the retargeting of the adenovirus to tumor cells [4–6].

* Corresponding author. Fax: +1 514 496 5143.
E-mail address: anne.lenferink@nrc-cnrc.gc.ca (A.E.G. Lenferink).

[1] *Abbreviations used:* EGF, epidermal growth factor; EGFR, EGF receptor; IBs, inclusion bodies; SPR, surface plasmon resonance; CBB, Coomassie Brilliant Blue; HPLC, high performance liquid chromatography; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; HEPES: *N*-(2-hydroxyethyl) piperazineethanesulfonic acid; PDEA, 2-(2-pyridinyldithio)ethaneamine; Guanidine–HCl, guanidine hydrochloride; EDTA, disodium ethylenediaminetetraacetate; RU, resonance unit; CH_3CN, Acetonitril; TFA, trifluoroacetic acid; PMSF, phenylmethylsulfonyl fluoride.

1046-5928/$ - see front matter Crown copyright © 2008 Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2008.11.005

The *de novo* designed E/K heterodimerizing coiled-coil system as previously described [7] is composed of two distinct peptides, designated E- and K-coil. Each peptide has five repeats of a seven amino acid sequence that contains both charged and hydrophobic residues. Individually, the E5- and K5-coil are highly soluble due to the large number of charged residues. When interacting, the E5- and K5-coil form a high affinity coiled-coil dimer [7,8] that has been proven to be useful for several applications, e.g. as a tag for purification purposes or for induced protein–protein interaction studies. We have shown previously using SPR-based biosensor studies that E5 or K5-coil-tagged receptor ectodomains (from the transforming growth factor (TGF)-β Types II and III receptors) bind TGF-β similarly to untagged receptor ectodomains, and that the coil tags can be used for receptor capture on the biosensor surface [9,10]. More recently, we used the E/K coiled-coil dimerization system to establish a virus retargeting scheme in which E5-coil-EGF was utilized to retarget K4-coil-expressing adenovirus to EGFR expressing cells [11].

Bacterial expression systems are frequently used to produce fusion proteins because of their simplicity in genetic manipulation and low production costs. However, protein refolding is often required after such productions. This can be a difficult and complicated process that requires many steps and often results in a very low recovery of the refolded protein. Here, we describe a straightforward method for the refolding and recovery of pure active E5/K5-coil-EGF fusion proteins using an *Escherichia coli* expression system. The biological activity of the E5/K5-coil-EGF fusion proteins was determined by their capacity to induce EGF receptor phosphorylation, and to stimulate the cell growth. The results obtained from both assays indicate that the biological activities of the E5/K5-coil-EGF fusion proteins are similar to those of native untagged murine submaxillary gland (m)EGF, and recombinant human (rh)EGF. In addition, analysis of binding using a cell-based competition assay, as well as the EGFR ectodomain on a SPR-based biosensor indicates that the EGFR binding characteristics of the E5/K5-coil-EGF versions are similar to that of non-tagged rhEGF.

In conclusion, our results show that *E. coli* offers a robust platform for the expression of E/K-coil-tagged proteins, such as E5/K5-coil-EGF, and that refolding as described here results in the recovery of a highly biologically active preparation of fusion protein. Due to the versatile and high affinity nature of the coiled-coil system, proteins tagged with such coils can be used in diverse applications. As shown here, the fusion of one coil to EGF enables it to act as a bifunctional adaptor molecule that can promote the retargeting of an adenovirus, which expresses the partner coil, through the EGF receptor. Additionally, immobilization of one of the coils on a biocompatible scaffold or nanoparticle would allow for the oriented and stable capture (grafting) of a protein fused to the partner coil, e.g. a growth factor such as EGF, thereby generating a homogenous surface that can be used for tissue regeneration purposes or nanoparticle targeting.

Materials and methods

The pGEMT vector was purchased from Promega Corporation (CA, USA), and the pET32c expression vector and recombinant enterokinase were from Novagen (CA, USA). QIAquick purification kit, the Miniprep kit and Ni–NTA superflow resin were purchased from Qiagen (ON, Canada). Isopropyl-β-D-1-thiogalactopyranoside (IPTG), ampicillin, guanidine HCl, L-cysteine were from Sigma (ON, Canada). PCR primers were purchased from IDT (ON, Canada) and restriction enzymes were from NEB (ON, Canada). Murine EGF (submaxillary glands) was purchased from Calbiochem (CA, USA) and recombinant human EGF was from Austral Biological (CA, USA). Sheep anti-human EGF antibody was a gift from Dr. H. Gregory and anti-phosphotyrosine monoclonal antibody was purchased from Upstate Biotechnology Inc. (NY, USA). All HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (PA, USA).

Cell culture

Human lung carcinoma cells (A549) and human glioblastoma cells (U87MG) were obtained from American Type Culture Collection and grown in Roswell Park Memorial Institute (RPMI-1640) media supplemented with 5% fetal bovine serum (FBS) or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 2 mM L-glutamine, respectively. Mouse mammary tumor cell line BRI-JM01 (as previously characterized [12]) was grown in DMEM/F12 media supplemented with 5% fetal bovine serum. All cell lines were cultured at 37 °C in a humidified incubator containing 5% CO_2.

Construction of the E5/K5-coil-EGF expression vector

Human EGF cDNA was amplified by PCR using the pEZZ18-EGF construct (gift from Dr. E.J.J. van Zoelen, University of Nijmegen, The Netherlands) with the following primers, $EGF_{forward}$: 5′-GATC GAATTCAACAGC GACTCTGAATGCCCG-3′ (EcoRI restriction site underlined) and $EGF_{reverse}$: 5′-GATCAAGCTTTTATTAACGGAGCTCC CACCA-3′ (HindIII restriction site underlined). E5-coil and K5-coil cDNA was amplified by PCR using the pcDNA3-E5-coil or pcDNA3-K5-coil construct (gift from Mrs. Myriam Banville, Biotechnology Research Institute, Montreal, Que., Canada). A 15-meramino acid linker $(Gly_4; Ser_1)_3$ was added C-terminally of the E5- and K5-coil using the following primers, $E5\text{-}coil_{forward}$: 5′-GATCCCATGGAGGTATCCGCTT TAGAGAAAGAA-3′ (NcoI restriction site underlined), $E5\text{-}coil_{reverse}$: 5′-GATCGAATTC***AGAGCCACCGCCACCGCTGCCACCGCCACCAGAGCCA CCGCCACC***CTTCTCAAGTGCTGATACTTCCTT-3′ (EcoRI restriction site underlined, linker in bold italic). $K5\text{-}coil_{for}$: 5′-GATCCCATGG GAAAGGTATCCGCTTTAAAGGAGAAA-3′ (NcoI restriction site underlined) and $K5\text{-}coil_{rev}$: 5′-GATCGAATTC***AGAGCCACCGCCACCGC TGCCACCGCCACCAGAGCCACCGCCACC***CTCTTTAAGTGCTGAAACCTT TTC-3′ (EcoRI restriction site underlined, linker in bold italic). These fragments were then directly cloned into the pGEMT vector, thus generating the pGEMT-EGF, pGEMT-E5-coil and pGEMT-K5-coil constructs. Human EGF cDNA, obtained by an EcoRI/NotI digest of the pGEMT-EGF construct, was ligated to pGEMT-E5-coil linker or pGEMT-K5-coil linker construct digested with the same enzymes, generating the pGEMT-E5-coil-EGF and pGEMT-K5-coil-EGF constructs. E5-coil-EGF and K5-coil-EGF fragments were obtained by digesting these constructs with NcoI and HindIII, after which they were inserted into the pET32c construct (in frame with the thioredoxin). The resulting gene expression plasmid used for the thioredoxin-E5-coil-EGF and thioredoxin-K5-coil-EGF fusion protein production was named pET-E5-coil-EGF and pET-K5-coil-EGF. The correct sequence of recombinant E5/K5-coil-EGF was verified by DNA sequence analysis.

Expression and purification of recombinant His-tagged E5/K5-coil-EGF

Escherichia coli BL21 (DE3) cells were transformed (by heat shock) with pET-E5-coil-EGF or pET-K5-coil-EGF and grown in LB medium containing ampicillin (100 μg/mL) at 37 °C. At an optical density of 0.7–1.0 (550 nm), IPTG was added to a final concentration of 1 mM to induce protein expression. Cultures were grown for another 4 h at 37 °C, harvested by centrifugation at 5000*g* (10 min) and stored overnight at −20 °C. The next day, cell pellets were washed once with Buffer A (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, and 1 mM PMSF, pH 8.0), resuspended in lysis buffer (Buffer A + 2% Triton-X100, and 0.4 mg/mL lysozyme) and incubated on ice (30 min) before being disrupted by sonication.

Cell lysates were centrifuged at 10,000*g* (4 °C, 30 min) to recover the His-E5/K5-coil-EGF inclusion bodies (IBs). IBs were washed twice with Buffer B (Buffer A + 2% Triton-X100) and twice with Buffer C (20 mM Tris, 150 mM NaCl, 1% Triton-X100; pH 8.0) with a centrifugation step in between each wash (4 °C, 10,000*g*, 10 min), after which the IBs were solubilized overnight (22 °C) in Buffer C containing 6 M Guanidine–HCl. The next day, solubilized IBs were centrifuged at 4 °C (10,000*g*, 60 min) and the coil-tagged EGF present in the supernatant was recovered and further purified by Ni–NTA affinity chromatography. Bound recombinant protein was eluted with 40 mL of elution buffer (20 mM Tris–HCl, 150 mM NaCl, 200 mM imidazole pH 8.0) and the His-E5/K5-coil-EGF fractions were analyzed by SDS–PAGE followed by Coomassie Brilliant Blue (CBB) staining. Finally, recombinant protein fractions were pooled, concentrated, subjected to a buffer exchange (20 mM Tris–HCl buffer; pH 8.0) using an Amicon Ultra device (cut-off: 10 kDa), and the protein concentration was determined by Bicinchoninic Acid (BCA) Protein Assay.

Denaturation and refolding

His-E5/K5-coil-EGF was refolded essentially as previously described [13]. Briefly, His-E5/K5-coil-EGF was diluted to 0.5 mg/mL and denatured at 22 °C (90 min) in 0.1 M Tris–HCl buffer (pH 8.4) containing 6 M guanidine hydrochloride and 5 mM L-cysteine. To initiate folding the denaturant was removed by passing the sample through a PD-10 column (Amersham Pharmacia Biotech) equilibrated in 0.1 M Tris–HCl (pH 8.4). Reduced and denatured recombinant protein was recovered in a volume of 3.5 mL, which was immediately diluted in the same Tris–HCl buffer supplemented with 2.5 mM L-cysteine to a final protein concentration of 0.5 mg/mL for oxidative refolding (24 h, 22 °C). Trifluoroacetic acid (TFA) was then added, at a final concentration of 1%, to the refolded His-E5/K5-coil-EGF before purification by high performance liquid chromatography (HPLC).

HPLC separation

Refolded His-E5/K5-coil-EGF was further purified by HPLC (Waters, ON, Canada) on a 4.6 × 250 mm Vydac C_{18} column using a linear gradient of CH_3CN in 0.1% (v/v) TFA at a flow rate of 1 mL/min. Fractions that, at a concentration of 100 ng/mL, were able to induced EGF receptor phosphorylation were pooled and freeze-dried. Recombinant enterokinase (1 unit per 50 μg of coil-tagged-EGF for 16 h at 22 °C) was used to cleave the His-tag from E5/K5-coil-EGF according to the manufacturer's instructions. After cleavage, 0.1 M PMSF was added to neutralize the enterokinase; the E5/K5-coil-EGF was separated from its His-tag by HPLC using the conditions described above, prior to lyophilization. Refolded E5/K5-coil-EGF protein was finally characterized by SDS–PAGE and western blot analysis using a specific anti-human EGF antibody. Immunoreactive bands were detected by a goat anti-sheep antibody linked to horseradish peroxidase (Jackson), and visualized by chemiluminescence (ECL, Roche Molecular Biochemicals).

Stimulation of receptor tyrosine phosphorylation

A549 were seeded in 24-well plates (80,000 cells/well) and incubated overnight under the growth conditions described. At 90% confluency, various amounts of rhEGF, mEGF, E5-coil-EGF or K5-coil-EGF were added and cells were incubated for 10 min at 37 °C. Subsequently, cells were washed once with tissue culture media and once with PBS (4 °C) and then immediately lysed in 100 μl of hot SDS (2%). Cell lysates were collected, mixed with reducing sample buffer and boiled for 5 min. Twenty microliters of these cell lysates were then analyzed on a 7% SDS–PAGE followed by western blotting with anti-phosphotyrosine monoclonal antibody. Immunoreactive bands were detected by an anti-mouse antibody linked to horseradish peroxidase (Jackson), and visualized by chemiluminescence (ECL, Roche Molecular Biochemicals). Experiments were repeated at least three times.

Mitogenic assays

BRI-JM01 cells (40,000 cells/well) were plated in 24-well plates and incubated overnight under the growth conditions described. The medium was then replaced with 1 mL serum-free DMEM/F12 and cells were incubated at 37 °C for another 4 h. Various amounts of mEGF, E5-coil- and K5-coil-EGF were added in 50 μl DMEM/F12 serum-free medium. After 24 h at 37 °C (5% CO_2), 100 μl serum-free media containing 0.5 μCi [^{3}H]-thymidine (Amersham, Piscataway, NJ, USA) was added per well. Cells were incubated for 3 h at 37 °C (5% CO_2), washed 3 times with PBS (RT), harvested in 250 μl of 0.05% trypsin–EDTA (37 °C, 30 min), and directly transferred to 5 mL scintillation fluid (Universol; ICN Biochemicals Inc.). Incorporated [^{3}H]-thymidine was determined using a β counter (LKB-Wallac). Experiments were performed in triplicates and repeated at least three times.

[^{125}I]-rhEGF binding competition assay

A549 cells (2.0×10^5 cells/well) were plated in 24-well plates and incubated overnight under the growth conditions described. The next day, cells were washed once with ice-cold tissue culture medium prior to incubation with 175 μl of medium containing 1 nM [^{125}I-rhEGF] and various concentrations of rhEGF or cleaved E5/K5-coil-EGF. Cells were washed twice with PBS, lysed by adding 250 μl 1% Triton-X100 in PBS per well followed by 1 h incubation at room temperature. Lysates were evaluated for their [^{125}I-rhEGF] levels using a γ-counter (Wallac Wizard, Perkin Elmer). Experiments were performed in triplicates and repeated at least three times.

SPR experiments

SPR analysis of the extracellular domain of the EGFR (EGFR-ED) and E5/K5-coil-EGF interaction was measured using a Biacore 3000 instrument (GE Healthcare Bio-Sciences Corporation). E5-coil and K5-coil surfaces used to capture E5/K5-coil-EGF were prepared on Biacore research grade CM-5 sensor chips using the thiol coupling method. The CM-5 surface was activated using a mixture of 0.2 M EDC and 0.05 M NHS in water followed by 80 mM PDEA in 0.1 M sodium borate (pH 8.5). Approximately 2 mg of E5-coil or K5-coil (containing a N-terminal cysteine) was dissolved in 200 μl peptide buffer (100 mM HEPES, 1.0 M NaCl; pH 7.5) and flown over the PDEA-activated surface using the MANUAL INJECT command until approximately 200 RUs of peptide were coupled to the surface. Remaining activated non-peptide bound thiol groups were quenched by injecting 50 mM cysteine and 1.0 M NaCl in 0.1 M sodium formate pH 4.0 followed by 5.0 M guanidine hydrochloride to remove any non-specifically bound peptide. Control surfaces were prepared in the same manner without ligand. All procedures were carried out at a flow-rate of 5 μl/min. E5-coil-EGF and K5-coil-EGF was diluted to approximately 1 μg/mL in HBS buffer (10 mM HEPES, 3.4 μM EDTA, 150 mM NaCl, 0.005% Tween 20) and injected over the K5- or E5-coil surface, respectively, using the MANUAL INJECT command until 70 RUs were captured. This surface remained extremely stable and was used without regenerating for the duration of the EGFR analysis.

To determine the E5/K5-coil-EGF affinity for EGFR-ED, EGFR-ED dilution series between 500 nM and 3.9 nM were injected using the KINJECT command. Every single injection was preceded by

one PRIME and five buffer injections and followed by an EXTRACLEAN command before the next injection cycle. Three independent EGFR-ED dilution series were analyzed over each of the EGF-coil surfaces. Generated sensorgrams were zero-ed to the injection start, blank surface subtracted, and double referenced using a buffer injection. These resulting binding sensorgrams were analyzed using BiaEvaluation version 4.1 (GE Healthcare Bio-Sciences Corporation) and the affinity of E5/K5-coil-EGF for EGFR-ED was determined by steady state analysis using the sensorgram's plateau values determined by the AVERAGE general fit command.

Generation of Adenovirus vectors

Adenovirus vectors (AdVs) expressing Green Fluorescent Protein (GFP) and wild-type fiber (Ad5FiberWt/GFP), or GFP and K5-coil-tagged fiber (Ad5FiberHIK5cDm/GFP), were produced by cleaving plasmids encoding the Adeno genome with PacI followed by transfection of 293E (cell line described in [11]) using polyethylenimine (PEI) [14]. After 21 days, when cytopathic effects were observed, cells were harvested by three cycles of freeze-thawing and the AdVs were propagated using standard methods. The infectious titer of the AdV was determined by measuring GFP expression in 293E cells by flow cytometry using a Cytomics FC500 MPL flow Cytometer as described previously [15], and is expressed in Transducing units ([TU]/ml). Stocks of AdV were purified through two consecutive centrifugation steps on CsCl gradients using standard procedures [16]. Titers were determined (OD at 260 nm) and expressed as physical particles/ml. Finally, purified AdV stocks were resuspended in buffer (20 mM Tris–HCl (pH 8.0), 2.5% glycerol, 25 mM NaCl) and stored at −80 °C.

Targeting assays

About 3×10^5 infectious viral particles of CsCl purified Ad5FiberHIK5cDm/GFP or Ad5FiberWt/GFP vectors in DMEM supplemented with 5% serum were incubated for 24 h at 37 °C in the absence or presence of 10 ng of rhEGF or 30 ng of purified E5-coil-EGF in a final volume of 50 μl. The AdVs were then used to infect U87MG cells at a MOI of 3. Four days later, cells were collected using Trypsin–EDTA (Sigma–Aldrich, St. Louis, MO), the enzymatic reaction was terminated by adding formaldehyde (Polysciences Inc., Washington, DC) to a final concentration of 2%, and cells were analyzed for GFP expression using a Cytomics FC500 MPL flow Cytometer (Beckman–Coulter, Miami, Fl) equipped with a 25 mW 488 nm argon ion laser as excitation source.

Viable cell populations were selected using forward and side scattering in combination with a 488 nm dichroic long pass filter. The GFP emission was detected using a 550 nm dichroic filter and a 525 nm band pass filter set.

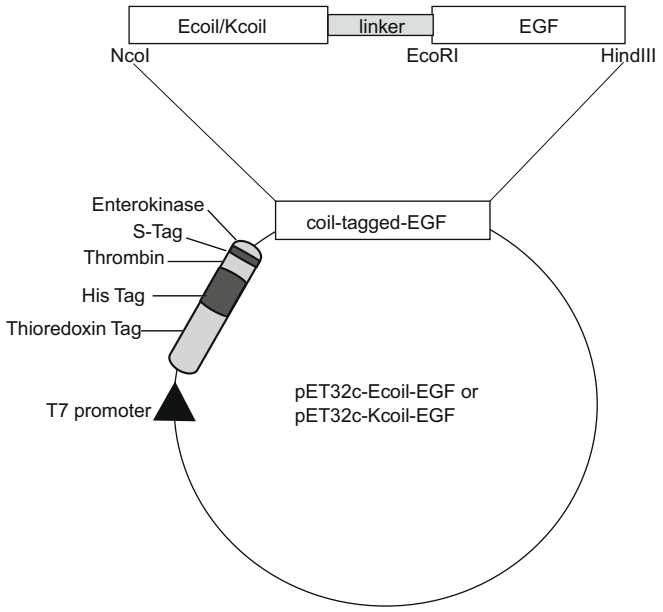

Fig. 1. Schematic structure of the gene and construct used for the expression of E5/K5-coil-tagged hEGF. Human EGF tagged with an E5- or K5-coil through a 15 a.a linker was cloned into the pET32c expression vector. This vector adds several additional tags to the fusion protein, which are used for purification (His tag), and cleavage (enterokinase) purposes.

Results

Construction, expression, and purification of coil-tagged human EGF fusion proteins

In this study, we produced plasmid constructs encoding for human EGF (hEGF) fusion proteins that are N-terminally tagged with either the E5-coil peptide or the K5-coil peptide (E5-coil-EGF or K5-coil-EGF). These EGF fusion proteins (Fig. 1), which have an apparent molecular weight of approximately 28 kDa, were engineered to include a series of functional domains: a His tag (for purification purposes), an enterokinase cleavage site (for removal of all the tags after the purification and refolding), and the E5-coil or K5-coil domain linked to hEGF by the 15 amino acid linker $((Gly_4; Ser_1)_3)$.

Using IPTG, *E. coli* cultures were induced to express high levels of His-E5-coil- or His-K5-coil-EGF, which accumulated in insoluble inclusion bodies (IBs). Approximately 30–50 mg of fusion protein was recovered from 6 M guanidine–HCl-dissolved IBs derived from 500 mL of bacterial culture (Table 1). His-E5/K5-coil-EGF fusion proteins were purified by Ni–NTA chromatography under reducing conditions and eluted using 250 mM imidazole. CBB staining of the SDS–PAGE showed that His-E5-coil-EGF migrated as a single band of approximately 37 kDa (Fig. 2A), while His-K5-coil-EGF migrated as a single band of about 30 kDa (Fig. 2B). The discrepancy in MW between the His-E5-coil-EGF and the His-K5-coil-EGF band is likely due to the opposite charges present on the E5- and K5-coils. Purification of the fusion proteins under reducing conditions improved binding to the Ni–NTA resin, and therefore prevented loss of protein during the wash steps (Fig. 2B). On average a 500 mL bacterial culture resulted in approximately 25 mg of His-E5-coil-EGF or 13 mg of His-K5-coil-EGF following Ni–NTA purification (Table 1).

Refolding and HPLC purification of coil-tagged EGF

Because of the reducing environment of the bacterial cytoplasm, proteins often accumulate in inclusion bodies in an inactive state [17]. Coil-tagged-EGF therefore requires refolding to recover its biological activities. We refolded His-E5/K5-coil-EGF essentially as previously described [13]. To avoid protein aggregation the

Table 1
Typical yield of E5/K5-coil-EGF at the different purification and refolding steps.

	E5-coil-EGF	K5-coil-EGF
Bacteria pellet (wet weight) from 500 mL culture	2.79 g	3.61 g
Solubilized IBs	50 mg	32 mg
After Ni–NTA purification and buffer exchange	25 mg	13 mg
After Refolding and the first HPLC purification	5 mg	2.7 mg
After the protein cleavage and the second HPLC purification	3 mg	1.5 mg

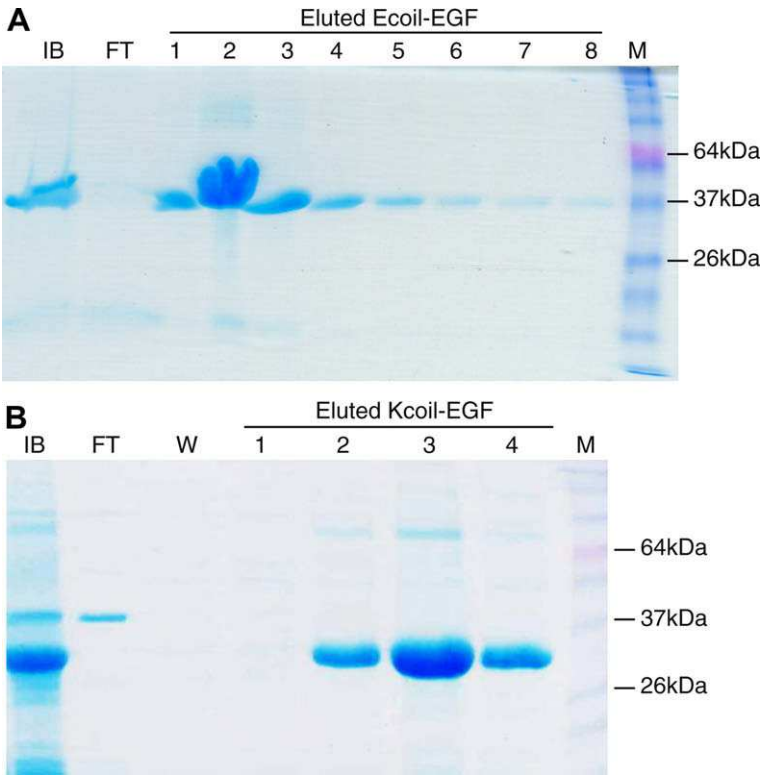

Fig. 2. Gel electrophoresis of purified His-E5-coil-EGF and His-K5-coil-EGF fusion proteins synthesized in *E. coli*. His-E5/K5-coil-EGF fusion proteins were purified by Ni–NTA chromatography under reducing conditions and eluted using 250 mM imidazole. Ten microliters (from eight fractions of 5 mL) of His-E5-coil-EGF (A) and 20 μl (from four fractions of 10 mL) of His-K5-coil-EGF (B) were separated using 12% SDS–PAGE under reducing conditions followed by CBB staining. His-E5-coil-EGF migrating at a molecular weight of ~37 kDa (A) and His-K5-coil-EGF at ~28 kDa (B). IB, inclusion body fraction; FT, flow-through; W, wash with 20 mM imidazole; M, molecular weight protein marker.

refolding was done immediately after protein purification by Ni–NTA chromatography. Imidazole was removed from Ni–NTA purified protein by buffer exchange, and recombinant E5/K5-coil-EGF was diluted to 0.5 mg/mL in Tris buffer (0.1 M, pH 8.4) containing 6 M guanidine hydrochloride and 5 mM cysteine to promote denaturation and reduction. Then, guanidine–HCl was rapidly removed by passing the samples through a PD-10 column. The refolding process was completed by incubating the coil-tagged-EGF in Tris buffer (0.1 M, pH 8.4) containing 2 mM cysteine for 24 h (RT).

HPLC can be used to separate the oxidized form of a protein from its reduced form [18]. We used this approach to purify the refolded E5/K5-coil-EGF. The A280 nm profile of the fractions that were collected indicated that His-E5-coil-EGF eluted in one major peak (retention time of approximately 36–37 min, Fig. 3A). Therefore, fraction 36–37 were pooled, lyophilized and reconstituted in distilled water. His-K5-coil-EGF eluted in two peaks (retention time of approximately 34–38 min, Fig. 3B). Fractions corresponding to these two peaks were separately analyzed by SDS–PAGE. No difference in the molecular weight was observed between the two peaks (data not shown). In addition, testing these two fractions for their ability to induce EGF receptor phosphorylation (at 100 ng/mL) in A549 cells did not show any difference in activity (data not shown). Therefore, these fractions were considered equally active and were pooled, lyophilized and reconstituted in distilled water.

Recombinant enterokinase was used to cleave all tags at the N-terminal of the E5- or K5-coil-EGF. This was followed by HPLC to separate E5- (Fig. 4A) and K5-coil-EGF (Fig. 4B) from its tags and uncleaved His-E/K-coil-EGF. CBB stained gels (Fig. 4C) indicated that the first peaks of the HPLC profiles shown in Fig. 4A and B represents cleaved K5-coil-EGF (~11 kDa) or E5-coil-EGF (~17 kDa), respectively, while the second peak represents a mixture of the purification tags (~17 kDa) and uncleaved His-E5-coil-EGF (~36 kDa) or His-K5-coil-EGF (~28 kDa). This was further confirmed by western blot using anti-hEGF antibody (Fig. 4D).

Biological activity of purified coil-tagged EGF

Different assays were performed to assess the biological activity of our bacterially produced E5/K5-coil-EGF using various cell lines. First, we evaluated the ability of E5-coil-EGF and K5-coil-EGF to induce EGFR phosphorylation in the EGFR expressing A549 cell line (Fig. 5A). Stimulation of this cell line with various concentrations of rhEGF, mEGF, E5-coil-EGF or K5-coil-EGF showed that both E5-coil-EGF and K5-coil-EGF are able to induce EGF receptor phosphorylation to the same extent as the rhEGF and mEGF. As previously reported by Lenferink et al. [12], EGF induces a clear mitogenic response in BRI-JM01 cells. Therefore, as a second assay, we evaluated the mitogenic activity of our purified E5-coil and K5-coil-EGF by measuring the incorporation of tritiated thymidine in the DNA of BRI-JM01 cells. Fig. 5B indicates that the presence of either an N-terminal E5-coil or K5-coil did not alter the biological activity as compared to mEGF. A concentration of 1 ng/mL of both mEGF and coil-tagged-EGF induced a 50% increase in thymidine incorporation, compared to non-stimulated control.

We next determined the affinity of the coil-tagged EGF for EGFR by performing a receptor binding competition assay on A549 cells using [^{125}I]-rhEGF. The binding competition curves of unlabeled rhEGF and E5/K5-coil-EGF are shown in Fig. 6A. Both E5-coil-EGF and K5-coil-EGF compete with [^{125}I]-rhEGF for binding to the EGFR to the same extent as rhEGF. We also used SPR-based biosensor analysis to determine the affinity of the interactions of both E5-

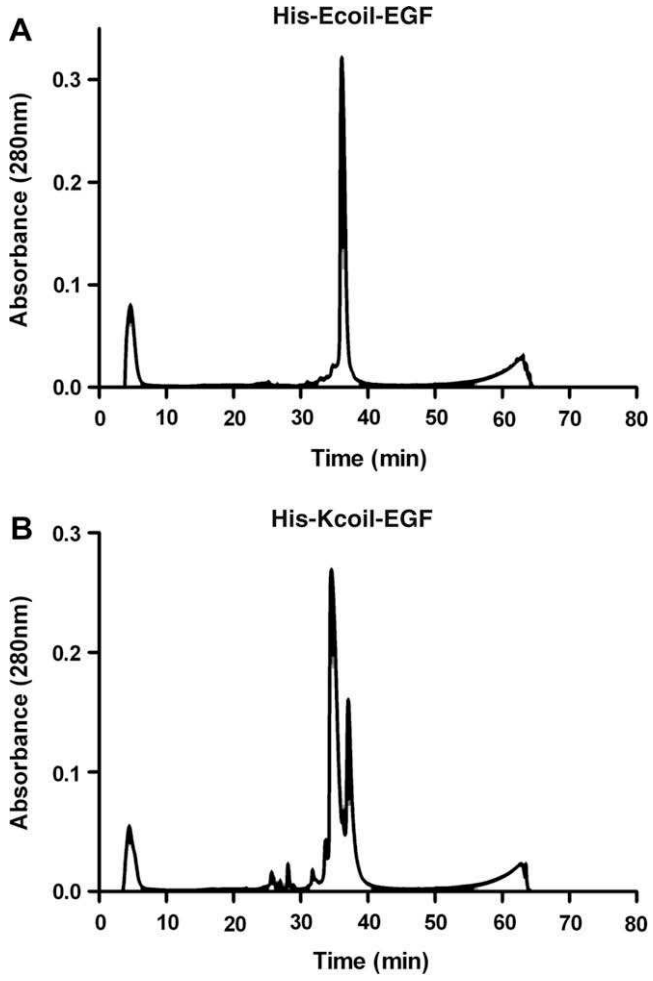

Fig. 3. HPLC profile of (A) His-E5-coil-EGF and (B) His-K5-coil-EGF. His-E5-coil-EGF eluted as one peak (retention time ~36–37 min), whereas His-K5-coil-EGF eluted in two peaks ~34–38 min after injection.

coil-EGF and K5-coil-EGF with the extracellular domain of the EGFR-ED. An E- or K-coil surface was used to capture the K5-coil and E5-coil-tagged EGF, respectively, after which various dilutions of the extracellular domain of EGFR-ED were injected. Steady state analysis demonstrated that E5-coil-EGF and K5-coil EGF exhibited similar average kDs (183 ± 12.1 and 146 ± 6.11 nM, respectively). These affinities are comparable to the values reported for mEGF and EGFR-ED (200 nM) using the SPR technology [19]. It can therefore be concluded from these experiments that the biological activities of rhEGF are not affected by the presence of an N-terminal E5- or K5-coil. Taken together, these results demonstrate that biologically active E5/K5-coil-EGF fusion proteins can be recovered from transformed bacteria by oxidative refolding.

E-coil-tagged EGF retargets K-coil AdVs to EGFR expressing U87MG cells

In order to demonstrate a functional advantage for coil-tagged EGF, as compared to untagged EGF, we compared the abilities of E-coil-EGF and rhEGF to retarget K-coil expressing adenovirus through the EGFR. Because EGFR is present on several cancer cell types, such as U87MG glioblastoma cells, EGFR is thought to be a good target for improving the tumor tropism of AdVs. We incubated an AdV that has the K5-coil fused in its fiber knob (Ad5FiberHIK5cDm/GFP), as well as an AdV that expresses wild type fiber (Ad5FiberWt/GFP), with rhEGF or the E-coil-EGF purified in this study. We then evaluated retargeting competency in U87MG cells. As shown in Fig. 7, Ad5FiberWt/GFP (white bars) and Ad5FiberHIK5cDm/GFP (black bars), alone or in the presence of rhEGF, did not efficiently transduce these cells. Notably, pre-incubation of E-coil-EGF with Ad5FiberHIK5cDm/GFP, which expresses the complementary K-coil, resulted in efficient transduction of the EGFR expressing U87MG cells. In contrast, pre-incubation of E-coil-EGF with the Ad5FiberWt/GFP control virus did not affect transduction efficiency. Together, these results indicate that the coiled-coil dependant interaction between E-coil-EGF and Ad5FiberHIK5cDm/GFP enables E-coil-EGF to act as a bispecific adaptor that promotes the retargeting of this AdV through the EGF receptor.

Discussion

In the manuscript presented here we describe the engineering of novel recombinant EGF fusion proteins that are N-terminally tagged with an E5-coil or a K5-coil peptide (E5/K5-coil-EGF), and their expression and isolation from *E. coli* inclusion bodies (IBs). The IBs, which contain the recombinant protein in a highly enriched form low in contaminants, can be easily recovered by cell lysis followed by centrifugation. Native EGF contains three disulfide bonds that are vital for its biological activity. Since the reducing environment of the bacterial cytosol prevents the formation of these disulfide bonds, it promotes the manifestation of protein aggregates in these IBs [20]. Therefore, the EGF recovered from these IBs requires refolding in order to regain its biological function.

It has been reported that, during refolding, approximately 43% of EGF becomes kinetically trapped in a single stable 2-disulfide form (EGFII). When performing refolding in the absence of redox agents, this form is unable to convert to the native EGF form. Furthermore, conversion of EGFII to the native EGF structure requires the crossing of an energy barrier that demands extensive unfolding of the EGFII [13]. In agreement with these reports, we found that refolding of E5/K5-coil-EGF in the presence of different concentrations of reduced/oxidized glutathione, a commonly used redox agent in protein refolding, resulted in less than 1% biologically active E5/K5-coil-EGF (data not shown). The addition of arginine which suppresses aggregation [21,22], glycerol or sucrose which enhance protein stability in solution [23,24], also did not significantly increase the yields of biologically active E5/K5-coil-EGF after refolding (data not shown). It has been reported that cysteine has a higher redox potential than glutathione [25,26]. Results published by Chang et al. [27] demonstrated that the use of cysteine increased not only the rate at which hEGF was refolded but also its yield [13,27]. Refolding of our recombinant E5/K5-coil-EGF in the presence of cysteine resulted in an approximately 20% yield after purification (Table 1), confirming that cysteine improves refolding of coil-tagged EGF, as well as untagged EGF. The yield of E5/K5-coil-EGF after the two other purification steps (from inclusion bodies and after cleavage) was approximately 50% (Table 1). This indicates that refolding remains a limiting step, even in the presence of cysteine. It has been reported by Lu et al. [28] that, when EGF is mutated to contain an extra cysteine, this residue is able to act as a "built-in" cysteine thiol catalyst that potently promotes disulfide scrambling and refolding. The use of such a mutant in the context of coil-tagged EGF could potentially increase yield.

Self-assembling oligopeptides, such as the coiled-coil motif, have been demonstrated to be useful in numerous applications, e.g. the generation of mini-antibodies [41,42], the assembly of receptor ectodomain subunits [9,19,43–45], and as an affinity tag system for purification and biosensor applications [8–10,46–48]. Immobilized EGF has been shown to be more mitogenic for CHO cells than soluble EGF [29], perhaps due to decreased ligand inter-

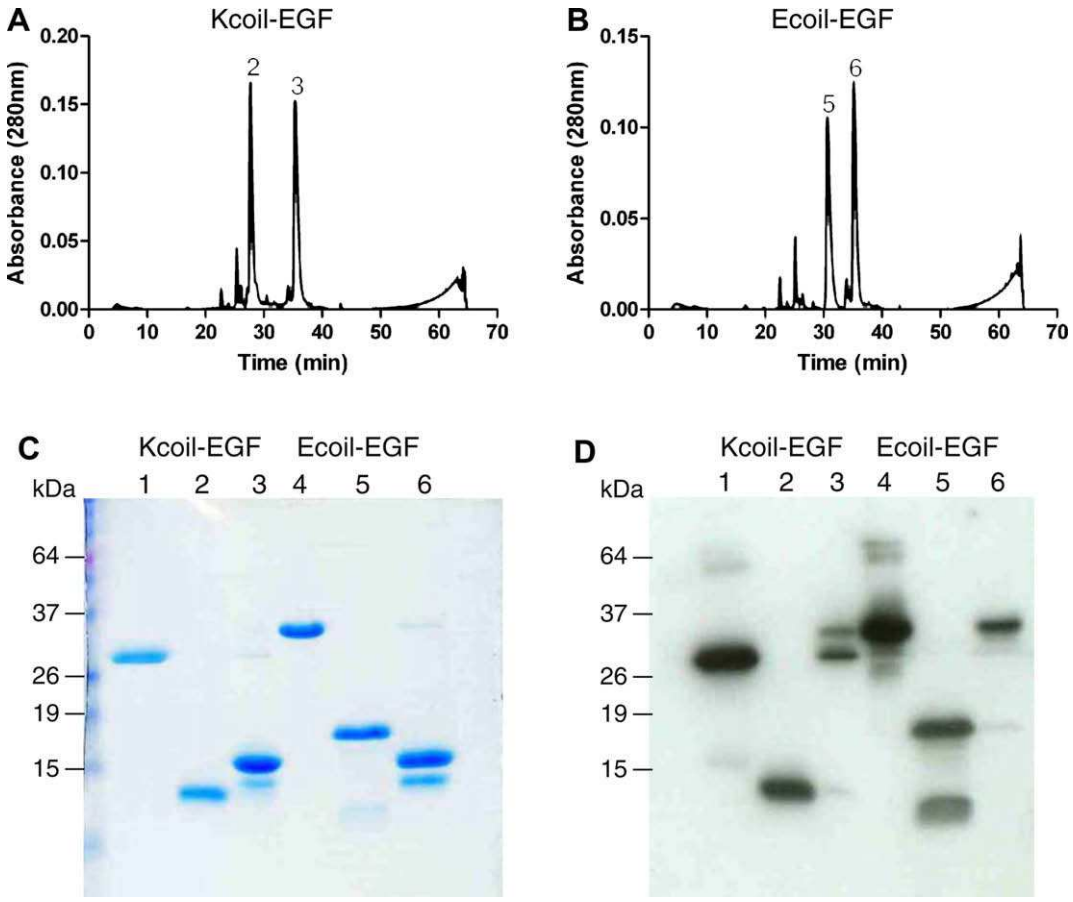

Fig. 4. HPLC profile of (A) K5-coil-EGF and (B) E5-coil-EGF after the purification tag was removed by recombinant enterokinase. CBB-stained SDS-PAGE shows that the K5-coil-EGF (~11 kDa; peak 2) eluted at ~28–29 min, while the E5-coil-EGF (~17 kDa; peak 5) eluted at ~31–32 min. (C) SDS–PAGE analysis K5-coil-EGF and E5-coil-EGF linked to the His tag prior to (lanes 1 and 4) and after cleavage with recombinant enterokinase, followed by HPLC separation (lanes 2 and 5). The purification tags are shown in lanes 3 and 6. (D) Corresponding western blot using sheep anti-hEGF antibody to detect purified E5/K5-coil-EGF.

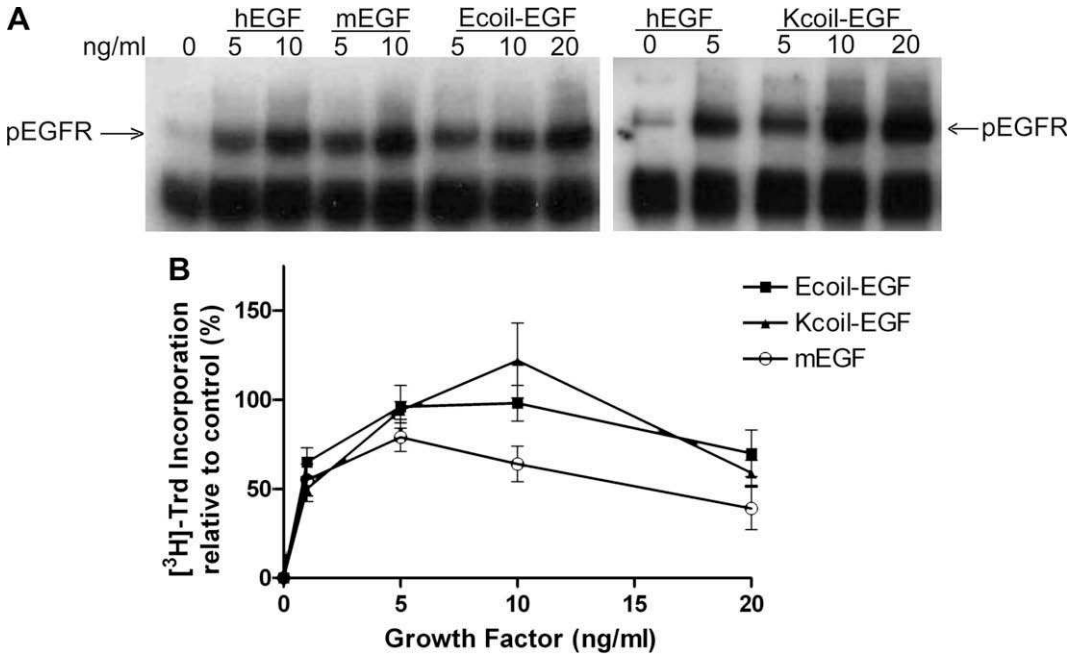

Fig. 5. Comparaison of E5/K5-coil-hEGF to rhEGF and mEGF biological activities (A) EGF receptor phosphorylation induced by E5/K5-coil-hEGF in A549 cell line. Cells were incubated with hEGF, mEGF, E5-coil-EGF or K5-coil-EGF as indicated for 10 min at 37 °C. Total cell extracts were prepared and separated on a 7% SDS–PAGE. EGFR phosphorylation was detected by western blotting using an anti-phosphotyrosine monoclonal antibody. Phosphorylation of the EGFR (arrows) was observed in cells treated with rhEGF, mEGF and E5/K5-coil-EGF. (B) Dose–response curves of tritium thymidine ([^{3}H]-TdR) incorporation of BRI-JM01 cells induced by mEGF and E5/K5-coil-EGF. [^{3}H]-TdR incorporation was measured 24 h after hours of exposure to serial dilutions of mEGF (open circles), E5-coil-EGF (closed squares) and K5-coil-EGF (closed triangles), and is expressed relative to the non-stimulated control.

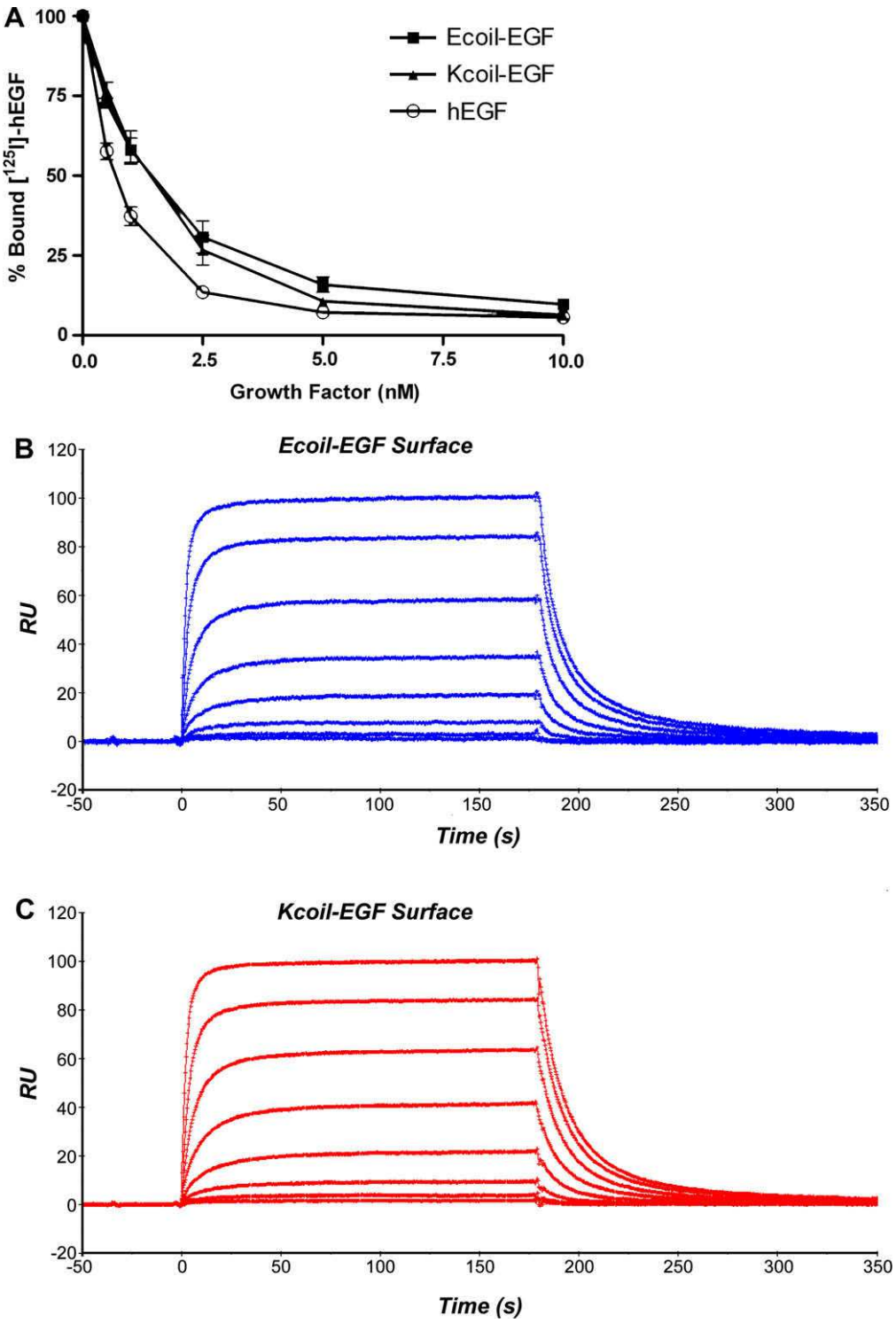

Fig. 6. Competitive binding assay between [^{125}I]-rhEGF and rhEGF or E/K-coil-EGF in A549 cells. (A) Various concentrations of rhEGF (open circles), E5-coil-EGF (closed squares) or K5-coil-EGF (closed triangles) were used to compete with 1 nM [^{125}I]-rhEGF for EGFR binding. The control value of 100% binding of [^{125}I]-rhEGF refers to the radioactivity bound in the absence of unlabeled rhEGF. Experiments were conducted in quadruplicate and repeated three times with similar results. SPR analysis of EGFR-ED interaction with captured E5-coil-EGF or K5-coil-EGF. Increasing concentrations of EGFR-ED ranging from 3.9 to 500 nM were injected over 70 RU of captured E5-coil-EGF (B) or K5-coil-EGF (C). E/K-coil-EGF binding to EGFR is comparable to that of rhEGF.

nalization and degradation [30]. Therefore, immobilization of EGF on an insoluble substrate in a non-endocytosible and non-diffusible manner could be very useful in processes such as wound healing, tissue regeneration and the growth of stem cells. Indeed, several groups have immobilized EGF on solid-phase surfaces by using polyethylene glycol (PEG) spacers [31,32], photo-immobilizing EGF [29,33] or by generating an EGF-collagen chimeric protein [34,35]. Other groups have designed EGF fusion proteins containing an immunoglobulin G (IgG) Fc region [36], or RGD sequence [37], both of which fusion proteins retained their cell adhesive and cell growth activity. More recently, Nakaji-Hirabayashi et al. [38] immobilized EGF in an oriented manner onto the Ni(II)-che-

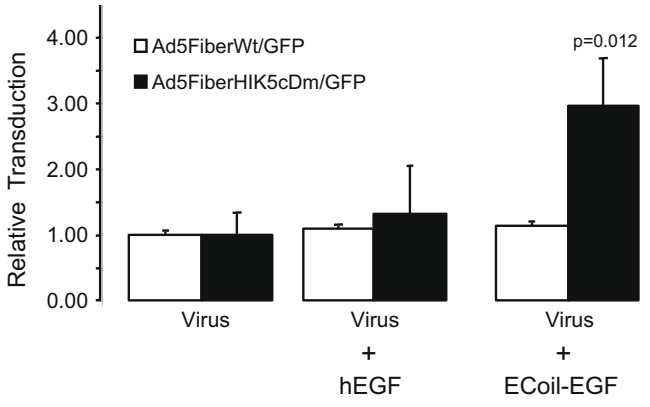

Fig. 7. Transduction of K-coil expressing Ad5FiberHIK5cDm/GFP can be increased using a bispecific E5-coil-EGF ligand. Ad5FiberWt/GFP and Ad5FiberHIK5cDM/GFP were incubated with or without ligand (rhEGF or E5-coil-EGF) at 37° for 24 h. EGF receptor expressing U87MG cells were then transduced this ligand associated virus (MOI = 3), and transduction efficiency was determined 96 h post-infection by measuring the GFP expression levels. The data plotted represent the average ± SD of the relative fluorescence index ($n = 6$). A relative value of 1 was set for cells transduced with each vector in absence of rhEGF or E5-coil-EGF.

lated surface through the binding of a His tag fused to the EGF C-terminus and used this system for the expansion of neural stem cells. In addition, tethering of EGF to a polymer substrate has been shown to improve the proliferation and survival of mesenchymal stem cells [39], and it has been demonstrated that immobilized EGF gradient surfaces induce accelerated and polarized migration of keratinocytes and therefore speed up the wound closure [40].

These reports suggest that, the coiled-coil domain could be used for the generation of functionalized surfaces through the immobilization of coil-tagged EGF (K-coil or E-coil) on a substrate in an oriented and stable manner via dimerization with the complementary coil motif (E-coil or K-coil, respectively). The SPR-based biosensor experiments presented here (Fig. 6B) indicate that E5/K5-coil-EGF remains biologically active following its immobilization on an E- or K-coil surface. Other groups have reported on the use of similar self-assembling peptide heterodimers based on human B-ZIP leucine zipper proteins for the generation of functionalized surfaces [49]. In this approach, one peptide was fused to a cysteine, which allowed for grafting to a polyacrylamide gel, whereas the other peptide was coupled to the RDGS cell adhesion binding domain. Combining the RGDS domain containing peptide with the modified polyacrylamide gel resulted in a functionalized hydrogel that supported the adhesion and spreading of endothelial cells [50]. The advantages for using the coil–coil motif for the generation of functionalized surfaces are (1) the simplicity and the stability of the method, (2) the possibility to design surfaces with multiple growth factors in controlled space-oriented manners and (3) the possibility to create a gradient of the immobilized molecules. Recently, we have demonstrated that the E/K coiled-coil motif can also be used in an adenoviral retargeting application. Gene delivery to specific tissues by AdVs relies on their natural tropism being ablated, followed by the introduction of new tropisms for both viral production and specific tissue targeting. We generated an AdV with K-coil fused to the fiber, and demonstrated that this AdV could be propagated in an E-coil-tagged receptor expressing cell line, and that it could be retargeted through the EGF receptor after pre-incubation with E-coil-tagged EGF [11]. Here, we further support this application by showing that an AdV with the K-coil fused at different position in the fiber can be retargeted, after incubation with E-coil-EGF produced in this study, to glioblastoma tumor cells expressing the EGF receptor (Fig. 7).

In conclusion, the data presented here show that E/K-coil-tagged proteins, such as the E5/K5-coil-EGF, can be produced using a bacterial expression system, and that, after refolding, a fusion protein with full biological activity can be recovered. The availability of interfacial assembly molecules, such as the E5- or K5-coil, will allow for the rapid systematic construction of functional surfaces using one, or a combination of, growth factors/ligands. The use of this type of biomaterial design will give researchers the ability to generate targeted particles for therapeutic and imaging applications, or to regulate cell physiology for tissue engineering and regeneration applications, in an unprecedented manner.

Acknowledgments

The authors acknowledge Myriam Banville for providing the E5-coil and K5-coil constructs, Suzanne Grothe for the iodination of all ligands used in this study and Edmund Ziomek for helpful advices during the HPLC purification. PUL was supported by a National Sciences and Engineering Council of Canada (NSERC) postdoctoral fellowship.

References

[1] Y. Yarden, The EGFR family and its ligands in human cancer signalling mechanisms and therapeutic opportunities, Eur. J. Cancer 37 (Suppl. 4) (2001) S3–S8.
[2] O. Dassonville, A. Bozec, J.L. Fischel, G. Milano, EGFR targeting therapies: monoclonal antibodies versus tyrosine kinase inhibitors. Similarities and differences, Crit. Rev. Oncol. Hematol. 62 (2007) 53–61.
[3] F. Morgillo, M.A. Bareschino, R. Bianco, G. Tortora, F. Ciardiello, Primary and acquired resistance to anti-EGFR targeted drugs in cancer therapy, Differentiation 75 (2007) 788–799.
[4] L. Pereboeva, S. Komarova, J. Roth, S. Ponnazhagan, D.T. Curiel, Targeting EGFR with metabolically biotinylated fiber-mosaic adenovirus, Gene Ther. 14 (2007) 627–637.
[5] J.E. Carette, H.C. Graat, F.H. Schagen, D.C. Mastenbroek, M.G. Rots, H.J. Haisma, G.M. Groothuis, G.R. Schaap, J. Bras, G.J. Kaspers, P.I. Wuisman, W.R. Gerritsen, V.W. van Beusechem, A conditionally replicating adenovirus with strict selectivity in killing cells expressing epidermal growth factor receptor, Virology 361 (2007) 56–67.
[6] N. Belousova, G. Mikheeva, J. Gelovani, V. Krasnykh, Modification of adenovirus capsid with a designed protein ligand yields a gene vector targeted to a major molecular marker of cancer, J. Virol. (2007) 630–637.
[7] G. De Crescenzo, J.R. Litowski, R.S. Hodges, M.D. O'Connor-McCourt, Real-time monitoring of the interactions of two-stranded de novo designed coiled-coils: effect of chain length on the kinetic and thermodynamic constants of binding, Biochemistry 42 (2003) 1754–1763.
[8] H. Chao, M.E. Houston Jr., S. Grothe, C.M. Kay, M. O'Connor-McCourt, R.T. Irvin, R.S. Hodges, Kinetic study on the formation of a de novo designed heterodimeric coiled-coil: use of surface plasmon resonance to monitor the association and dissociation of polypeptide chains, Biochemistry 35 (1996) 12175–12185.
[9] G. De Crescenzo, P.L. Pham, Y. Durocher, M.D. O'Connor-McCourt, Transforming growth factor-beta (TGF-beta) binding to the extracellular domain of the type II TGF-beta receptor: receptor capture on a biosensor surface using a new coiled-coil capture system demonstrates that avidity contributes significantly to high affinity binding, J. Mol. Biol. 328 (2003) 1173–1183.
[10] G. De Crescenzo, P.L. Pham, Y. Durocher, H. Chao, M.D. O'Connor-McCourt, Enhancement of the antagonistic potency of transforming growth factor-{beta} receptor extracellular domains by coiled coil-induced homo- and heterodimerization, J. Biol. Chem. (2004) 26013–26018.
[11] Y. Zeng, M. Pinard, J. Jaime, L. Bourget, P. Uyen Le, M.D. O'Connor-McCourt, R. Gilbert, B. Massie, A ligand-pseudoreceptor system based on de novo designed peptides for the generation of adenoviral vectors with altered tropism, J. Gene Med. 10 (2008) 355–367.
[12] A.E. Lenferink, J. Magoon, C. Cantin, M.D. O'Connor-McCourt, Investigation of three new mouse mammary tumor cell lines as models for transforming growth factor (TGF)-beta and Neu pathway signaling studies: identification of a novel model for TGF-beta-induced epithelial-to-mesenchymal transition, Breast Cancer Res. 6 (2004) R514–R530.
[13] J.Y. Chang, L. Li, P.H. Lai, A major kinetic trap for the oxidative folding of human epidermal growth factor, J. Biol. Chem. 276 (2001) 4845–4852.
[14] Y. Durocher, S. Perret, A. Kamen, High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells, Nucleic Acids Res. 30 (2002) E9.
[15] J. Cote, L. Bourget, A. Garnier, A. Kamen, Study of adenovirus production in serum-free 293SF suspension culture by GFP-expression monitoring, Biotechnol. Prog. 13 (1997) 709–714.

[16] B. Massie, F. Couture, L. Lamoureux, D.D. Mosser, C. Guilbault, P. Jolicoeur, F. Belanger, Y. Langelier, Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette, J. Virol. 72 (1998) 2289–2296.
[17] K.W. Walker, H.F. Gilbert, Effect of redox environment on the in vitro and in vivo folding of RTEM-1 beta-lactamase and *Escherichia coli* alkaline phosphatase, J. Biol. Chem. 269 (1994) 28487–28493.
[18] J.Y. Lee, C.S. Yoon, I.Y. Chung, Y.S. Lee, E.K. Lee, Scale-up process for expression and renaturation of recombinant human epidermal growth factor from *Escherichia coli* inclusion bodies, Biotechnol. Appl. Biochem. 31 (Pt. 3) (2000) 245–248.
[19] G. De Crescenzo, S. Grothe, R. Lortie, M.T. Debanne, M. O'Connor-McCourt, Real-time kinetic studies on the interaction of transforming growth factor alpha with the epidermal growth factor receptor extracellular domain reveal a conformational change model, Biochemistry 39 (2000) 9466–9476.
[20] H. Lilie, E. Schwarz, R. Rudolph, Advances in refolding of proteins produced in *E. coli*, Curr. Opin. Biotechnol. 9 (1998) 497–501.
[21] D. Arora, N. Khanna, Method for increasing the yield of properly folded recombinant human gamma interferon from inclusion bodies, J. Biotechnol. 52 (1996) 127–133.
[22] M. Suenaga, H. Ohmae, S. Tsuji, T. Itoh, O. Nishimura, Renaturation of recombinant human neurotrophin-3 from inclusion bodies using a suppressor agent of aggregation, Biotechnol. Appl. Biochem. 28 (Pt. 2) (1998) 119–124.
[23] E. De Bernardez Clark, E. Schwarz, R. Rudolph, Inhibition of aggregation side reactions during in vitro protein folding, Methods Enzymol. 309 (1999) 217–236.
[24] K. Tsumoto, D. Ejima, I. Kumagai, T. Arakawa, Practical considerations in refolding proteins from inclusion bodies, Protein Expr. Purif. 28 (2003) 1–8.
[25] J.Y. Chang, Controlling the speed of hirudin folding, Biochem. J. 300 (Pt. 3) (1994) 643–650.
[26] B. Raman, T. Ramakrishna, C.M. Rao, Refolding of denatured and denatured/reduced lysozyme at high concentrations, J. Biol. Chem. 271 (1996) 17067–17072.
[27] J.Y. Chang, P. Schindler, U. Ramseier, P.H. Lai, The disulfide folding pathway of human epidermal growth factor, J. Biol. Chem. 270 (1995) 9207–9216.
[28] B.Y. Lu, C. Jiang, J.Y. Chang, Isomers of epidermal growth factor with Ser → Cys mutation at the N-terminal sequence: isomerization, Stability, unfolding, refolding, and structure, Biochemistry 44 (2005) 15032–15041.
[29] G. Chen, Y. Ito, Y. Imanishi, Photo-immobilization of epidermal growth factor enhances its mitogenic effect by artificial juxtacrine signaling, Biochim. Biophys. Acta 1358 (1997) 200–208.
[30] H.S. Wiley, Trafficking of the ErbB receptors and its influence on signaling, Exp. Cell Res. 284 (2003) 78–88.
[31] P.R. Kuhl, L.G. Griffith-Cima, Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase, Nat. Med. 2 (1996) 1022–1027.
[32] B.J. Klenkler, M. Griffith, C. Becerril, J.A. West-Mays, H. Sheardown, EGF-grafted PDMS surfaces in artificial cornea applications, Biomaterials 26 (2005) 7286–7296.
[33] Y. Ito, J.S. Li, T. Takahashi, Y. Imanishi, Y. Okabayashi, Y. Kido, M. Kasuga, Enhancement of the mitogenic effect by artificial juxtacrine stimulation using immobilized EGF, J. Biochem. 121 (1997) 514–520.
[34] F.L. Hall, A. Kaiser, L. Liu, Z.H. Chen, J. Hu, M.E. Nimni, R.W. Beart Jr., E.M. Gordon, Design, expression, and renaturation of a lesion-targeted recombinant epidermal growth factor-von Willebrand factor fusion protein: efficacy in an animal model of experimental colitis, Int. J. Mol. Med. 6 (2000) 635–643.
[35] M. Hayashi, M. Tomita, K. Yoshizato, Production of EGF-collagen chimeric protein which shows the mitogenic activity, Biochim. Biophys. Acta 1528 (2001) 187–195.
[36] K. Ogiwara, M. Nagaoka, C.S. Cho, T. Akaike, Construction of a novel extracellular matrix using a new genetically engineered epidermal growth factor fused to IgG-Fc, Biotechnol. Lett. 27 (2005) 1633–1637.
[37] I. Elloumi, R. Kobayashi, H. Funabashi, M. Mie, E. Kobatake, Construction of epidermal growth factor fusion protein with cell adhesive activity, Biomaterials 27 (2006) 3451–3458.
[38] T. Nakaji-Hirabayashi, K. Kato, Y. Arima, H. Iwata, Oriented immobilization of epidermal growth factor onto culture substrates for the selective expansion of neural stem cells, Biomaterials 28 (2007) 3517–3529.
[39] V.H. Fan, K. Tamama, A. Au, R. Littrell, L.B. Richardson, J.W. Wright, A. Wells, L.G. Griffith, Tethered epidermal growth factor provides a survival advantage to mesenchymal stem cells, Stem cells 25 (2007) 1241–1251.
[40] T.J. Stefonek, K.S. Masters, Immobilized gradients of epidermal growth factor promote accelerated and directed keratinocyte migration, Wound Repair Regen. 15 (2007) 847–855.
[41] P. Pack, A. Pluckthun, Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric FV fragments with high avidity in *Escherichia coli*, Biochemistry 31 (1992) 1579–1584.
[42] P. Pack, K. Muller, R. Zahn, A. Pluckthun, Tetravalent miniantibodies with high avidity assembling in *Escherichia coli*, J. Mol. Biol. 246 (1995) 28–34.
[43] H.C. Chang, Z. Bao, Y. Yao, A.G. Tse, E.C. Goyarts, M. Madsen, E. Kawasaki, P.P. Brauer, J.C. Sacchettini, S.G. Nathenson, et al., A general method for facilitating heterodimeric pairing between two proteins: application to expression of alpha and beta T-cell receptor extracellular segments, Proc. Natl. Acad. Sci. USA 91 (1994) 11408–11412.
[44] Z. Wu, S.F. Eaton, T.M. Laue, K.W. Johnson, T.R. Sana, T.L. Ciardelli, Coiled-coil molecular recognition: directed solution assembly of receptor ectodomains, Protein Eng. 7 (1994) 1137–1144.
[45] Z. Wu, K.W. Johnson, B. Goldstein, Y. Choi, S.F. Eaton, T.M. Laue, T.L. Ciardelli, Solution assembly of a soluble, heteromeric, high affinity interleukin-2 receptor complex, J. Biol. Chem. 270 (1995) 16039–16044.
[46] B. Tripet, L. Yu, D.L. Bautista, W.Y. Wong, R.T. Irvin, R.S. Hodges, Engineering a de novo-designed coiled-coil heterodimerization domain off the rapid detection, purification and characterization of recombinantly expressed peptides and proteins, Protein Eng. 9 (1996) 1029–1042.
[47] H. Chao, D.L. Bautista, J. Litowski, R.T. Irvin, R.S. Hodges, Use of a heterodimeric coiled-coil system for biosensor application and affinity purification, J. Chromatogr. B. Biomed. Sci. Appl. 715 (1998) 307–329.
[48] B. Catimel, M.C. Faux, M. Nerrie, J. Rothacker, L.J. Otvos, J.D. Wade, E.C. Nice, A.W. Burgess, The use of coiled-coil interactions for the analysis and micropreparative isolation of adenomatous polyposis coli protein complexes, J. Pept. Res. 58 (2001) 493–503.
[49] J.R. Moll, S.B. Ruvinov, I. Pastan, C. Vinson, Designed heterodimerizing leucine zippers with a ranger of p*I*s and stabilities up to 10(-15) M, Protein Sci. 10 (2001) 649–655.
[50] P.J. Willcox, C.A. Reinhart-King, S.J. Lahr, W.F. DeGrado, D.A. Hammer, Dynamic heterodimer-functionalized surfaces for endothelial cell adhesion, Biomaterials 26 (2005) 4757–4766.