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Construction and characterization of a fusion protein with epidermal growth factor and the cell-binding domain of fibronectin

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An efficient expression system was constructed for C-EGF, a fusion protein made of a fragment of the cell-binding domain of human fibronectin (FN) bound with epidermal growth factor (EGF). C-EGF was produced in *Escherichia coli* HB101 cells carrying the recombinant plasmid pCE102 as inclusion bodies, which were solubilized and refolded after purification. C-EGF had both cell-adhesive and EGF activities, so it might be more effective than EGF in therapeutic applications. This fusion system would be useful for the construction of a recombinant drug delivery system for cells that have fibronectin receptors (integrins).

Fibronectin; Epidermal growth factor; Fusion protein; Recombinant plasmid; Drug delivery system

1. INTRODUCTION

Fibronectins (FN) are multifunctional cell-adhesive glycoproteins present in plasma and the extracellular matrix. FN contains several functional domains, including the cell-binding domain, which contributes to the cell-adhesive function of FN. The tetrapeptide Arg-Gly-Asp-Ser (RGDS) has been identified as a recognition signal for cell adhesion [1]. The strength of cell-adhesive activity is related to the number of type III homology repeats within the cell-binding domain, and the effect of contribution by the repeats is inversely related to their distance from the RGDS sequence [2]. Based on these findings, we have constructed an efficient system for the expression in *Escherichia coli* of a 30-kDa fragment of the cell-binding domain of FN, and used the system to express a fusion protein of the cell-binding domain and the heparin-binding domain of FN [3]. Fusion proteins of the cell-binding domain and biologically active peptides such as growth factors, lymphokines, neuropeptides, or hormones could be useful as a drug delivery system (DDS) for target cells that have FN receptors (integrins).

Here, we constructed an expression plasmid for a fusion protein of the cell-binding domain of human FN and epidermal growth factor (EGF), which might be more useful in therapeutic applications than unfused EGF. *E. coli* HB101 cells carrying the plasmid overproduced the fusion protein as inclusion bodies, which were solubilized and refolded in active form. This expression system allows the efficient expression of unstable smaller polypeptides as fusion proteins and will be useful for the construction of a DDS, particularly for growth factors.

Abbreviations: FN, fibronectin; EGF, epidermal growth factor; DDS, drug delivery system; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Construction of a recombinant plasmid (Fig. 1)*

Four synthetic DNAs (EGF-1, -2, -3, and -4) were designed and synthesized to construct the human EGF gene with an DNA synthesizer (Applied Biosystems Inc., Foster, CA). EGF-1 (sense strand, 84-mer) and EGF-2 (antisense strand, 86-mer), which together correspond to the N-terminal-half of EGF, were annealed (1.5 μg each) after phosphorylation of the 5′-terminus of EGF-2. In a similar way, EGF-3 (sense strand, 88-mer) and EGF-4 (antisense strand, 86-mer), which together correspond to the C-terminal half of EGF, were annealed. The two resultant double-stranded DNAs were mixed and ligated in 80 μl of solution with 450 units of T4 DNA ligase at 16°C for 30 min. The resultant 172-bp fragment was isolated by agarose gel electrophoresis, giving the EGF gene with compatible ends; 1.0 μg of this was then phosphorylated and ligated with 1.0 μg of a expression vector, pUC118NT [3], previously digested with *Nco*I and *Eco*RI. A recombinant plasmid, pUC118NT-EGF, that gave a 172-bp *Nco*I-*Eco*RI fragment was isolated from *E. coli* cells transformed with the ligation products. The nucleotide sequence of the EGF gene was confirmed by the dideoxy chain termination method [4]. A 1.07-kb *Nco*I-*Sal*I fragment was isolated from pUC118NT-EGF and inserted into the *Nco*I-*Sal*I site of pTF7520 [2], which expresses the cell-binding domain of human FN, giving a plasmid, pCE102.

2.2. *Protein purification and refolding*

E. coli HB101 cells carrying pCE102 were grown in L broth supplemented with 50 μg/ml ampicillin as described elsewhere [2]. The cell pellet obtained from a 2-liter culture was suspended in 50 ml of buffer

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A (50 mM Tris-HCl, pH 8.0, 25% sucrose, and 1 mM EDTA) and incubated with 4 mg of lysozyme at 4°C for 30 min followed by sonication. To the disrupted cell suspension, 150 μl of 1.0 M $MgCl_2$ and 7,500 units of DNase I were added and the mixture was incubated at 37°C for 30 min. After the addition of 10 ml of buffer B (20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1.0% deoxycholate, and 1.0% NP-40), the insoluble material was isolated as inclusion bodies by centrifugation. The inclusion bodies were washed first with buffer C (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, and 0.5% Triton X-100), secondly with 20% ethylene glycol, and finally with 2.0% NP-40. The resultant inclusion body (450 mg as wet weight) was dissolved in 25 ml of buffer D (20 mM Tris-HCl, pH 8.0, 250 mM DTT, and 6 M urea), and put on a column of DEAE-Toyopearl 650S (15 ml) equilibrated with 20 mM Tris-HCl (pH 8.0) containing 10 mM DTT. After the column was washed with the same buffer, C-EGF was eluted with a stepwise increase in NaCl concentrations. Two monoclonal antibodies, FN12-8 [5] and anti-EGF (Wakunaga, Osaka, Japan), were used for the detection of the fusion protein by immunoblotting. Pure fractions by SDS-PAGE were pooled and dialyzed for 40 h at 4°C against 20 mM Tris-HCl (pH 7.5) containing 1.0 mM reduced glutathione and 0.1 mM oxidized glutathione. The refolded protein thus obtained was then dialyzed against phosphate-buffered saline (PBS) and lyophilized.

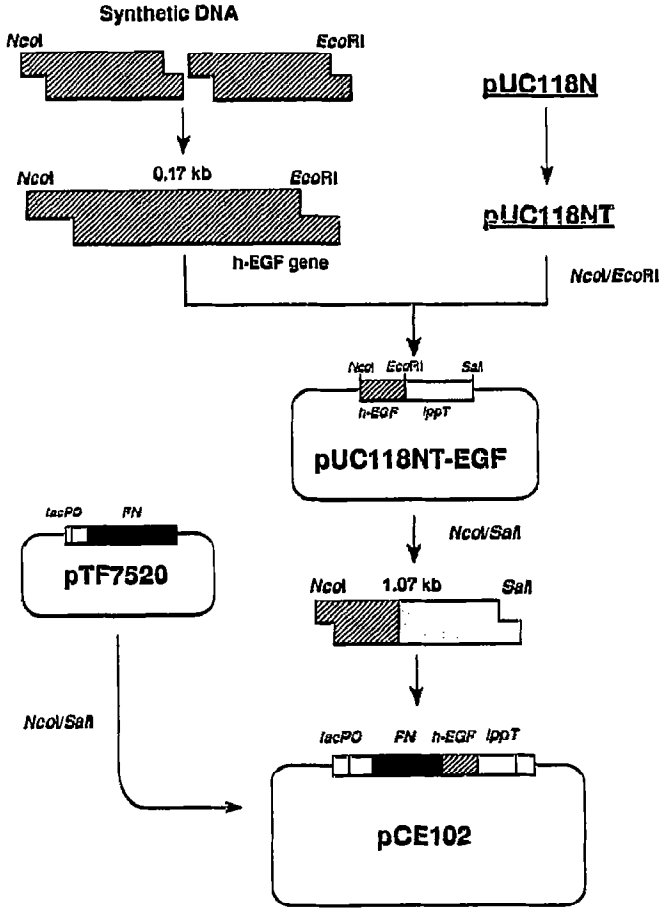

Fig. 1. Scheme for the construction of the expression plasmid. A synthetic human EGF gene with cloning sites at both ends was constructed from four synthetic oligonucleotides and subcloned into pUC118N with a transcription terminator (*lppT*), giving pUC118NT-EGF. This was digested with *NcoI-SalI* and the resultant 1.07-kb fragment was inserted into the *NcoI-SalI* site of pTF7520, giving pCE102, which expresses C-EGF under the control of the *lac* promoter (*lacPO*).

2.3. *Assay of EGF activity*

EGF activity was assayed essentially as described by Cohen and Carpenter [6]. Briefly, cultured NRK-49F cells were diluted to 5×10^4/ml with DMEM supplemented with 10% FCS and put into a 96-well microtiter plate (200 μl per well). After incubation of the plate at 37°C for 3 days, 40 μl of a PBS solution of the sample was added to each well. After incubation of the plate for 18 h longer, 20 μl of [^{3}H]thymidine (0.1 μCi) was added to each well and the plate was incubated for 6 h. The plate was washed twice with PBS and then incubated with 100 μl per well of 0.1% trypsin for 2 h. The trypsinized cells were trapped on a glass filter and dried, and the radioactivity of the cells was counted by a liquid scintillation counter.

2.4. *Other*

Identification of the N-terminal amino acid sequence [2] and the C-terminal amino acid [3] were described elsewhere, as was the cell-adhesion assay [2].

3. RESULTS AND DISCUSSION

The strategy for the construction of the expression plasmid is shown in Fig. 1. The plasmid pCE102 coded for a fusion protein of the cell-binding domain of human FN (Pro^{1239}–Ser^{1515}, with 277 amino acids, numbered by the system of Kornblihtt et al. [7]) bound via Met-Ala with human EGF. *E. coli* HB101 cells carying the plasmid pCE102 produced a 35-kDa protein, which was detected by SDS-PAGE of the whole-cell lysate and by immunoblotting with use of two monoclonal antibodies as described in Materials and Methods. The 35-kDa protein, which we named C-EGF, accounted for about 20% of the total cell protein by densitometric analysis and was produced as inclusion bodies. The inclusion bodies were washed extensively, solubilized with a buffer that contained 6 M urea, and then purified by ion-exchange chromatography.

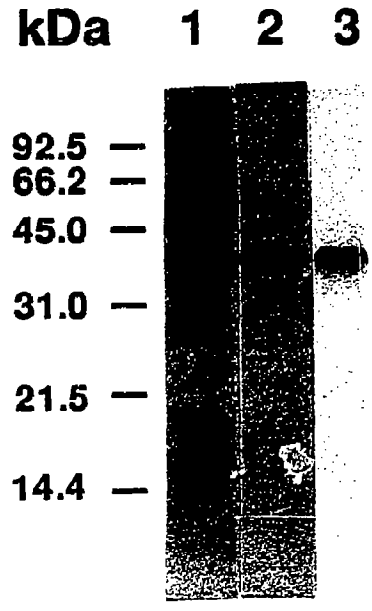

Fig. 2. Purification profile of C-EGF by SDS-PAGE. A portion of the sample in each purification step was put on a 15% gel and stained with Coomassie brilliant blue. Lane 1, whole-cell lysate; lane 2, inclusion bodies; lane 3, purified C-EGF.

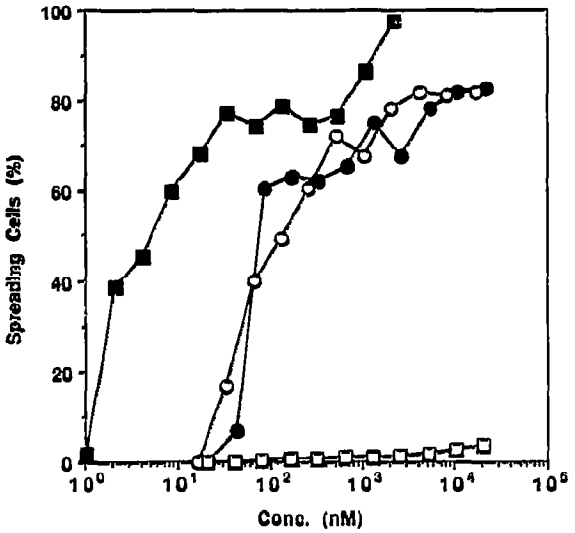

Fig. 3. Cell-adhesive activity of C-EGF. Cell-adhesive activity of C-EGF was assayed as described elsewhere [2] and compared with activities of native FN and C-279. Open circles, C-279; closed circles, C-EGF; closed squares, FN; open squares, EGF.

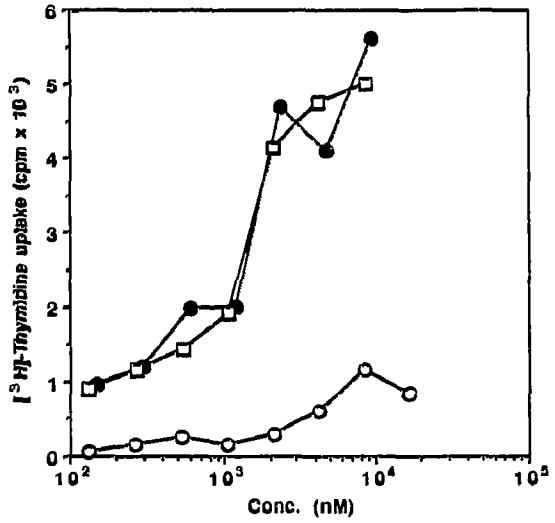

Fig. 4. EGF activity of C-EGF. The EGF activity of C-EGF was assayed as described in Materials and Methods and compared with the activities of C-279 and native EGF. Open circles, EGF; closed circles, C-EGF; open squares, C-279.

The purification profile of C-EGF is shown in Fig. 2. The N-terminal amino acid sequence and the C-terminal amino acid of the purified protein were identical with those predicted. The purified C-EGF was refolded as described in Materials and Methods and assayed for biological activities. C-EGF had the same amount of cell-adhesive activity as an unfused fragment of the cell-binding domain, C-279 (Pro^{1239}–Met^{1517}, with 279 amino acids [2]; Fig. 3). The EGF activity of C-EGF was also compared with that of recombinant human EGF (Wakunaga; Fig. 4). C-EGF was almost the same as human EGF in its stimulation of [^{3}H]thymidine uptake, suggesting that C-EGF contains the native form of EGF. EGF had no cell-adhesive activity, and C-279 had no EGF activity (Figs. 3 and 4).

These results indicate that C-EGF had both cell-adhesive activity and EGF activity, each of which was indistinguishable from that of the corresponding unfused protein. Small biologically active peptides such as growth factors, peptide hormones, or neuropeptides are often unstable in host cells when produced directly in *E. coli*; EGF is particularly unstable. Stable and efficient expression of EGF was obtained by fusion of the sequente to the C-terminus of the cell-binding domain of FN. This fusion protein would probably be more effective in therapeutic uses (including wound healing) than native EGF, because the moiety of the cell-binding domain could act as an anchor by binding to target cells that have FN receptors (integrins). Our expression system should be useful for the construction of other DDS for biologically active peptides.

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