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IDENTIFICATION OF A POLYPEPTIDE SECRETED BY HUMAN BREAST CANCER CELLS (MCF-7) AS THE HUMAN ESTROGEN-RESPONSIVE GENE (pS2) PRODUCT

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Human epidermal growth factor-like immunoreactive factor (designated as EGF-LI) synthesized and secreted by human breast cancer cells, strain MCF-7, was isolated in pure form. Thirty-seven micrograms of EGF-LI was purified by anion-exchange, gel permeation, and reverse-phase high-performance liquid chromatography from 2 liters of serum-free medium conditioned by the cells. The sequence of the first 36 amino acids from the N-terminus was determined with a gas-phase protein sequencer. Computer-assisted screening revealed, quite unexpectedly, this sequence to be completely identical to that of the translational product encoded by pS2, the human estrogen-responsive gene, over the region extending from residue 25 to 60 (Jakowlew, S. B. et al.(1984) Nucleic Acids Res., 12, 2861-2878).

For many years, transformed cells have been shown to require fewer serum or exogenous growth factors for optimal proliferation than do their normal counterparts. The relatively autonomous growth of transformed cells has been postulated to result from their production of and response to their own growth factors. Thus this mechanism, which underlies autocrine systems, has received much attention (1). For instance, MCF-7 cells, a strain of human breast cancer cells, are known to secrete transforming growth factor (both type-α and type-β) (2), insulin-like growth factor I (3), and platelet-derived growth factor (4).

We recently developed a sensitive enzyme immunoassay system for human epidermal growth factor (hEGF) (5) in order to obtain more information about

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Abbreviations used: EGF, epidermal growth factor; EGF-LI, epidermal growth factor-like immunoreactive factor; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; PTH-, phenylthiohydantoin derivatives

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the relationship between EGF and malignant transformation. With this assay we screened conditioned media of various cancer cells for the presence of materials immunoreactive to anti-hEGF antibodies and found that certain cancer cells, i.e., MCF-7 cells and human gastric cancer cells (MKN-45 and KATO-III), synthesized and secreted a large amount of hEGF-like immunoreactive factor (EGF-LI) in culture (6-8). To investigate the role of EGF-LI in cancer cells, EGF-LI was isolated in pure form from serum-free medium conditioned by MCF-7 cells. Amino acid sequence analysis revealed that EGF-LI is a secretory protein encoded by pS2, the human estrogen-responsive gene (9).

MATERIALS AND METHODS

Materials.

DEAE-Sephadex A-25 and Sephadex G-50 superfine were obtained from Pharmacia; μBondapak C_{18} column (10 μm pore size, 3.9 x 300 mm) from Waters; fetal calf serum, from Bocknek; insulin, from Novo; β-estradiol, from Tokyo Kasei, Co., Ltd. (Tokyo); Dulbecco's modified Eagle's minimum essential medium (DMEM) and HAM F12, from Nissui Pharmaceutical Co., Ltd. (Tokyo). All glasswares were siliconized to prevent nonspecific adsorption.

Cell Culture.

MCF-7 cells were cultured in DMEM supplemented with 10% fetal calf serum, insulin (2×10^{-6}M), β-estradiol (10^{-8}M), glutamine (0.3 mg/ml), penicillin (50 units/ml), and streptomycin (0.05 mg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Medium was changed every other day. When the cells reached the confluent state in Nunc plastic flasks (175 cm^2), the cells were washed with phosphate-buffered saline containing calcium and magnesium ions; and then serum-free medium consisting of DMEM and HAM F12 (1 : 1) supplemented with β-estradiol (10^{-8}M) was added. After a 24 h-incubation, the serum-free medium was collected and clarified by centrifugation at 5,000 rpm for 30 min. The supernatant was stored at -20°C.

Assay for EGF-LI.

Assay of EGF-LI samples was performed using the two site enzyme immunoassay for hEGF described previously (5,6). The limit of detection of EGF-LI was as low as 1 pg/assay tube.

Protein Determination.

Protein was determined by the dye-binding method of Bradford (10) utilizing a Bio-Rad Protein Assay kit. Bovine serum albumin was used as a standard.

Purification of EGF-LI.

The serum-free conditioned medium was dialyzed extensively against 0.05 M ammonium acetate buffer, pH 5.5, using Spectrapor 3 dialysis tubing and applied at a flow rate of 50 ml/hr to a column (1.7 x 43.5 cm) of DEAE-Sephadex A-25 previously equilibrated with the same buffer. The column was washed with the same buffer and then EGF-LI was eluted at the same flow rate with a linear gradient prepared from 500 ml of 0.05 M ammonium acetate buffer (pH 5.5) and 500 ml of 2 M ammonium acetate buffer (pH 5.5). The fractions with hEGF-like immunoreactivity eluted from the DEAE-Sephadex A-25 column were pooled and lyophilized. The lyophilized material was dissolved in 10 ml of 1% acetic acid and applied at a flow rate of 12 ml/hr to a column (2.5 x 100 cm) of Sephadex G-50 superfine equilibrated with 1% acetic acid. The fractions with hEGF-like immunoreactivity were then eluted with the same eluent and at the same flow

rate from the column, pooled, and evaporated to dryness in vacuo. The final purification of EGF-LI was achieved by reverse-phase high-performance liquid chromatography (RP-HPLC) on Shimazu LC-7A system. The evaporated material was dissolved in 100 µl of 10% trifluoroacetic acid (TFA) and applied to a µBondapak C_{18} column at a flow rate of 0.7 ml/min at room temperature. EGF-LI was eluted from the column with a linear 100-min gradient of acetonitrile (0% - 50%) containing 0.1% TFA. The eluate from the column was monitored at 210 nm.

Amino Acid Sequence Analysis.

EGF-LI was automatically sequenced by means of an Applied Biosystem Sequencer (model 470A) equipped with an online PTH-analyzer (model 129A). Polybrene was used as a carrier.

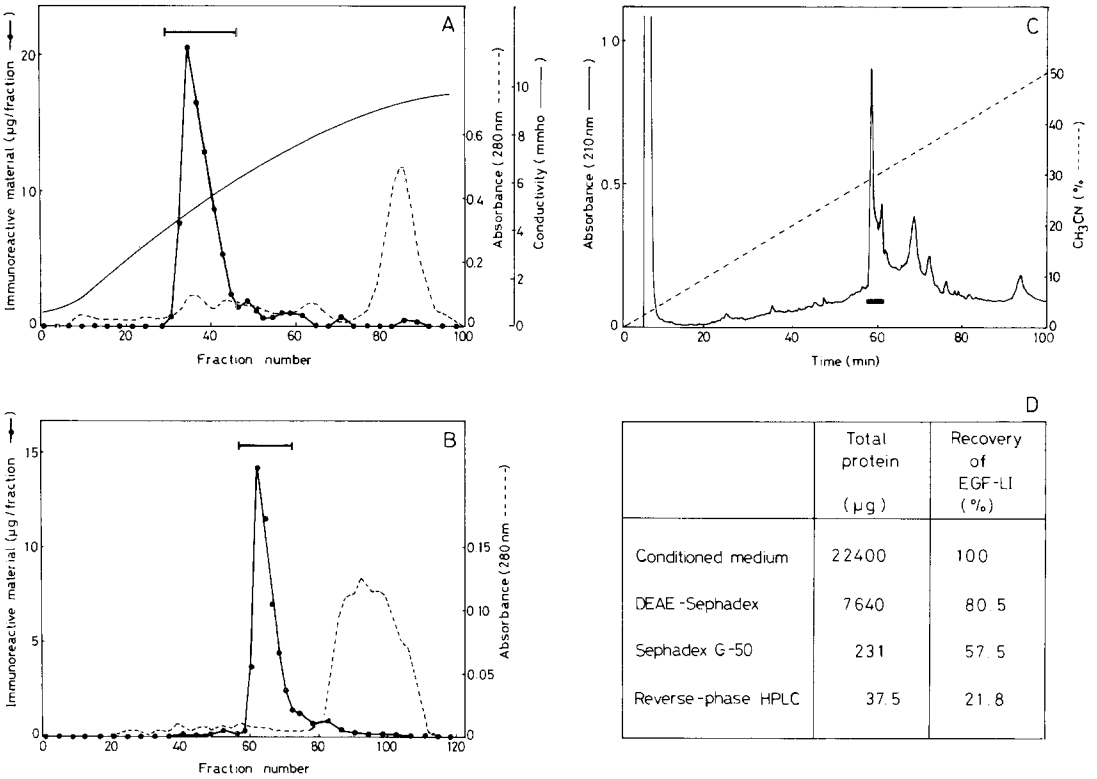
RESULTS AND DISCUSSION

Isolation of EGF-LI.

EGF-LI was isolated from the serum-free medium conditioned by human breast cancer cells, strain MCF-7, because the level of EGF-LI secreted by these cells was highest among that secreted by various cancer cells so far examined. As β-estradiol was found to have an inductive effect on EGF-LI production by MCF-7 cells (11), β-estradiol (10^{-8} M) was added to the medium of serum-free cultures. Since the molecular weight and isoelectric point of EGF-LI were already determined (6), we utilized anion-exchange chromatography using DEAE-Sephadex A-25 at pH 5.5 (Fig. 1A) and gel permeation chromatography using Sephadex G-50 (Fig. 1B) for purification. Final purification was achieved by reverse-phase high-performance liquid chromatography using a µBondapak C_{18} column and acetonitrile containing trifluoroacetic acid as the elution buffer (Fig. 1C). Fig. 1D shows the recovery of protein and EGF-LI at each step. After these procedures, 37 µg of EGF-LI was obtained from 2 liters of the medium. The purity of EGF-LI was more than 97% as evidenced by amino acid sequence analysis.

Amino Acid Sequence of EGF-LI.

The N-terminal amino acid sequence of EGF-LI was determined automatically by applying intact EGF-LI onto a gas-phase protein sequencer. This sequence, determined with 6 µg of EGF-LI obtained by RP-HPLC as a peak with a retention time of 59 min (Fig. 1C), is shown in Fig. 2. We detected only one major peak at each cycle and assigned it as the indicated amino acid. We did not detect any PTH-amino acid at cycles 7, 17, 27, 32, and 33 and assumed them to be Cys residues. The purity of EGF-LI was more than 97% as estimated by relative peak areas of PTH-amino acids liberated at each cycle. The shoulder peak (from the RP-HPLC run) having a retention time of 62 min (Fig. 1C) had the same N-terminal amino acid sequence over 36 amino acid residues and probably resulted from C-terminal heterogeneity of EGF-LI generated during the isolation procedure.



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	Total protein (μg)	Recovery of EGF-LI (%)
Conditioned medium	22400	100
DEAE-Sephadex	7640	80.5
Sephadex G-50	231	57.5
Reverse-phase HPLC	37.5	21.8

Fig. 1. Purification of EGF-LI from Serum-Free Medium Conditioned by MCF-7 Cells.

(A) Anion-exchange chromatography on DEAE-Sephadex A-25. The column eluate was monitored by absorbance at 280 nm (dotted line) and conductivity (thin line). Closed circles show hEGF-like immunoreactive material and the horizontal bar indicates the pooled fractions. The elution profile is shown.

(B) Gel permeation chromatography on Sephadex G-50 superfine. The column eluate was monitored at 280 nm (dotted line). Closed circles show hEGF-like immunoreactivity and the horizontal bar indicates the pooled fractions.

(C) Reverse-phase high-performance liquid chromatography on μ Bondapack C_{18}. The column eluate was monitored at 210 nm (solid line), and the dotted line shows the percentages of acetonitrile in the elution medium. EGF-LI obtained in pure form is indicated by the black bar.

(D) Recovery of protein and EGF-LI at each step of purification. Immunoreactivity present in the 2 liters of serum-free medium is taken as 100%.

Detail conditions of isolation and chromatography were described in MATERIALS AND METHODS.

Identification of EGF-LI as pS2 Protein.

To get a clue as to the functions of EGF-LI, the sequence was compared with data base protein sequences for homologies with known growth factors or oncogene products. As shown in Fig. 3, we found quite unexpectedly that the sequence of the first 36 amino acids from the N-terminus of EGF-LI was completely identical to that of the translational product encoded by pS2 over the

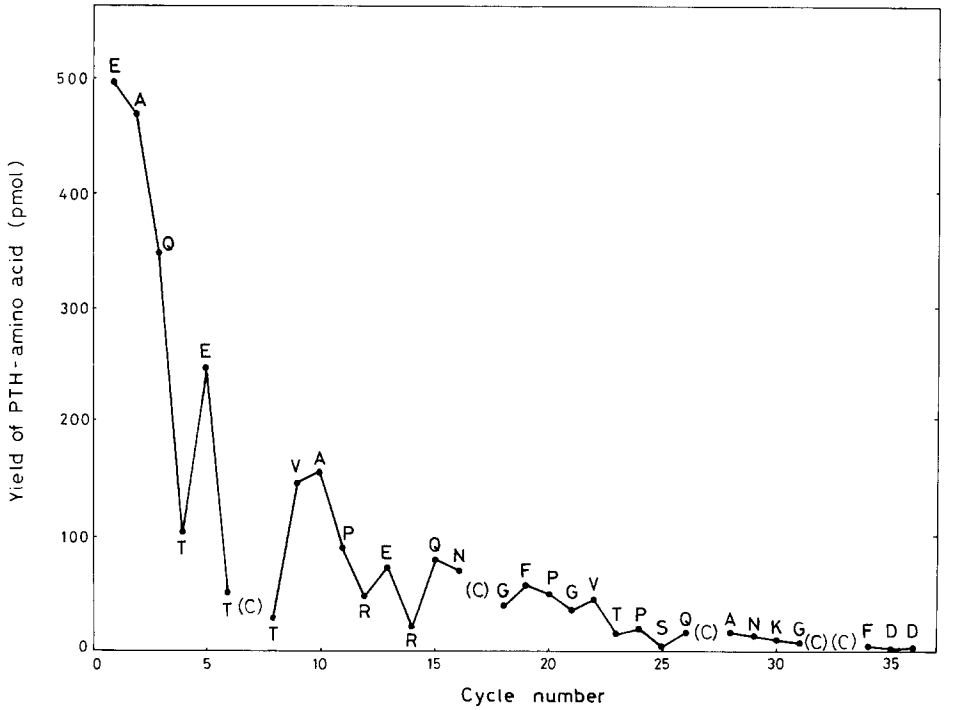

Fig. 2. N-Terminal Sequence Analysis of EGF-LI

The ordinate represents the amount of PTH-amino acid liberated at each cycle of Edman degradation, from which the amount of PTH-amino acid detected at the previous cycle was subtracted. The one-letter amino acid notation is used.

region extending from residue 25 to 60. pS2 is a human gene whose transcription is directly induced by β-estradiol in MCF-7 cells (9). As shown by Jakowlew and co-workers (9) the translational product encoded by pS2 gene is a polypeptide composed of 84 amino acids. The N-terminal sequence of the product contains the typical signal sequence present in secretory proteins. Since the most frequent signal peptide cleavage site has been observed to be that following Ala-X-Ala or that located after the sixth amino acid following

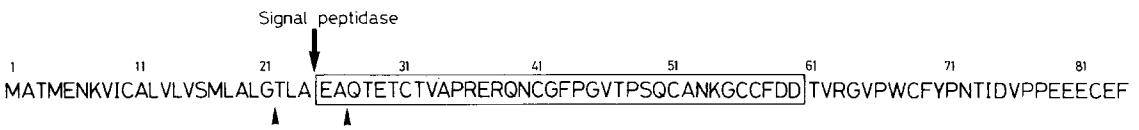

Fig. 3. Identification of EGF-LI as pS2 Protein.

Complete amino acid sequence of the translational product encoded by pS2 gene is given. The open box shows the sequence determined with EGF-LI, which corresponds to residues 25 to 60 of the whole pS2 protein. The arrowheads indicate the signal peptide cleavage sites proposed by Jakowlew, S. B. et al., and the arrow notes the site indicated by our results.

the core sequence, the above group thought that the cleavage by signal peptidase occurs between positions 26 and 27 or between 21 and 22, resulting in a secreted protein composed of 58 or 63 amino acids, respectively (sites marked by arrowheads in Fig. 3). Recently they characterized the pS2 protein in more detail using antibody raised against synthetic polypeptide corresponding to 31 amino acids from the C-terminus of the pS2 protein. Because pS2 protein immunoprecipitated from the medium conditioned by MCF-7 cells was labelled with ^{35}S-cysteine but not with ^{35}S-methionine and ^{14}C-leucine, they proposed that the pS2 protein is secreted as a 58-amino acid polypeptide (12). EGF-LI purified by us always extended from position 25; however, the signal peptide cleavage site is between the first Ala and Glu of the Ala-X-Ala sequence (marked by the arrow in Fig. 3). This cleavage site is not contradictory to their differential amino acid labelling experiments at all. Thus our results indicate that the pS2 gene product is secreted as a 60-amino acid polypeptide in MCF-7 cells. The value explains satisfactorily the behavior of EGF-LI on a column of Sephadex G-50, where EGF-LI eluted slightly faster than hEGF, which is composed of 53 amino acids. EGF-LI contains more acidic amino acids (7 Glu + 3 Asp) than basic amino acid (1 Lys + 3 Arg), being in good agreement with its isoelectric point (pI 4.3) estimated by isoelectric focusing analysis (11). EGF-LI contains 7 half-cystines which are distributed throughout the sequence. If 6 of the 7 half-cystines form three disulfide linkages, the resulting molecular weight of EGF-LI is 6,661.

The hEGF used for immunization was purified by RP-HPLC, and the purity was checked by polyacrylamide gel electrophoresis, amino acid composition analysis, and radioreceptor assay. The detection of EGF-LI (pS2 protein) by our enzyme immunoassay for hEGF is probably due to minor contamination (less than 5%) of EGF-LI in the hEGF sample used for immunization because both the molecular weight and isoelectric point of EGF-LI and hEGF are quite similar. Recently we found that hEGF but not EGF-LI is bound to Benzamidine-Sepharose 6B column and antibody raised against hEGF purified from urine by procedures including Benzamidine-Sepharose 6B chromatography did not crossreact with EGF-LI at all. So Benzamidine-Sepharose 6B chromatography is a critical step for purification of hEGF from urine.

Davidson *et al.* (13) examined the expression of the pS2 gene in two variants of MCF-7 cells. In the variant cell line I13, cell growth was inhibited but the pS2 mRNA was still induced by β-estradiol. And in variant cell line LY2, the cells were resistant to the growth-inhibitory effects of the antiestrogen, LY117018, but expression of the pS2 gene was inhibited by LY117018. From these results, they concluded that the pS2 protein is not the major autocrine growth-stimulating agent. The physiological role of pS2 protein in breast cancer cells still remains unclear.

This report is the first identification of the translational product of pS2, human estrogen-responsive gene, as a 60-amino acid polypeptide in human breast cancer cell strain MCF-7. Investigation of the biological activity and determination of the secondary structure of pS2 protein are now in progress.

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