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Expression of Recombinant Epidermal Growth Factor in *E. coli*

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Epidermal growth factor (EGF) known as a urgastrone is a powerful mitogen with a wide variety of possibilities for medical usages. A mature EGF coding region was isolated from human prepro-EGF sequence by a conventional PCR and cloned into pQE vector in which the gene product was supposed to be expressed with 6× His tag for the subsequent purification. The recombinant mature EGF was expressed in M15[Rep4], an *Escherichia coli* host strain, in amount of 30-40% of total proteins present in *E. coli* extract by the addition of isopropyl-thio-β-galactopyranoside (IPTG). The recombinant EGF purified using a Ni^{2+}-NTA affinity column chromatography was active in its ability to induce phosphorylation on tyrosine residues of several substrate proteins when murine NIH3T3 and human MRC-5 fibroblast cells were stimulated with it. This work may provide the basic technology and information for the production of recombinant EGF.

Key words: recombinant EGF, pQE, tyrosinephosphorylation

INTRODUCTION

EGF, a single-chain polypeptide of 53 amino acid residues with molecular weight of approximately 7,300 Da, was first isolated from submaxillary glands of adult mice and is presently well characterized as a mitogen of epithelial cells [1, 2]. EGF stimulates proliferation of many cell types, mainly those of epithelial and epidermal tissues [3, 4]. Several metabolic events triggered by the growth factor include stimulation of protein and DNA synthesis, stimulation of glucose uptake, induction of ion fluxes and Ca^{2+} transport. Human EGF (hEGF) known as a urogastrone also inhibits gastric acid secretion *in vivo* [2].

In mammalian cells EGF is synthesized as a part of a larger precursor displaying the features of a transmembrane protein, prepro EGF. This precursor contains, in addition to a portion of mature EGF, eight EGF-like repeats which conceivably might have a potential to function as mitogen. PreproEGF is processed in most cases to yield the mature EGF although preproEGF from kidney is also expressed as a transmembrane protein which does not seem to be processed to EGF or other peptides [5].

Here we report production of the recombinant EGF in *E. coli* and establishment of its biological assay system. To this end, a portion of the mature EGF cDNA was isolated and cloned into a pQE vector in which the recombinant EGF protein with 6 x His tag was expressed by adding IPTG and subsequently purified using Ni^{2+}-NTA column. The purified recombinant protein was able to induce *in vivo* stimulation of tyrosine-phosphorylation of intracellular protein substrates.

MATERIALS AND METHODS

Cloning of a Mature EGF cDNA

The human preproEGF cDNA was obtained from Dr. M. Heidaran, NIH, USA and used as the template. To isolate a portion of mature EGF, polymerase chain reaction (PCR) was performed using two primers: upstream primer, ccggatccAATAGTGACTCTGAATG-T (3347-3364) and downstream primer, ccaagcttGCGC-AGTT CCCACCACTT (3505-3488) [5], where lower case letters indicate restriction sites for cloning. PCR reaction contained template DNA (10 ng/μl), 100 pmol upstream primer, 100 pmol downstream primer, 2.5 mM dNTPs each, 1.5 mM $MgCl_2$, and 2.5 units of Taq polymerase (Boehringer Mannheim). The reaction was carried out using thermocycler (Perkin Elmer) with 30 cycles (1 min, 95°C, 2 min, 55°C, and 3 min, 72°C). The expected PCR product (175 bp) was cloned into *Bam*H1 and *Hin*dIII site of pQE30 vector (Qiagen). The cloned gene was sequenced to confirm that there is no base substitution or deletion.

Induction and Purification of Recombinant EGF

A recombinant plasmid, pQE30(hEGF), was transformed into a *E. coli* strain, M15[Rep4] [6], which overexpresses lac repressor (*lac* I^q, *amp*^{r}) and the cells were grown at 37°C in Luria-Bertani (LB) medium containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml) to A_{600} of 0.7-0.9. The cells were then treated with or without 2 mM IPTG (Sigma), grown for an additional 3 h at 37°C and harvested by centrifugation at 5,000 rpm for 20 min. The cell pellet was dissolved in lysis buffer (100 mM Tris · HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 20% glycerol) for SDS-PAGE. Alternatively, the cell pellet was suspended in 8M urea buffer (100 mM PO_4, 10 mM Tris · HCl, pH 8.0) and stirred at room temperature for 1 hr. The supernatant was collected by centrifugation at 10,000× g for 15 min, added to a 50% slurry of Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) agarose (Qiagen) which has been preequilibrated in the same buffer, and binding was carried out under stirring con-

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dition at room temperature for 45 min. Then the mixture was loaded into a 10 mm diameter glass column (Bio-Rad). After being washed sequentially with 3 column volumes of pH 8.0 and pH 6.3 8M urea buffer (100 mM PO_4, 10 mM Tris·HCl), the proteins were eluted with 8M urea buffer containing 250 mM imidazole (pH 6.3) (Sigma). The pooled fractions were dialyzed to remove urea for assay of biological activity.

Tissue Culture

Mouse NIH3T3 fibroblast cells and human lung fibroblast cells, MRC-5 cells, have been utilized to measure event of tyrosine-phosphorylation [6, 7], and become quiescent upon starvation of serum [5]. These cells were grown to confluency at $0.5\text{-}1.0\times 10^5$ cells per cm^2 on 60 mm dish (Costar) in Dubecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GibcoBRL) and shifted to serum free medium overnight. The cells were stimulated at various concentrations of the purified recombinant EGF and incubated for different time periods. Commercial recombinant EGF and platelet-derived growth factor (PDGF-BB) (GibcoBRL) were used as controls. The cells were lysed in lysis buffer (50 mM Tris pH 7.4, 1% NP-40, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF) and then the cell lysates were subjected on SDS-PAGE.

Immunoblot Analysis of the Recombinant EGF and Phosphorylated Substrates

The recombinant EGF and the cell lysates from cells treated with or without the recombinant EGF were transferred electrophoretically to 0.45 μm nitrocellulose membrane (Schleicher & Shuell). The membrane was then incubated overnight with blocking solution 5% (w/v) nonfat dried milk, 0.01% antifoam A, 0.02% sodium azide, 0.02% Tween 20 in Tris-buffered saline (TBS), washed with TBS containing 0.1% Tween 20 (TTBS), and incubated for 2 h at room temperature with anti-phosphotyrosine antibody (200 fold dilution) or anti-hEGF antibody (Boehriner Mannheim). The protein bands were visualized in the presence of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) following incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (diluted 1/1000 in blocking solution) (Zymad).

RESULTS AND DISCUSSION

Isolation, Cloning and Expression of a Mature EGF cDNA

A portion of mature EGF cDNA (159 bp) (lane 1 in Fig. 1) was isolated from human prepro EGF cDNA [5] by a PCR and was ligated with pQE30, an *E. coli* expression vector used in this experiment. The recombinant plasmid (lane 2 in Fig. 1) was introduced into M15[Rep4]. The recombinant EGF protein was then expressed by addition of 2 mM IPTG (lane 2 in Fig. 2a) for 3 h. No detectable expression was observed from uninduced cells (lane 1 in Fig. 2a). The protein species was immunoreactive to anti-EGF antibody as evidenced by the immunoblotting analysis (lane 2 in Fig. 2b), confirming that the expressed protein is EGF. The molecular weight of the recombinant protein was approximately 7,000 Da which is the same as a commercial recombinant EGF (GibcoBRL) used as a control

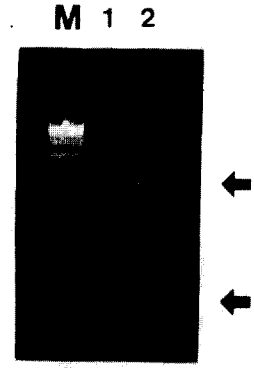

Fig. 1. PCR of mature EGF cDNA and cloning the PCR product into pQE30. M: λ *Hind*III marker, lane 1: PCR product, 159 bp, lane 2: digestion of recombinant EGF cDNA with *Bam*H1 and *Hind*III.

(lane 3 in Fig. 2b). In addition, the production kinetics showed that the protein was expressed optimally in 3 h postinduction (data not shown).

Purification of Recombinant EGF

The recombinant EGF contains 6× His tag on N-terminus which allows us to purify the protein through a single affinity chromatography using Ni^{2+}-NTA resin ($Kd=10^{-13}$, pH 8.0). The purification was followed as manufacturers' instruction. The bacterial cells were cultured in 500 ml scale of LB medium containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml) in 4 liter-flask. The cell pellet was treated with 8 M urea buffer

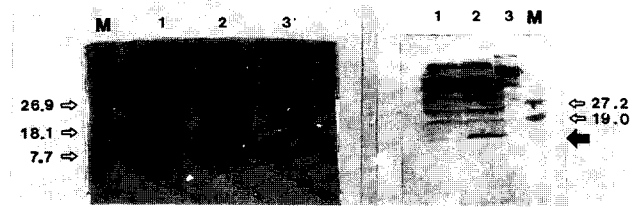

Fig. 2. SDS-PAGE of recombinant EGF: The cells harboring the recombinant plasmids were treated with IPTG (2 mM) for 3 h, and the cell lysates were subjected to SDS-PAGE followed by (a) Coomassie Brilliant Blue staining and (b) immunoblot analysis. M: prestained molecular weight marker (Bio-Rad), lane 1: uninduced cells, lane 2: induced cells, lane 3: commercial recombinant EGF (GibcoBRL).

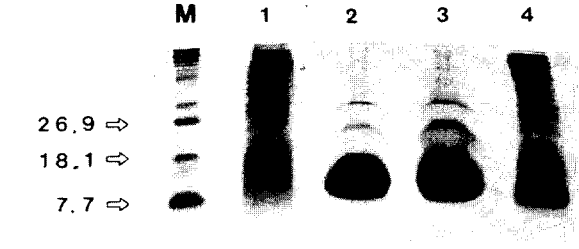

Fig. 3. Purification of the recombinant EGF protein. 500 ml of cultured cells was treated 8M urea buffer and the cell extract was applied to Ni^{2+}-column chromatography. M: prestained molecular weight marker, lane 1: uninduced cells, lane 2: fraction 3, lane 3: fraction 4, lane 4: induced cells

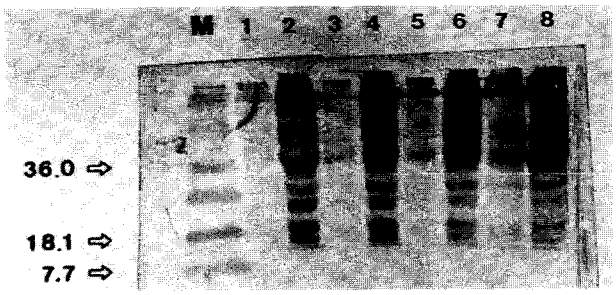

Fig. 4. Immunoblot analysis of phosphotyrosine proteins from cells stimulated with the recombinant EGF for 10 min. The complete monolayered cells (1×10^6 cells/dish) were incubated in serum-free DMEM medium overnight and treated with the purified recombinant EGF (10 μl), PDGF-BB (10 ng/ml), and commercial rEGF (10 ng/ml) for 10 min. The cells were then lysed with lysis buffer and the cell lysates (20 μl) were subjected to immunoblot analysis with anti-phosphotyrosine antibody. Even number of lanes: NIH3T3 cells, odd number of lanes: MRC-5 cells, lane 1 and 2: unstimulated cells, lane 3 and 4: PDGF-BB, lane 5 and 6: commercial-rEGF, lane 7 and 8: purified-recombinant EGF.

(100 mM PO_4, 10 mM Tris·HCl, pH 8.0) and the proteins were fractionated through Ni^{2+}-NTA column by eluting with 250 mM imidazole (pH 6.3). The selected fractions of eluted protein were subjected to SDS-PAGE (lane 2 and 3 in Fig. 3), demonstrating that the recombinant EGF was of high purity more than 90% as measured by densitometer. However, more than 90% of protein fractions were insoluble when the pooled fractions were dialyzed in urea free buffer, suggesting that the eukaryotic protein is stored in inclusion body once it is synthesized.

Biological Activity of the Recombinant EGF

The biological activity of the recombinant EGF is measured by its ability to induce tyrosine-phosphorylation of intracellular protein substrates upon exposure of EGF to cells. NIH3T3 and MRC-5 fibroblast cells were cultured in DMEM containing 10% FBS and then starved overnight by shifting to serum free medium. The cultures were treated at various concentrations of the purified recombinant EGF for different time intervals. After being treated, the cells were lysed, and the cell lysates were subjected to immunoblot analysis using an anti-phosphotyrosine antibody. Tyrosine-phosphorylated proteins were much more intense numerous in NIH3T3 cells than in MRC-5 cells when the cells were exposed to growth factors for 10 min, yet they did not seem to be strictly dependent on the presence of growth factors used (even lanes in Fig. 4). However, considerable increases in number and amount of tyrosine-phosphorylated proteins were observed in the purified recombinant-treated cells (lane 7 in Fig. 4) as compared with the commercial recombinant EGF (10 ng/ml)-treated cells (lane 5 in Fig. 4). This result seems to be significant because only small fraction (less than 10%) of the recombinant EGF purified in our experiment was soluble and the small amount (10 μl) of it (6000 μl) was used to stimulate cells. Little difference in phosphorylation patterns between stimulated and unstimulated cells allowed us to examine whether a longer exposure (5 days) to the growth factors might make difference in extent of

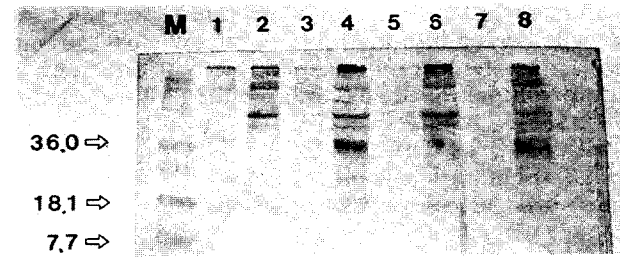

Fig. 5. Immunoblot analysis of phosphotyrosine proteins from cells stimulated with the recombinant EGF for 5 days. The procedure was the same as in Fig. 4 except for incubation period 5 days in place of 10 min. Even number of lanes: NIH3T3 cells, odd number of lanes: MRC-5 cells, lane 1 and 2: unstimulated cells, lane 3 and 4: PDGF-BB, lane 5 and 6: commercial-rEGF, lane 7 and 8: purified-recombinant EGF.

stimulation. As shown in Fig. 5, the number and intensity of tyrosine-phosphorylated proteins were indeed increased when NIH3T3 cells were stimulated with the purified recombinant EGF (compare lane 8 and lane 4 in Fig. 5). PDGF-BB was used as a control and also had a substantial effect on the phosphorylation of proteins in NIH3T3 cells (compare lane 6 and lane 4 in Fig. 5). This data indicate that the purified recombinant EGF is produced as biologically active form. Further study, however, is needed to optimize rEGF production.

Like most of bacterially expressed recombinant proteins derived from eukaryotes, the recombinant EGF was expressed in a form of inclusion body and 90% of the protein was not solubilized. In order to maximize the solubility of the recombinant EGF, the refolding of the protein should be carried out by gradual dilution of the denaturing agents, together with careful reformation of the disulfide bridges. It is also necessary to establish the optimal physiological conditions for the refolding of the recombinant EGF using a commercial recombinant EGF. Furthermore induction of expression in our system is carried out by use of IPTG which is too expensive to scale up as the case of economic production. EGF gene, therefore, should be cloned to other expression vectors with promoters such as heat shock promoter and tryptophan promoter for the purpose of mass production.

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