J. Biochem. Mol. Biol. Vol. 28, No. 2 pp. 87~93 March 31, 1995

Chemical Synthesis and Determination of Biological Activity of the Epidermal Growth Factor-Like Domain of Mouse Betacellulin

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(Received July 29, 1994)

Abstract: To investigate the biological functions of the EGF-like domain of mouse betacellulin (BTC), mouse BTC(33-80), a 48-residue peptide corresponding to the EGF-like domain, was synthesized by stepwise solid-phase methods using a 9-fluorenylmethoxycarbonyl (Fmoc) strategy. The homogeneity of synthetic mouse BTC(33-80) was confirmed by analytical reversed phase (RP)-HPLC, amimo acid analysis, and fast atom bombardment mass spectrometer (FAB-MS). Three disulfide bond pairings of synthetic mouse BTC(33-80) were established by amino acid analysis of cysteine-containing fragments derived from thermolytic digestion. These were consistent with the pairings of EGF and transforming growth factor (TGF-α). The EGF-like domain of mouse BTC showed equipotent activity in both EGF-receptor binding on A-431 epidermoid carcinoma cells, and mitogenesis on NIH-3T3 fibroblast cells, as compared with authentic h-EGF. Results suggest that the EGF-like domain of BTC plays a significant role in mitogenic activity with an EGF-receptor mediated system.

Key words: betacellulin, binding activity, EGF-like domain, mitogenic activity, peptide synthesis.

Betacellulin (BTC) is a novel peptide growth factor isolated from a conditioned medium of the pancreatic β tumor cell line, which was derived from a transgenic mouse expressing the SV40 large T gene under the control of the insulin promoter (Shing *et al.*, 1993). Mouse BTC is a 32-KDa glycosylated protein composed of 80 amino acid residues, and a new member of the epidermal growth factor (EGF) family of peptides including EGF itself (Cohen, 1962; Savage *et al.*, 1972; Gregory, 1975; Carpenter and Wahl, 1990), and the transforming growth factor (TGF-α) (DeLarco and Todaro, 1978; Marquardt *et al.*, 1983; Derynck *et al.*, 1984). Mouse BTC is a potent mitogen for Balb/c 3T3 fibroblast cells, retinal pigment epithelial cells, and vascular smooth muscle cells (Shing *et al.*, 1993; Sasada *et al.*, 1993). This growth factor contains the EGF-like domain that shares a high sequence homology with members of the EGF family in the carboxy terminal portion. Six conserved cysteine residues that are shared by growth factors belonging to the EGF family of peptides are involved in the EGF-like domain (Schoyab *et al.*, 1989; Kimura *et al.*, 1990; Higashiyama *et al.*, 1991). However, little is known about the functional role of the EGF-like domain of BTC.

In this investigation of the biological properties of the EGF-like domain of BTC, mouse BTC(33-80) corresponding to the EGF-like domain was synthesized by stepwise solid-phase method (Merrifield, 1963) based on a 9-fluorenylmethoxycarbonyl (Fmoc) strategy. The biological activities of mouse BTC(33-80) were evaluated by a colorimetric MTT (tetrazolium) assay (Mosmann, 1983; Scudiero *et al.*, 1988) in NIH-3T3 fibroblast cell lines and by binding assay (Kuppuswamy and Pike, 1989) to the EGF receptor on human epidermoid moid carcinoma A-431 cells.

Materials and Methods

Chemical reagents

Fmoc-amino acids were purchased from Watanabe Chemical (Osaka, Japan). Benzotriazole-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP) was obtained from Kokusan Chemical Works (Tokyo, Japan). Diisopropylethylamine (DIEA), 1-methyl-2-pyrrolidone (NMP), 4-dimethylaminopyridine (DMAP), O-

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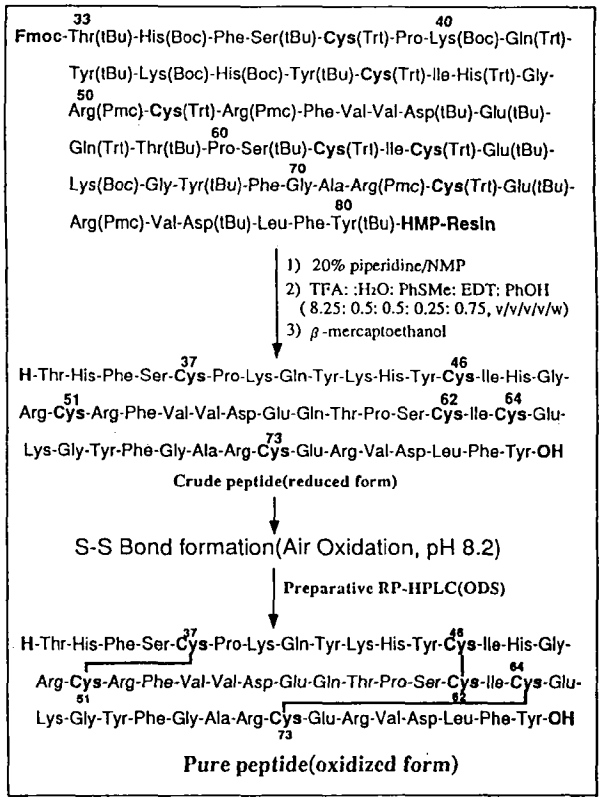
benzotriazole-1-yl-tetramethyluronium hexafluorophosphate (HBTU), hydroxymethylphenoxy (HMP)-resin, and piperidine were purchased from Applied Biosystems (Foster city USA). Human-EGF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phenol, 1,2-ethanedithiol (EDT), thioanisole, and benzonic anhydride were supplied by Wako Pure Chemical (Osaka, Japan). Dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) were obtained from Peptide Institute (Osaka, Japan).

Cell lines

Mouse fibroblast cells (NIH/3T3) and human epidermoid carcinoma (A-431) cells were purchased from the American Type Culture Collection and Riken Cell Bank, Japan, respectively. NIH/3T3 and A431 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-deactivated fetal calf serum (FCS), 50 U/ml penicillin G, and 100 mg/ml of streptomycin sulfate.

Peptide synthesis

Peptide was manually prepared by the stepwise solid phase method, which are shown in Scheme 1 using Fmoc-Tyr(tBu)-HMP-resin (400 mg, 0.45 mmol/g) as a starting material. The Fmoc group, a protecting group for the α-amino group of amino acids, was removed with 20% piperidine in NMP. The side chain protecting groups of Fmoc-amino acids used were; *tert*-butyl (tBu) for Asp, Ser, Glu, Thr, and Tyr, trityl(Trt) for Asn, Cys, and Gln; *tert*-butyloxycarbonyl (Boc) for Lys and His, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg. Loading of Fmoc-Tyr(tBu)-OH onto the HMP-resin was carried out by the DCC/DMAP method [Fmoc-Tyr (tBu)-OH : DCC : DMAP = 1.5 : 1.2 : 0.1 equi.]. Coupling of Fmoc-amino acids in each step was performed using either the BOP/HOBt or the HBTU/HOBt method in NMP. Coupling completion was checked by means of the Kaiser's ninhydrin test (Kaiser *et al.*, 1970). Incomplete coupling reactions were either recoupled until a negative Kasier result was obtained, or acetylated by treatment with benzoic anhydride in DMF-DCM (1 : 3, v/v). After completion of chain elongation, the protected peptide resin (200 mg) was treated with a mixture of trifluoroacetic acid (TFA)/thioanisole/phenol/EDT/H_2O (8.25 ml; 500 μl; 750 mg; 250 μl; 500 μl) for 2 h at room temperature. After removing volatile materials under a vacuum, the crude residue was washed with cold ethyl ether to remove trace amounts of scavengers, then extracted with 30 ml of 30% CH_3CN containing 0.1% β-mercaptoethanol. The extract was diluted with H_2O and lyophilized to give fuffly powder



Scheme 1. Synthetic route of mouse BTC(33-80) by solid phase method.

(120 mg). To form the three disulfide linkages of synthetic peptides, the reduced crude peptide (final peptide concentration: 20 μM) was oxidized in 0.1 M Tris-HCl buffer (pH 8.2) containing 0.1 mM ethylenediaminetetraaceticacid (EDTA), and 0.30 mM oxidized and 0.15 mM reduced glutathione for 3 days at room temperature. The progress of oxidation was monitored by analytical RP-HPLC (Beckman Ultrasphere ODS, 4.6×250 mm). The crude oxidized peptide was purified by preparative RP-HPLC (Inertsil ODS-2, 20.0×250 mm) eluted with a H_2O-CH_3CN gradient containing 0.1% TFA (flow rate; 5 ml/min) to give the pure peptide (6.9 mg). The homogeneity of the synthetic peptide was confirmed by analytical RP-HPLC (Beckman Ultrasphere ODS, 4.6×250 mm). The amino acid composition of synthetic peptides was determined by amino acid analysis after 6 N-HCl acid hydrolysis containing trace amounts of phenol at 110℃ for 22 h. The number of Cys residues was determined by performic acid oxidation of amino acid hydrolysate. The molecular masses of synthetic peptides were determined using a fast atom bombardment mass spectrometer (FAB-MS) (JEOL JMS-HX110 double-focusing mass spectrometer).

The determination of disulfide bond pairings of synthetic peptides

Synthetic mouse BTC(33-80) (200 μg) was suspended in 0.5 ml of 0.1 M pyridine-AcOH buffer (pH 6.5). Thermolysin (20 μg, Boehringer Mannheim) was added and the solution was incubated at 45℃ for 24 h (DeFoe-Jones *et al.*, 1989). Digestion mixtures were fractionated by analytical RP-HPLC (Nucleosil ODS-2, 4.6 ×250 mm), then eluted with a linear gradient of acetonitrile containing 0.1% TFA. Peaks were collected and hydrolyzed with 6 N HCl for 24 h at 110℃. The sequences of peptide fragments containing cystine were determined by amino acid analysis

Radioreceptor competition binding assay

A431 cells were plated on 24-well plates at a density of 5.0×10^4 cells/well and grown at 37℃ in a 5% CO_2 atmosphere for 2 days, after the cells were approximately 80% confluent. The medium was decanted and replaced with 500 μl of serum-free DMEM medium containing 1 mg/ml of bovine serum albumin, followed by incubation for 2 h and cooling by placement for 1 h in ice-cold Hank's balanced salt solution (HBSS). A binding medium [0.5 ml of bicarbonate-free DMEM supplemented with 40 mM 4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2, and 1 mg/ml BSA] containing ^{125}I-h-EGF (20 pM) and synthetic mouse BTC(33-80) was added to each well. Plates were incubated at 4℃ for 3 h. The medium was aspirated and the monolayers were washed three times with 0.5 ml of HBSS. The cells were then dissolved by treatment with 0.5 ml of 0.4 M NaOH. The resulting solutions were then transferred to vials and counted for ^{125}I in an automatic well GAMMA system (Aloka, ARC-1000 M). Nonspecific binding was determined from triplicate wells containing 160 nM of unlabeled h-EGF, and normally represented less than 60 cpm. This value was subtracted from the total binding to obtain the value of specific cell-bound ^{125}I-h-EGF.

Colorimetric MTT (tetrazolium) assay

NIH/3T3 cells were plated on a 96 well plate (Costar) at a density of 5.0×10^3 cells/well in all except peripheral wells with 100 μl of 10% FCS/DMEM, and incubated at 37℃ in a 5% CO_2 atmosphere for 24 h. The medium was decanted and replaced with 90 μl of DMEM medium containing 0.8% FCS, and incubated for 24 h. Replicates of 6 samples were added in a standard volume of 10 μl, and incubated for 3 days. MTT (a 5 mg/ml solution of MTT in phosphate-buffered saline, 10 μl) was added to each well and the plates were incubated at 37℃. After 6 h, the medium and MTT were removed from the wells and 10 μl of acidic isopropanol (0.04 M HCl in isopropanol) was added and mixed thoroughly using a multi-channel pipette to dissolve the dark blue crystals which were formed (MTT-formazan product). The absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Corona electric: MTP-22, 2-wavelength microplate photometer) at a wavelength of 550 nm.

Results and Discussion

Mouse BTC is a new peptide growth factor belonging to the EGF family of peptides (Fig. 1). Mouse BTC has an EGF-like domain that exhibits a high sequence homology with EGF in its carboxyl-terminal region. To elucidate the biological activity of the EGF-like domain of mouse BTC, mouse BTC(33-80) (Fig. 2) was synthesized by the Fmoc-based solid-phase method. Mouse BTC(33-80) contains three His, four Tyr, five Arg and six Cys residues which are susceptible to side reactions which occur during TFA peptide-resin cleavage and side-chain deprotection. In this study, the protected-peptide resin of mouse BTC(33-80) was deprotected by Reagent K (82.5% TFA/5% phenol/5% water/5% thioanisole/2.5% EDT) (King *et al.*, 1990; Choi *et al.*, 1993), which is an effective cleavage reagent used in

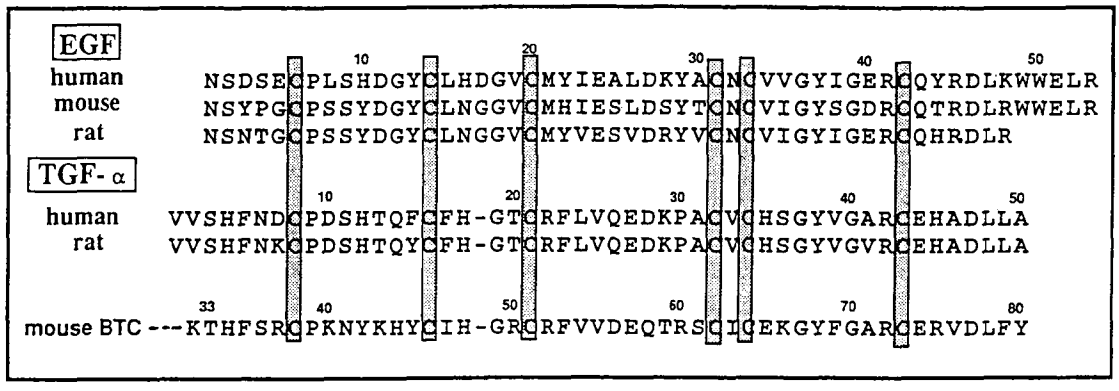


Fig. 1. Amino acid sequences of EGF, TGF-α and mouse BTC.

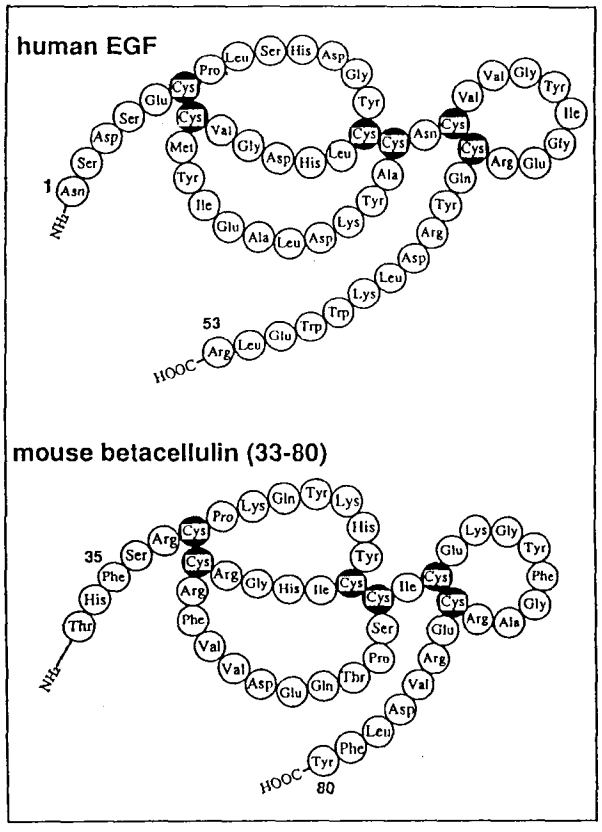


Fig. 2. Schematic representation of the primary structure of human-EGF and mouse BTC(33-80).

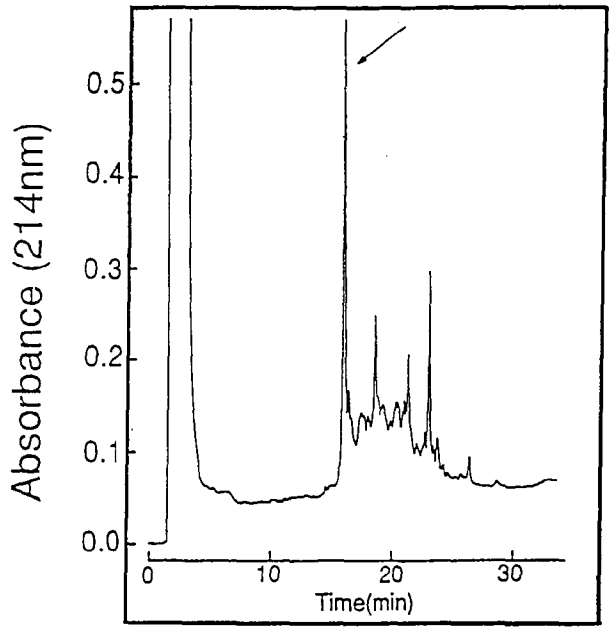


Fig. 3. Analytical RP-HPLC profile of the crude oxidized mouse BTC(33-80) after air oxidation.
Column : Beckman Ultrasphere ODS (4.6×250 mm), Flow rate : 1 ml/min, Detection : 214 nm, Solvent A : 0.1% TFA, Solvent B : 80 % CH_3CN, 0.1% TFA, Eluent : Solvent B (0%)→Solvent B (80%) (linear gradient : 1%/min).

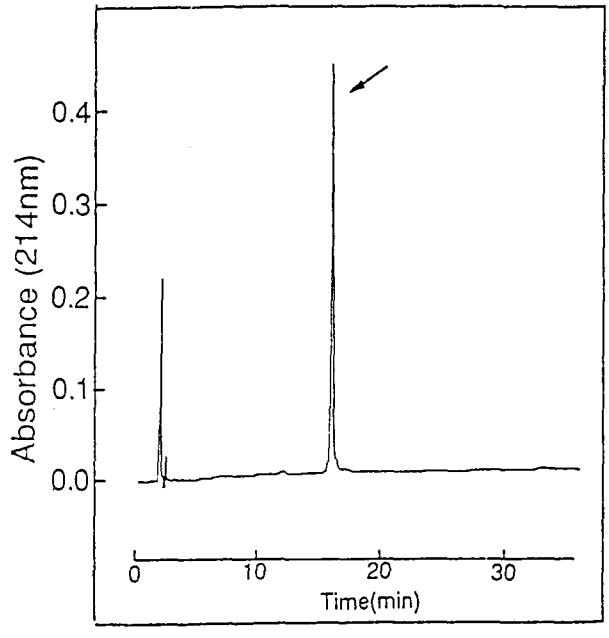


Fig. 4. Analytical RP-HPLC profile of the pure oxidized mouse BTC(33-80) after purification.
Column : Beckman Ultrasphere ODS (4.6×250 mm), Flow rate : 1 ml/min, Detection : 214 nm, Solvent A : 0.1% TFA, Solvent B : 80 % CH_3CN, 0.1% TFA, Eluent : Solvent B (0%)→Solvent B (80%) (linear gradient : 1%/min).

the synthesis of complex peptides.

The six cysteinyl residues in the crude linear peptides of mouse BTC(33-80) were refolded by air oxidation (Health and Merrifield, 1986; Tam *et al.*, 1986; Shin *et al.*, 1992) in a highly dilute basic solution (pH 8.2) in the presence of redox reagents to form intramolecular disulfide bridges. After air oxidation the oxidized form of mouse BTC(33-80) appeared as a main peak in analytical RP-HPLC, as shown in Fig. 3. This main peak was collected by preparative RP-HPLC. Analytical RP-HPLC showed that the oxidized form of mouse BTC(33-80) was eluted as a single symmetrical peak (Fig. 4).

The results of amino acid hydrolysis of the purified oxidized form of mouse BTC(33-80) with 6 N HCl were agreement with following the predicted composition: (theoretical values in parentheses) [Asx, 2.00(2); Thr, 1.85(2), Ser, 1.75(2); Glx, 5.43(5); Pro, 1.76(2); Gly, 3.08(3); Ala, 1.07(1); Cys, 5.63(6); Val, 2.40(2); Ile, 1.64(2); Leu, 1.00(1); Tyr, 4.04(4); Phe, 4.34(4); His, 3.12(3); Lys, 3.00(3); Arg, 4.74(5)]. Synthetic mouse BTC(33-80) was analyzed by positive ion FAB-mass spectrometry and found to exhibit an average molecular ion value $(M+H)^+$ of 5773.5, corresponding to the calculated mean value m/z of 5772.6. A strong peak corresponding to $(M+2H)^{2+}$ was detected in the range of 2887.2 (calculated value is 2887.3) (Fig. 5).

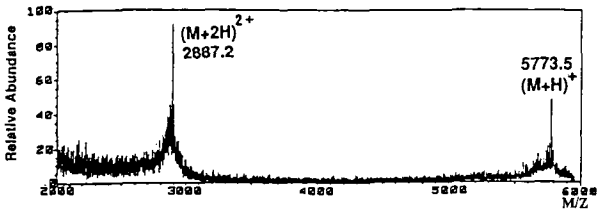


Fig. 5. FAB-mass spectrum of synthetic mouse BTC(33-80).

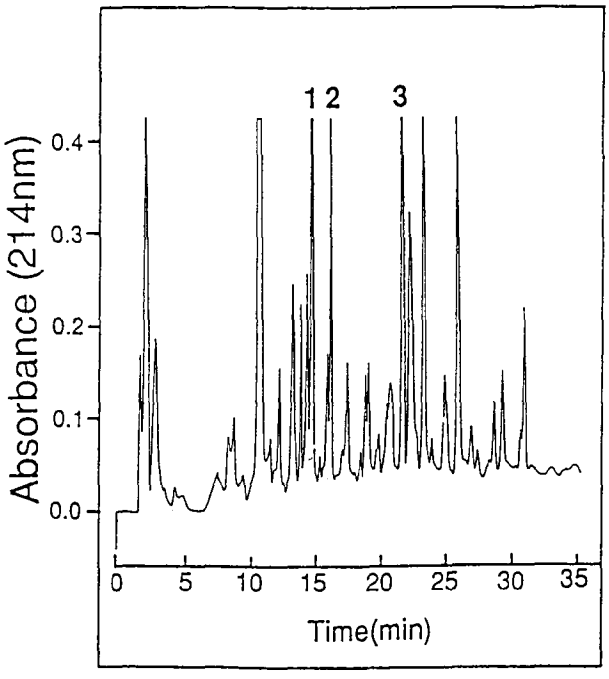


Fig. 6. Analytical RP-HPLC profile of the thermolytic digest of synthetic mouse BTC(33-80). Peaks 1, 2 and 3 indicate cysteine-containing fragments.
Column : Beckman Ultrasphere ODS (4.6×250 mm), Flow rate : 1 ml/min, Detection : 214 nm, Solvent A : 0.1% TFA, Solvent B : 80 % CH_3CN, 0.1% TFA, Eluent : Solvent B (0%)→Solvent B (80%) (linear gradient : 1%/min).

Table. Amino acid composition of peak 1, peak 2, and peak 3 obtained from thermolytic digestion of synthetic mouse BTC (33-80).

Peak 1			Peak 2			Peak 3		
Amino acid composition			Amino acid composition			Amino acid composition		
			Ser	(1)	1.10			
Glx	(2)	1.68	Glx	(1)	1.07	Asx	(1)	0.93
Gly	(2)	2.20	Glx	(1)	1.08	Thr	(1)	0.86
Ala	(1)	1.09	Ile	(1)	1.00	Ser	(1)	0.81
Ile	(1)	1.00	Tyr	(1)	1.87	Glx	(2)	2.01
Phe	(1)	1.07	Phe	(2)	2.10	Val	(1)	1.10
Lys	(1)	1.11	Lys	(1)	0.86	Tyr	(1)	1.10
Arg	(2)	1.79	His	(1)	0.88	Pro	(1)	0.85
Cys	(2)	1.89	Arg	(3)	2.27	Cys	(2)	1.78
			Pro	(1)	0.79			
			Cys	(2)	1.78			

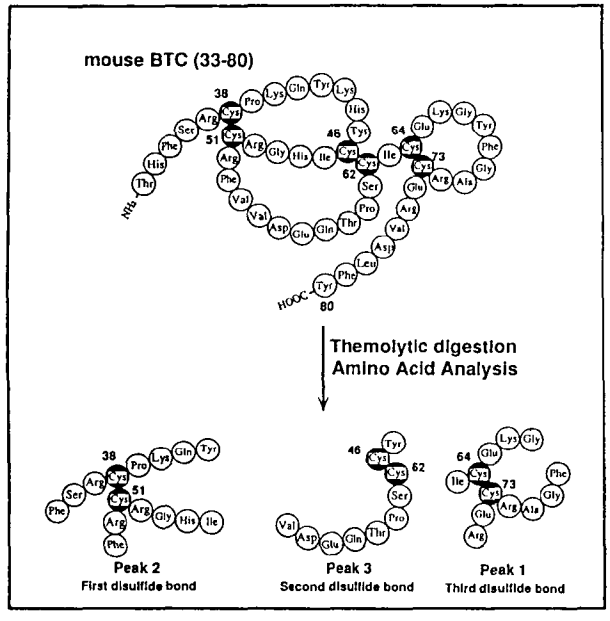


Fig. 7. Amino acid sequence of cysteine-containing peptide fragments after thermolytic digestion of synthetic mouse BTC(33-80).

These correct values indicate that synthetic mouse BTC (33-80) is monomeric.

The correct disulfide linkages in the EGF family of peptides are essential for biological activity as well as conformational stability. To investigate the pattern of the three disulfide bond pairings of mouse BTC(33-80), mouse BTC(33-80) was digested with thermolysin, then fractionated by analytical RP-HPLC (Fig. 6). It was confirmed by amino acid analysis that peaks 1, 2, and 3 are cysteine-containing peptide fragments (Table). Peak 1 contained two sequences corresponding to Ile^{63}-Cys-Glu-Lys-Gly^{67} and Arg^{69}-Glu-Cys-Arg-Ala-Gly-Phe^{75}. Peak 2 contained two sequences encompassing Phe^{35}-Ser-Arg-Cys-Pro-Lys-Gln-Tyr^{42}, and peak 3 involved two sequences corresponding to Val^{55}-Asp-Glu-Gln-Thr-Pro-Ser-Cys^{62} and Tyr^{45}-Cys^{46} (Fig. 7). The six Cys residues of synthetic mouse BTC(33-80) are linked by the three disulfide bonds of Cys^{38}-Cys^{51}, Cys^{46}-Cys^{62}, and Cys^{62}-Cys^{73}. These three disulfide pairings of synthetic mouse BTC(33-80) was consistent with the pairings of EGF. The EGF-like motif ---$CX_7CX_{4-5}CX_{10}CX_1CX_8C$---, is identified in the EGF-like domain of mouse BTC. These results suggested that the spacing pattern of the six cysteines in the EGF-like domain play an important role in the formation of intramolecular disulfide bridges of patterns 1~3, 2~4, and 5~6 in oxidative refolding.

The affinity of mouse BTC(33-80) for the EGF-receptor was determined by a radioreceptor competition

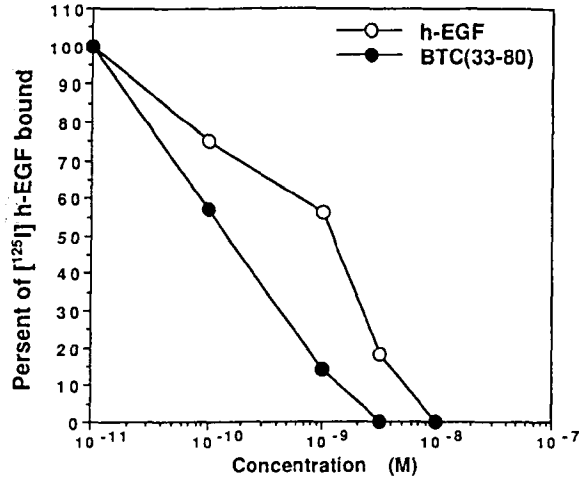


Fig. 8. Displacement of receptor-bound [^{125}I]h-EGF by human EGF and synthetic mouse BTC(33-80) in human epidermoid carcinoma A431 cells.

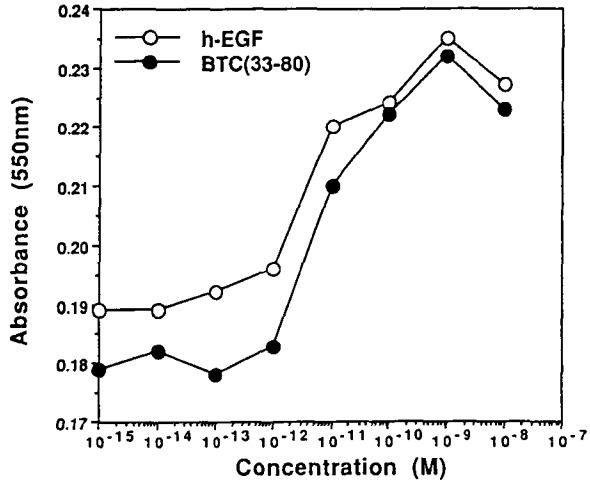


Fig. 9. Concentration-response curves of mitogenesis induced by human EGF and synthetic mouse BTC(33-80) in NIH/3T3 fibroblast cells colorimetric MTT assay.

binding assay on A-431 epidermoid carcinoma cells. The competition binding curves of authentic h-EGF and mouse BTC(33-80) are shown in Fig. 8. The affinity of the EGF-like domain of mouse BTC was almost equipotent to authentic h-EGF in receptor binding. The cell proliferation activity of mouse BTC(33-80) was measured by colorimetric MTT (tetrazolium) assay in NIH/3T3 fibroblast cells. Fig. 9 shows concentration-response for h-EGF and synthetic mouse BTC(33-80). The EGF-like domain of mouse BTC was as active as human-EGF. These results suggested that the EGF-like domain of mouse BTC plays an important role in mitogenic activity and binding with the EGF-receptor. From structure-activity relationships of EGFs and TGF-αs it has been reported that Tyr-13, Leu-15, Ile-23, Arg-41, and Leu-47 (EGF numbering system) are critical in the binding of EGF to the EGF-receptor and its mitogenic response (Burgess *et al.*, 1989; Gregory *et al.*, 1988; Dudgeon *et al.*, 1990; Matsunami *et al.*, 1990; Engler *et al.*, 1992; Campion *et al.*, 1993; Tadaki *et al.*, 1993; Shin *et al.*, 1994). In mouse BTC(33-80), Leu at position 15 was replaced with Ile and Ile at position 23 was replaced with Val (positions 47 and 54 in mouse BTC correspond to positions 15 and 23 in EGF, respectively). The hydrophobicity of these two positions are highly conserved. Also, Tyr, Arg, and Leu at positions 13, 41, and 47 are absolutely conserved (positions 45, 72, and 78 of BTC correspond to positions 13, 41 and 47 of EGF, respectively). These results obtained from the structure-activity study of the EGF-like domain in mouse BTC are consistent with results from EGFs and TGF-αs.

In summary, disulfide bond pairings of synthetic mouse BTC(33-80) corresponding to the EGF-like domain of mouse BTC was homologous to those in EGF and TGF-α. Mouse BTC(33-80) and h-EGF were equipotent in stimulating NIH/3T3 cell proliferation and in binding human epidermoid carcinoma A431 cells. These results suggest that BTC is one of the specific ligands for the EGF-receptor and the EGF-like domain of BTC is essential for the biological activity and receptor recognition.

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