Identification of betacellulin as a major peptide growth factor in milk: purification, characterization and molecular cloning of bovine betacellulin

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Betacellulin (BTC), a member of the epidermal growth factor (EGF) family of peptide growth factors, was purified from a growth-factor-enriched whey fraction of bovine milk by a combination of ion-exchange chromatography, gel-filtration chromatography, affinity chromatography and reverse-phase HPLC. Bovine BTC (bBTC) had an apparent molecular mass of 21–22 kDa on SDS/PAGE and exists in a glycosylated form. The cDNA encoding bBTC was obtained by a combination of 5' and 3' rapid amplification of cDNA ends ('RACE'). The primary translation product consists of 178 amino acid residues containing a putative signal sequence, a transmembrane domain, the

mature BTC domain and a cytoplasmic domain containing a highly hydrophilic Arg-Lys-rich region similar to that of mouse BTC and human BTC. The amino acid sequence of the bBTC precursor was 88 % identical with human BTC and 79 % identical with mouse BTC. The bBTC gene was found to be expressed in a wide range of tissues, including the mammary gland. The identification of BTC in milk raises the possibility that it has a major role in the growth and development of the neonatal gastrointestinal tract.

Key words: epidermal growth factor, ErbB receptor, whey.

INTRODUCTION

The regulation of cellular growth and differentiation is mediated in part by the binding of peptide growth factors to specific cell-surface receptors, triggering a cascade of intracellular signals controlling gene expression and DNA replication. The characterization of these peptide growth factors and the sequence analysis of their genes have permitted their grouping into a relatively small number of families on the basis of sequence similarity [1]. One of these is the epidermal growth factor (EGF) family. Members of the EGF family are characterized by extensive sequence similarity, including a six-cysteine consensus motif (CX₇CX₄CX₁₀CXCX₈C) that forms three intramolecular disulphide bonds (C1–C3, C2–C4 and C5–C6; where C1 is the first cysteine residue and so on) and a core arginine residue that stabilizes protein orientation, and their ability to activate a family of four receptor tyrosine kinases encoded by the *erbB* gene family (*erbB*-1/EGF receptor, *neu*/*erbB*-2/*HER*-2, *erbB*-3/*HER*-3 and *erbB*-4/*HER*-4). A common feature of the EGF family members is that they are synthesized as larger transmembrane precursor molecules that can be cleaved proteolytically to release the soluble form of the growth factor or they can function as membrane-anchored growth factors in juxtacrine signalling. Members of the mammalian EGF family include EGF [2], transforming growth factor α (TGF- α) [3], heparin-binding EGF-like growth factor (HB-EGF) [4], epiregulin [5], amphiregulin (AR) [6], neural- and thymus-derived activator for ErbB kinases ('NTAK') [7], the neuregulin subfamily, which includes the products of two alternately spliced genes *NRG1* [8] and *NRG2* [9,10] and a third gene, *NRG3* [11], and betacellulin (BTC) [12].

BTC was originally identified as a growth-promoting factor in the conditioned medium of a mouse pancreatic β -cell carcinoma

(insulinoma) cell line [12] and has since been identified in humans [13]. The mature form of mouse BTC (mBTC) exists as a 32 kDa glycoprotein composed of 80 amino acid residues derived from a 178-residue membrane-bound precursor. The amino acid sequence of mature mBTC is 82.5 % identical with that of human BTC (hBTC); both exhibit significant overall similarity with other members of the EGF family. Interspecific backcross analysis has mapped BTC to mouse chromosome 5, where it is tightly linked with the gene for AR [14], raising the possibility that these two proteins might have arisen through a tandem gene duplication event.

BTC was initially shown to compete with EGF for binding to A431 cells, which overexpress *erbB*-1, suggesting that *erbB*-1 is the receptor for BTC [15]. Subsequently, BTC was found to bind to human mammary carcinoma MDA-MB-453 cells, which lack *erbB*-1 [16], leading to the conclusion that BTC is also able to bind directly to, and induce the tyrosine phosphorylation of, *erbB*-4 [16–19]. More recently, with the use of cell lines engineered to express pairwise combinations of *erbB* receptors ectopically, it has become apparent that BTC, in addition to stimulating homodimers of *erbB*-1 and *erbB*-4, is capable of binding and activating all possible combinations of heterodimeric *erbB* receptors including the oncogenic *erbB*-2/*erbB*-3 complex [20,21].

Although BTC has been shown to be a potent mitogen for Balb/c 3T3 fibroblasts, vascular smooth-muscle cells and retinal pigment epithelial cells [12], few clues exist to the function(s) of BTC *in vivo*. Elevated expression levels of BTC mRNA in the pancreas has led to the notion that BTC might have a physiological role in the development of pancreatic islet cells [22]. This is supported by observations that BTC, together with activin-A, can convert populations of exocrine AR42J rat pancreatic tumour

Abbreviations used: AR, amphiregulin; b, h, m and r, bovine, human, mouse and recombinant respectively; BTC, betacellulin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGF-RRA, EGF radioreceptor assay; FGF, fibroblast growth factor; HB-EGF, heparin-binding EGF-like growth factor; HFBA, heptafluorobutyric acid; IGF, insulin-like growth factor; MDBK, Madin–Darby bovine kidney; PDGF, platelet-derived growth factor; RACE, rapid amplification of cDNA ends; RP, reverse-phase; TFA, trifluoroacetic acid; TGF- α , transforming growth factor α ; WGFE, whey growth factor extract.

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cells into insulin-secreting cells [23] and that BTC is required for the induction of insulin gene expression in PDX-1-expressing α TC1.6 cells [24]. Additionally, BTC has recently been shown to mediate the proliferation and differentiation of the rat insulinoma cell line INS-1 [25].

Milk is not only a rich source of nutrients for the developing infant but also contains a variety of peptide growth factors. These milk-derived growth factors are thought to regulate both the development and maturation of the neonatal gut and immune system as well as the growth and secretory functions of the maternal mammary gland [26]. In bovine milk and colostrum, cell growth stimulatory activity has been attributed to a number of peptide growth factors including platelet-derived growth factor (PDGF) [27] and insulin-like growth factor I and II (IGF-I and IGF-II) [28]. EGF, although abundant in human milk, has not been unequivocally detected in bovine milk or colostrum [29].

A novel method, using cation-exchange chromatography, that effectively concentrates the growth factor component of bovine milk has been reported [30]. This whey growth factor extract (WGFE) has been shown to be remarkably potent in stimulating the growth *in vitro* of cells of mesodermal origin, including rat L6 myoblasts and Balb/c 3T3 fibroblasts [31]. Several growth factors have been identified in this extract, including fibroblast growth factors 1 and 2 (FGF-1 and FGF-2) [32], IGF-I and IGF-II, PDGF and TGF- β [33]. Interestingly, however, a cocktail of recombinant growth factors containing IGF-I, IGF-II, PDGF, TGF- β and FGF-2 stimulates the growth of Balb/c 3T3 cells to a level equivalent to only 50% of that of WGFE [34], suggesting that much of the Balb/c 3T3 cell-stimulating activity present in WGFE remains uncharacterized. By using an EGF radioreceptor assay (EGF-RRA) we were able to monitor a hitherto undescribed growth-stimulating activity in WGFE that was capable of displacing 125 I-EGF from AG2804 cells. Here we describe the purification, characterization and molecular cloning of this growth factor, and its identification as the bovine homologue of BTC (bBTC).

EXPERIMENTAL

Materials

Recombinant human EGF (rhEGF), IGF-I and IGF-II were purchased from GroPep Pty. Ltd. (Adelaide, SA, Australia). Recombinant human BTC (rhBTC) was from R&D Systems (Minneapolis, MN, U.S.A.) and PDGF-AB, FGF-2, TGF- β 2 were from Austral Biologicals (San Ramon, CA, U.S.A.). 125 I-rhEGF was prepared to a specific radioactivity of approx. 30–60 μ Ci/ μ g with carrier-free Na 125 I by a modification of the chloramine-T method [35]. Carrier-free Na 125 I and iodo[2- 3 H]acetic acid were from Amersham Pharmacia Biotech (Sydney, NSW, Australia). Goat anti-rabbit immunoglobulin was from Dako Pty. Ltd. (Botany, NSW, Australia). Bovine tissues from freshly slaughtered cattle were obtained from Agro abattoir (Gepps Cross, Adelaide, Australia) and were snap-frozen in liquid N $_2$ before being stored at -80°C . Reverse-phase (RP) HPLC columns were from Brownlee Lab (Santa Clara, CA, U.S.A.) and Millipore-Waters (Lane Cove, NSW, Australia). Q-Sepharose, heparin-Sepharose, Superdex-75 (35/600) and HiTrap Protein G-Sepharose were purchased from Amersham Pharmacia Biotech. (Sydney, NSW, Australia). HPLC-grade acetonitrile and propan-1-ol were purchased from Merck (Kilsyth, VIC, Australia) and trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) from Sigma-Aldrich (Castle Hill, NSW, Australia). [α - 32 P]dCTP (2000 Ci/mmol) was from GeneWorks Ltd. (Adelaide, SA, Australia). All other reagents were of analytical grade.

Cell culture

Balb/c 3T3 fibroblasts (ATCC CCL 163) and Madin–Darby bovine kidney (MDBK) cells (ATCC CCL 22) were obtained from the American Type Culture Collection. AG2804 Simian-virus-transformed human lung fibroblasts were kindly provided by Dr J. M. Gunn (Texas A and M University, College Station, TX, U.S.A.). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 60 mg/ml penicillin, 100 mg/ml streptomycin, 1 mg/ml fungizone and 10% (v/v) fetal bovine serum and propagated at 37°C in a humidified air/CO $_2$ (19:1) atmosphere.

Purification of bBTC

Ultrafiltration

WGFE (6 litres) prepared from pasteurized bovine cheese whey as described previously [35], was acidified to pH 2.5 with HCl and microfiltered against a 100 kDa polysulphonate exclusion membrane fitted to a Sartocoon II crossflow filtration unit (Sartorius, Oakleigh, VIC, Australia). The permeate obtained from this step was diafiltered with an Amicon DC-10 ultrafiltration unit (Amicon, Beverly, MA, U.S.A.) equipped with a 0.1 μ m hollow-fibre cartridge and then concentrated to approx. 1.8 litres against a 3 kDa cellulose triacetate membrane with the same unit.

Anion-exchange chromatography

The desalted and concentrated permeate was made 20 mM with respect to Tris/HCl, pH 7.5, filtered through a 1 μ m pore-size membrane and applied to a Q-Sepharose column (5 cm $\times$ 15 cm) attached to an FPLC system (Amersham Pharmacia Biotech.) at a flow rate of 5 ml/min. After extensive washing with 20 mM Tris/HCl, bound protein was eluted with a 2.1-litre linear salt gradient of 0–0.6 M NaCl in 20 mM Tris/HCl at a flow rate of 5 ml/min. Fractions of 30 ml were collected and analysed for their ability to displace 125 I-rhEGF from specific EGF receptors present on AG2804 cells (see below). Fractions containing 'EGF-like' activity were pooled, dialysed for 16 h against water (2 $\times$ 20 litres) and freeze-dried.

Size-exclusion chromatography

The freeze-dried EGF-like activity pool from the above step was reconstituted in 15 ml of 150 mM NaCl/1 M acetic acid/10% (v/v) acetonitrile, filtered through a 0.22 μ m pore-size membrane and applied to a Superdex-75 35/600 (3.5 cm $\times$ 60 cm) column attached to the FPLC system at a flow rate of 3.5 ml/min. Fractions of 17.5 ml were collected and analysed for EGF-like activity.

C $_4$ RP-HPLC

Fractions containing EGF-like activity were pooled, diluted 1:4 with 0.1% (v/v) TFA and applied to a Delta-Pack C $_4$ RP-HPLC column (15 μ m bead size, 300 Å pore size, 25 mm $\times$ 100 mm) equilibrated with 0.1% (v/v) TFA. Bound protein was eluted with a linear gradient of 0–80% acetonitrile/0.08% (v/v) TFA over 80 min at a flow rate of 5 ml/min. Fractions of 10 ml were collected and those containing EGF-like activity were pooled and freeze-dried.

Affinity chromatography

The freeze-dried pool from the above step was reconstituted in 20 mM Tris/HCl, pH 7.5, and applied to a 5 ml HiTrap heparin–

Sepharose affinity column (attached to the FPLC system) at a flow rate of 0.5 ml/min. The column was washed with 20 mM Tris/HCl, pH 7.5, until the A_{280} returned to the baseline; bound proteins were then eluted with 75 ml of a linear salt gradient of 0–1 M NaCl in 20 mM Tris/HCl, pH 7.5. Fractions of 1 ml were collected and those containing EGF-like activity were pooled.

C₁₈ RP-HPLC, step 1

The heparin–Sepharose pool was diluted 1:4 with 10% (v/v) propan-1-ol/0.13% (v/v) HFBA and applied to a Nova-Pack C₁₈ RP-HPLC column (4 µm bead size, 60 Å pore size, 8 mm × 100 mm) at a flow rate of 1 ml/min. The column was washed with 10% (v/v) propan-1-ol/0.13% (v/v) HFBA and the bound protein was eluted with a two-step gradient of 10–24% (v/v) propan-1-ol/0.13% (v/v) HFBA over 10 min and then 24–40% (v/v) propan-1-ol/0.13% (v/v) HFBA over 160 min. Fractions of 1 ml were collected and analysed for EGF-like activity.

C₁₈ RP-HPLC, step 2

Fractions containing EGF-like activity from the above step were pooled, diluted 1:3 with 0.1% (v/v) TFA and applied to the same C₁₈ column as above, at a flow rate of 1 ml/min. The column was washed with 0.1% (v/v) TFA and bound protein was eluted with a two-step gradient of 0–10% (v/v) acetonitrile over 10 min and then 10–35% (v/v) acetonitrile over 250 min at a flow rate of 1 ml/min. Fractions of 2 ml were collected and analysed for EGF-like activity.

Production and affinity purification of rabbit anti-bBTC antibody

A polyclonal antiserum directed against recombinant bBTC was generated in Semi Lop rabbits by standard procedures (details available from the authors) and affinity-purified with a HiTrap Protein-G–Sepharose column in accordance with the manufacturer's instructions. The resulting affinity-purified antiserum does not cross-react with either EGF or TGF-α (Dr. Sue Bastian, personal communication).

EGF-RRA

bBTC was measured by competitive displacement of ¹²⁵I-rhEGF from specific EGF receptors present on AG2804 fibroblasts. In brief, AG2804 cells were grown to 70–80% confluence in DMEM supplemented with 10% (v/v) fetal bovine serum in 24-well plates. The cells were washed twice with binding buffer [100 mM Hepes (pH 7.6)/120 mM NaCl/5 mM KCl/1.2 mM MgSO₄·7H₂O/8 mM glucose/0.1% BSA] and then incubated with column fractions and ¹²⁵I-rhEGF (10000 c.p.m.) in binding buffer for 18 h at 4 °C. At the end of this period, cells were washed three times with Hanks buffered salt solution and lysed with 1 ml of 0.5 M NaOH/0.1% (v/v) Triton X-100 for 30 min at room temperature. Radioactivities of cell lysates were determined with a γ-counter. Total binding was determined by the addition of binding buffer in place of column fractions. Non-specific binding was determined by adding an excess (100 ng) of unlabelled rhEGF in place of sample and was usually approx. 5% of total binding. Standard curves for EGF displacement were obtained by using increasing amounts of unlabelled rhEGF (0.2–100 ng) in the assay.

Assay for biological activity

Mitogenic activity of bBTC on Balb/c 3T3 cells was determined by a cell proliferation assay. Balb/c 3T3 cells were subcultured in

96-well plates at a density of 2×10^5 cells/ml and incubated overnight at 37 °C in air/CO₂ (19:1). The plates were then washed extensively with DMEM to remove any residual medium after which bBTC, recombinant rhBTC or rhEGF was added at the indicated concentrations (final volume 100 µl). After incubation for a further 48 h, the plates were washed twice with 150 mM NaCl, the monolayers of cells were fixed and the cell masses were quantified by the addition of 1% (w/v) Methylene Blue and measuring A_{655} . Experimental data for both the EGF-RRA and the cell proliferation assay were fitted to a sigmoidal dose–response model with a variable slope by using GraphPad Prism (GraphPad, San Diego, CA, U.S.A.). Experiments with recombinant growth factors were undertaken in DMEM/0.1% BSA (RIA grade; Sigma). Cell growth data generated in response to the individual growth factors or antibodies were analysed by one-way analysis of variance; post-hoc comparisons were undertaken with Dunnett's test (SigmaStat; Jandel Scientific Software, San Rafael, CA, U.S.A.).

Amino acid sequence analysis

The amino acid sequence of bBTC and of peptide fragments generated by endoproteinase Lys-C digestion were determined with a Hewlett-Packard G1000A protein sequencer. In brief, 20 µg of purified bBTC was reduced with 100 µl of 4 mM dithiothreitol in 400 µl of denaturation buffer [6 M guanidinium chloride/100 mM Tris/HCl (pH 8.5)/5 mM EDTA] for 30 min at room temperature in the dark. The denatured and reduced bBTC was S-carboxymethylated by adding 1 µmol of iodoacetic acid containing 200 µCi of iodo[2-³H]acetic acid in denaturation buffer, then incubated as above. Subsequently 16 µmol of iodoacetic acid dissolved in denaturation buffer was added and the incubation was continued for a further 15 min. The reaction was stopped by the addition of 5 µl of TFA and the [³H]carboxymethylated bBTC was recovered by RP-HPLC. The [³H]carboxymethylated bBTC was dried under vacuum and sequenced from the N-terminus or digested with 0.5 µg of endoproteinase Lys-C (Promega, Madison, WI, U.S.A.) in 100 µl of Tris/HCl, pH 8.5, at 37 °C for 16 h. The reaction was stopped by the addition of 5 µl of TFA and the resulting peptide fragments were separated on a Brownlee C₁₈ RP-HPLC column (2.1 mm × 30 mm) with a linear gradient of 0–50% (v/v) acetonitrile and 0.08% (v/v) TFA over 70 min at a flow rate of 0.25 ml/min. Peptide-containing fractions were dried under vacuum and sequenced.

bBTC deglycosylation

The removal of N- and O-linked oligosaccharide chains from bBTC was performed with endo-α-acetylglactosaminidase (O-glycosidase), α2-3,6-neuraminidase (NANase II), and peptide-N⁴-(acetyl-β-glucosaminyl)-asparagine aminidase (PNGase F) (Bio-Rad, Richmond, CA, U.S.A.). For this reaction, 5 µg of pure bBTC was dried in a 1.5 ml polypropylene Microfuge tube, resuspended in 16 µl of 50 mM sodium phosphate, pH 6.0; 0.2 unit of NANase II and 0.002 unit of O-glycosidase were added and the reaction was incubated at 37 °C for 1 h. The reaction volume was then increased to 40 µl with 500 mM sodium phosphate (dibasic) and 2.5 µl of 2% (w/v) SDS/1 M 2-mercaptoethanol. The reaction was then incubated at 95 °C for 5 min and placed on ice; then 2.5 µl of Nonidet P40 and 0.005 unit of PNGase F were added and incubated at 37 °C for 3 h. Finally, the reaction was dried under vacuum and analysed by SDS/PAGE.

cDNA amplification of the mature and precursor forms of bBTC

Total RNA from MDBK cells was isolated with an RNeasy Mini Kit (Qiagen, Clifton Hill, VIC, Australia) and oligo(dT)-primed cDNA synthesized with Superscript II (Gibco BRL, Gaithersburg, MD, U.S.A.) in accordance with the manufacturers' instructions. cDNA corresponding to the mature form of bBTC was amplified by PCR with specific primers corresponding to the amino acid sequences (single-letter codes) DGNSTRSPE (5'-GATGGGAATTCAACCAGAAGTCCTG-AA-3') and RCERVDLFY (5'-GTAAAACAAGTCAACTCTCTCACACCT-3'). PCR was performed in 50 µl of 60 mM Tris/H₂SO₄/18 mM (NH₄)₂SO₄/1.5 mM MgSO₄ (pH 9.1)/0.2 mM dNTPs containing 200 ng of each primer, 1 unit of eLONGase (Gibco BRL) and 1 µl of cDNA. After an initial incubation at 94 °C for 3 min, 30 cycles of amplification were performed as follows: 94 °C for 1 min, 50 °C for 1 min and 68 °C for 1 min, followed by a final extension at 68 °C for 3 min.

cDNA encoding precursor bBTC (pro-bBTC) was obtained by the rapid amplification of cDNA ends (RACE) technique with 5' and 3' RACE kits (Gibco BRL). For 5' RACE, first-strand cDNA was synthesized from MDBK total RNA as described above with a primer complementary to the amino acid sequence CCHPLRK (5'-GTTTCCGAAGAGGGTGACAGCA-3') of hBTC. This cDNA was used in a first-round PCR with the RCERVDLFY primer (see above) and an oligo(dC) anchor primer (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3') was added to the 5'-end by terminal deoxynucleotidyltransferase. PCR was performed as described above except that 40 cycles were performed and the annealing temperature was raised to 55 °C. After amplification, a second round of PCR was performed with a primer complementary to the bBTC amino acid sequence HAENCPATT (5'-GGTAGCTGGCAGTTTTCTGCGTG-3'), identified by sequence analysis of mature bBTC, and oligo(dC). For 3' RACE, first-strand cDNA was synthesized from MDBK total RNA with an adapter primer [5'-GGCCACGCGTCGACTAGTAC(T)₁₇-3']. PCR was performed under the conditions described above, with a primer corresponding to the bBTC amino acid sequence PSCVCDEG (5'-CCCTCCTGCGTCTGTGATGAAGGC-3') and a commercially supplied abridged universal amplification primer (5'-GGCCACGCGTCGACTAGTAC-3'). A second round of PCR was then performed with 5'-CCCTCCTGCGTCTGTGATGAAGGC-3' and a primer based on a conserved region within the 3' untranslated region of mBTC and hBTC (5'-CTAGCTGTTTTCTGAGACA-3'). The annealing temperature was lowered to 50 °C for the second-round PCR.

All PCR products were separated by electrophoresis on a 2% (w/v) agarose gel and revealed by staining with ethidium bromide. The mature bBTC PCR product and PCR products generated by 5' and 3' RACE were cloned into pCR-Blunt vector (Invitrogen, Leek, The Netherlands) in accordance with the manufacturer's instructions and sequenced by the dideoxynucleotide chain-terminator method with an Amplicycle sequencing kit (Perkin-Elmer). PCR products were sequenced in both directions twice from two independent clones to confirm the sequence.

bBTC expression analysis

The expression of bBTC in a variety of tissues was examined by Northern blotting. Poly(A)⁺ RNA was isolated from total RNA prepared from the indicated tissues by using Oligotex (Qiagen) in accordance with the manufacturer's instructions. For Northern blot analysis, 2 µg of poly(A)⁺ RNA was resolved by electrophoresis through a formaldehyde/1% (w/v) agarose gel, and

transferred and fixed to positively charged nylon membrane (Boehringer Mannheim, Castle Hill, NSW, Australia) in 50 mM NaOH [36]. The membrane was prehybridized for 1 h at 42 °C in 5 × Denhardt's solution/5 × SSPE [SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA]/0.1% SDS/50 µg/ml salmon sperm DNA/50% (v/v) formamide and then hybridized for 16 h in the same solution containing full-length mature ³²P-labelled bBTC cDNA. Non-specifically bound probe was removed by washing the membrane in SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS for 20 min, followed by three 10 min washes at 42 °C in 0.2 × SSC/0.1% SDS. The membranes were then exposed to Kodak X-Omat film at -80 °C. To ensure that equal amounts of RNA were loaded in each lane, blots were stripped and rehybridized with a 461 bp bovine β-actin probe amplified by PCR from oligo(dT)-primed kidney cDNA, with 5' primer (5'-ACCAACTGGGACGACATGGAGAAG-3') and 3' primer (5'-CATCTCTGCTCGAAGTCCAAGG-3'). bBTC and β-actin probes were radioactively labelled with [α-³²P]dCTP (2000 Ci/mmol) by random priming with a Gigaprime kit (GeneWorks Ltd.).

SDS/PAGE

For SDS/PAGE analysis of purified bBTC, 2.5 µg of protein was freeze-dried and resuspended in a minimal amount of 4% (w/v) SDS/200 mM Tris/5% (w/v) Bromophenol Blue in the presence or absence of 1% (v/v) 2-mercaptoethanol and denatured by heat (90 °C for 5 min). Purified protein was analysed on a 10–20% precast Tricine gel (Novex, San Diego, CA, U.S.A.) in accordance with the manufacturer's instructions.

RESULTS

Purification of bBTC

Cation-exchange chromatography of bovine cheese whey provides a suitable method for the enrichment of cell-growth promoting activity which is free from abundant whey proteins [30]. The WGFE obtained by this method was used as the source of starting material for the purification of bBTC from bovine milk. The first step in the purification of bBTC from WGFE involved ultrafiltration size exclusion under acidic conditions to remove high-molecular-mass contaminating proteins such as lactoperoxidase. The permeate from this step was subsequently applied to a Q-Sepharose column and collected fractions were assayed for their ability to displace ¹²⁵I-EGF in an EGF-RRA. A single well-defined peak of 'EGF-like' activity was eluted from this column (Figure 1A) and resulted in the removal of approx. 83% of contaminating protein (Table 1). After a gel-filtration step (Superdex-75), fractions containing EGF-like activity were pooled and then subjected to C₄ RP-HPLC with a 0–80% (v/v) acetonitrile gradient (Figure 1B). The pooled fractions containing EGF-like activity exhibited salt-dependent binding to heparin-Sepharose; this interaction was used for further purification (Figure 1C). This step eliminated most contaminating proteins and after two C₁₈ RP-HPLC steps a single peak of protein coincident with a peak of EGF-like activity was obtained (Figure 1D). Table 1 summarizes the results of the purification. Approx. 29 µg of bBTC was recovered from 209 g of WGFE. The overall purification was 37000-fold, with a recovery of 0.5%. Purified bBTC migrated as a single diffuse band at an apparent molecular mass of 21–22 kDa on SDS/PAGE (Figure 2, lane 2).

Amino acid sequence analysis of bBTC

The amino acid sequence of bBTC was determined by automated Edman degradation of reduced and S-carboxymethylated protein

Figure 1 Purification elution profiles of bBTC

(A) Q-Sepharose chromatography; (B) C_4 RP-HPLC; (C) heparin-Sepharose chromatography; (D) C_{18} RP-HPLC, step 2. See the Experimental section for details.

Table 1 Summary of the purification of bovine BTC

One unit of activity is defined as the amount of factor required to displace 50% of ^{125}I -rhEGF from AG2804 cells.

Purification step	Total protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
WGFE	209560*	5366.0	0.025	1	100.0
Permeate	16326*	2808.0	0.172	7	52.3
Q-Sepharose	2820*	924.9	0.328	13	17.2
Superdex-75	228*	372.2	1.630	63	6.9
C_4 RP-HPLC	16.5*	157.6	9.502	371	2.9
Heparin-Sepharose	0.372†	138.7	376.0	14690	2.5
C_{18} RP HPLC	0.152‡	48.0	320.0	12500	0.9
C_{18} RP HPLC	0.029‡	27.7	958.0	37400	0.5

* Estimated by bicinchoninic acid protein assay kit (Pierce).

† Estimated by micro-bicinchoninic acid protein assay kit (Pierce).

‡ Estimated by N-terminal sequence analysis.

Figure 2 SDS/PAGE analysis and deglycosylation of bBTC

Lane 1, molecular mass markers (molecular masses indicated at the left); lane 2, 5 μg of purified bBTC; lane 3, 5 μg of purified bBTC digested with O-glycosidase, peptide-N-glycosidase F (PNGase F), and neuraminidase II (NANase II) as described in the Experimental section; lane 4, deglycosylation enzymes.

and of peptide fragments generated by digestion with endoproteinase Lys-C. N-terminal sequence analysis identified the purified protein as bBTC (Figure 3). Four peptides were recovered by C_{18} RP-HPLC after digestion with endoproteinase Lys-C (Figure 3). As expected, the amino acid sequences of these peptides were attributable to fragments theoretically processed after each lysine residue. The peptide sequence QYK, which was not recovered by C_{18} RP-HPLC, was identified by cDNA cloning (see below). The amino acids at positions 3 and 5 in the mature molecule could not be identified by Edman degradation but were assigned as an Asp residue and a Thr residue respectively by cDNA cloning (see below). These two residues have been suggested as potential sites for N- and O-linked glycosylation in

hBTC and mBTC [12,15]. The glycosylation of bBTC was examined by the enzymic digestion of purified protein with a mixture of O-glycosidase, peptide-N-glycosidase F, and neuraminidase II. This resulted in a shift in the apparent molecular mass of bBTC from approx. 21 to 15 kDa (Figure 2).

cDNA cloning and molecular structure of bBTC

The nucleotide sequence encoding the mature form of bBTC was determined by PCR amplification of bovine kidney cDNA with oligonucleotide primers based on the N- and C-terminal amino acid sequences of purified bBTC. Like other members of the

Deduced Sequence:

10 20 30 40 50 60 70 80

DGXSXSPEDDGLGCGHAENCPATTTPKRRGHFSRCPK---HYCIKGRCRFVVAEQTPSCVDEGYAGARCERVDLFY

N-terminal sequence:

DGXSXSPEDDGLGCGHAENCPATTTPKRRGHF

Endoproteinase Lys-C peptides:

DGXSXSPEDDGLGCGHAENCPATTTPKRRGHFSRCPK

HYCIK

GRCRFVVAEQTPSCVDEGYAGARCERVDLFY

Figure 3 Amino acid sequence determination of bBTC

Reduced and denatured bBTC (20 µg) was S-carboxymethylated and sequenced directly from the N-terminus or digested with endoproteinase Lys-C. Endoproteinase Lys-C peptide fragments were separated and recovered after C₁₈ RP-HPLC and sequenced. X indicates an amino acid that was not identified by N-terminal sequencing.

Figure 4 Amino acid sequence and hydropathy analysis of bBTC

(A) Nucleotide sequence and deduced amino acid sequence of the cDNA for bBTC. The boxed sequence indicates mature bBTC. The two underlined regions represent the putative signal peptide and the transmembrane domain. The arginine-lysine-rich domain is marked with a double underline. Potential sites for N- and O-linked glycosylation are in bold. Numbers at the right and the left indicate the positions of amino acids (upper) and nucleotides (lower). (B) Hydropathy profile of the deduced amino acid sequence of the precursor bBTC, based on the algorithm of Kyte and Doolittle [48].

EGF family, BTC seems to be derived from a precursor sequence that includes a signal peptide, a hydrophobic transmembrane domain and a highly basic cytoplasmic domain [12]. 5' and 3' RACE were used to obtain the nucleotide sequence and the deduced amino acid sequence of pro-bBTC. The complete nucleotide sequence and deduced amino acid sequence for bBTC are shown in Figure 4(A). Hydropathy analysis (Figure 4B) revealed two strongly hydrophobic regions within the precursor

bBTC: MARAAPGSGASPLPPLPALGLVILHCVVA DGNSTRSPEDDGLGCGHAENCPATTTPKRR 62

hBTC: MDRAARCSGASSLPPLALGLVILHCVVA DGNSTRSPETNGLGCGDPEENCAATTTQSKR 62

mBTC: MDPTAPGSSVSLPPLLVIALGLAILHCVVA DGNSTRTPETNGLGCGDPEENCAATTTQSKR 62

bBTC: RGHFSRCPKQYKHYCIKGRCRFVVAEQTPSCVDEGYAGARCERVDLFY LRGDRGQILVICL 124

hBTC: KGHFSRCPKQYKHYCIKGRCRFVVAEQTPSCVDEGYAGARCERVDLFY LRGDRGQILVICL 124

mBTC: KTHFSRCPKQYKHYCIKGRCRFVVAEQTPSCVDEGYAGARCERVDLFY LQDGRGQILVICL 124

bBTC: IAVMVPIILVVSICTCHPLRKRKRKRKEEMETLGKDTIPINDIQTSTIA 178

hBTC: IAVMVPIILVIGVCTCHPLRKRKRKRKEEMETLGKDTIPINDIQTSTIA 178

mBTC: IAVMVPIILVIGVCTCHPLRKRKRKRKEEMETLGKDTIPINDIQTSTIA 177

Figure 5 Amino acid sequence alignment of bBTC, hBTC and mBTC precursors

Vertical lines indicate amino acid residues conserved in all three species. The boxed region indicates mature BTC.

Figure 6 Biological and receptor binding activity of bBTC

(A) Binding of BTC to EGF receptors present on AG2804 cells. ¹²⁵I-rhEGF was added to 70–80% confluent AG2804 cells with various amounts of bBTC (○) or rhEGF (●). (B) Promotion of Balb/c 3T3 cell growth; cell proliferation is expressed as the percentage increase in absorbance (655 nm) above the control lacking growth factor. Symbols: ●, rhEGF; ○, bBTC; ▲, rhBTC. See the Experimental section for details. Results are means ± S.D. for triplicate determinations (S.D. not shown where smaller than the symbols).

protein; one was a presumptive secretion signal peptide, based on the −3 −1 rule for signal peptide cleavage [37], the other was a likely transmembrane domain. A unique highly hydrophilic stretch of arginine/lysine residues, also present in the precursor forms of mouse and hBTC, was present in bBTC. In addition, an

Figure 7 Northern blot analysis of bBTC mRNA

Poly(A)⁺ RNA (2 µg) isolated from various bovine tissues was subjected to electrophoresis, transferred to nylon membrane and probed with full-length bBTC cDNA (A) or bovine β-actin cDNA (B). Lanes 1, MDBK cells; lanes 2, kidney; lanes 3, mammary gland; lanes 4, liver; lanes 5, heart; lanes 6, pancreas; lanes 7, lung; lanes 8, small intestine.

Arg-Gly-Asp (RGD) integrin binding motif was present in the short stretch of amino acid residues adjacent to the putative transmembrane domain, which might mediate cell-cell adhesion. Comparison of the precursor amino acid sequence of hBTC, mBTC and bBTC reveals substantial similarity (Figure 5). Overall, the hBTC and bBTC precursors share 88% sequence identity; however, between the bovine precursor and the mouse precursor there is only 79% sequence identity.

Biological activity

Purified bBTC competed with ¹²⁵I-rhEGF for binding to EGF receptors on AG2804 cells in a dose-dependent fashion; 50% inhibition of binding of ¹²⁵I-rhEGF (mean ± S.D.) was observed at 3.7 ± 0.61 nM bBTC, in comparison with 1.8 ± 0.12 nM for rhEGF (Figure 6A). These findings suggest that bBTC has a slightly lower affinity for EGF receptors on AG2804 cells than does EGF. The mitogenic activity of the purified bBTC preparation was analysed on Balb/c 3T3 fibroblasts: bBTC stimulated the proliferation of Balb/c 3T3 cells in a dose-dependent manner that was very similar to that of rhBTC and EGF (Figure 6B). Half-maximal stimulation of cell proliferation was observed at 73.4 ± 13.6, 83.5 ± 12.2 and 125.7 ± 21.6 pM for bBTC, rhBTC and rhEGF respectively. These results are consistent with previous studies on hBTC [15,22].

Expression of bBTC mRNA

To examine the distribution of bBTC gene expression, full-length mature BTC cDNA was used to probe Northern blots of poly(A)⁺ RNA derived from a variety of tissues. A single transcript of approx. 3 kb was detected in several tissues, particularly kidney and, to a smaller extent, liver, pancreas, small intestine and mammary gland. Relatively little transcript could be detected in heart tissue (Figure 7). These results are consistent with the patterns of BTC expression in human and mouse tissues [12,13].

Contribution of bBTC to the overall mitogenic effect of WGFE

A previous study has shown that the known growth factors present in WGFE (namely IGF-I and IGF-II, PDGF, TGFβ2 and FGF-2) in combination account for only approx. 50% of the growth factor activity in Balb/c 3T3 cells [34]. The extent to which bBTC contributes to the overall growth factor activity of

Figure 8 Contribution of bBTC to the growth factor activity of WGFE

(A) Balb/c 3T3 cells were cultured in DMEM/0.1% BSA plus the indicated growth factor or growth factor combination. Results are expressed as percentages of the response obtained with WGFE (1 mg/ml), and are means ± S.D. for triplicate determinations (S.D. not shown where smaller than the symbols). Growth factors were added at the following concentrations: IGF-I, 20 ng/ml; IGF-II, 20 ng/ml; PDGF-AB, 10 ng/ml; TGF-β2, 1 ng/ml; FGF-2, 1 ng/ml; bBTC, 10 ng/ml; WGFE, 1 mg/ml. 'All -bBTC' refers to the growth-factor cocktail containing IGF-I, IGF-II, PDGF-AB, TGF-β2 and FGF-2; 'All +bBTC' refers to the growth-factor cocktail containing IGF-I, IGF-II, PDGF-AB, TGF-β2, FGF-2 and bBTC. (B) Effect of anti-bBTC on Balb/c 3T3 cell growth in response to bBTC. Cells were cultured in DMEM/0.1% BSA containing bBTC (2.5 ng/ml) in the presence of increasing concentrations of either anti-bBTC antibody (●) or control antibody (IgG) (○) (0–100 µg/ml) added at the time of culture. Results are expressed as percentages of the growth factor response obtained in the absence of either antibody, and are means ± S.D. for triplicate determinations (S.D. not shown where smaller than the symbols). (C) Effect of anti-bBTC on Balb/c 3T3 cell growth in response to WGFE. Cells were cultured in DMEM/0.1% BSA containing WGFE (1 mg/ml) in the presence of 0, 0.1, 1, 10 or 100 µg/ml anti-bBTC antibody (filled columns) or 100 µg/ml control antibody (IgG) (open columns) added at the time of culture. Results are expressed as percentages of the WGFE response obtained in the absence of either antibody, and are means ± S.D. for triplicate determinations.

WGFE was assessed in two ways. First, recombinant forms of the known growth factors in WGFE were added to Balb/c 3T3 cells at concentrations approximating that present in 1 mg/ml WGFE either alone or as a 'cocktail' in the presence or absence of bBTC. Balb/c 3T3 cell growth in a combination of IGF-I, IGF-II, PDGF, TGF- β 2 and FGF-2 was equivalent to 56 % of that observed with WGFE. Importantly, however, the same cocktail containing 10 ng/ml (450 pM) bBTC stimulated the proliferation of Balb/c 3T3 cells to a level equivalent to that of WGFE (Figure 8A). On the basis of the purification data in Table 1, the concentration of bBTC in WGFE was estimated to be approx. 12 ng/mg of WGFE. In a separate experiment the extent to which the growth factor activity in WGFE could be attributed to bBTC was investigated by using a specific anti-bBTC neutralizing antibody. The ability of anti-bBTC to neutralize bBTC activity was confirmed by using Balb/c 3T3 cells (Figure 8B). Thus anti-bBTC (1–100 μ g/ml) completely inhibited Balb/c 3T3 cell proliferation in response to 2.5 ng/ml (114 pM) bBTC. Control antibody (goat anti-rabbit IgG) was without effect. In the presence of 100 μ g/ml anti-bBTC the WGFE-induced growth of Balb/c 3T3 cells was decreased by 49 % ($P < 0.05$) compared with the growth observed in the absence of antibody (Figure 8C). The control antibody at the same concentration did not affect cell growth. These results suggest that bBTC is a major growth factor in WGFE accounting for approx. 50 % of its growth-stimulatory activity, at least in Balb/c 3T3 cells.

DISCUSSION

In addition to nutrients, vitamins and minerals, milk contains a variety of growth factors that are thought to be important in the regulation of growth and secretory functions of maternal mammary tissue and, in the newborn, regulation of growth, development, and maturation of the gut and immune system [26]. In human milk, EGF has been identified as a major growth-promoting factor [38], whereas there seems to be very little, if any, EGF in bovine milk [29]. Instead, the major growth-promoting factors identified in bovine milk seem to be PDGF [27] and IGF-I and IGF-II [28]. Work in our laboratory has shown that the growth factor component in bovine milk is retained in the whey fraction, where it can be enriched 100–200-fold by a single cation-exchange chromatography step [30]. Characterization of the growth-promoting factors in this extract led us to discover the presence of a Balb/c 3T3 cell growth-promoting factor capable of displacing 125 I-rhEGF from EGF receptors present on AG2804 cells. We have identified this growth factor from bovine whey, and by implication from bovine milk, as BTC, a recently described member of the EGF family.

bBTC was purified from WGFE to homogeneity by a seven-step procedure involving ion exchange, gel filtration, RP-HPLC and affinity chromatography. Affinity chromatography on heparin-Sepharose has been used to purify a number of growth factors of the EGF family, including HB-EGF, AR and mBTC. bBTC was eluted from the heparin-Sepharose column at approx. 0.5 M NaCl, which is similar to that for mBTC [12]. In contrast, 1 M NaCl is required to elute AR and HB-EGF from heparin-Sepharose [4]. Clusters of basic amino acid residues (particularly lysine and arginine) in the N-terminal portion of HB-EGF and AR [39] have been implicated in high-affinity binding to heparin. Interestingly, mBTC, hBTC and bBTC do not contain such basic residues at the N-terminus and, unlike AR and HB-EGF, the mitogenic activity of BTC on Balb/c 3T3 cells is not inhibited by heparin [15]. In this respect it is unclear whether the interaction between BTC and heparin *in vitro* is a

true reflection of a physiologically relevant association between BTC and heparan sulphate proteoglycans *in vivo*.

Purified bBTC from WGFE migrated on SDS/PAGE at approx. 21–22 kDa. This is in contrast with mBTC, which has a molecular mass of approx. 30–32 kDa. This difference is probably due to variable states of glycosylation between the two molecules. The likely sites of N- and O-linked glycosylation in mature hBTC and mBTC have been suggested as Asn³ and Thr⁵ [12,15]. These sites are also present in bBTC; in support of this, neither Asn³ nor Thr⁵ could be detected in N-terminal sequencing. Asn¹¹, which is present in mBTC and hBTC, might also be a further site for N-linked glycosylation. In this context it is interesting that in bBTC this position corresponds to an Asp residue. Therefore the difference in molecular mass between mouse and bBTC might reflect additional glycosylation at Asn¹¹.

Treatment of mBTC with N- and O-glycanase results in a shift in the molecular mass from 30–32 kDa to 18 kDa, suggesting that sugar moieties contribute approx. 12 kDa to the overall molecular mass. Digestion of bBTC with a combination of NANase II, O-glycosidase and PNGase F shifted the molecular mass from 21 kDa to approx. 15 kDa, suggesting that the contribution of sugar complexes to the overall molecular mass of bBTC is much smaller. The predicted molecular mass of bBTC and mBTC is approx. 9 kDa, clearly different from the sizes generated by enzymic deglycosylation. This discrepancy might be due to a retarded mobility of BTC on SDS/PAGE as a result of primary structure. This type of SDS/PAGE anomaly has also been reported for the non-glycosylated form of recombinant HB-EGF, which is predicted to be approx. 8.3 kDa but migrates on SDS/PAGE with an apparent molecular mass of 12–14 kDa [40].

The open reading frame of bBTC cDNA encodes a 178-residue primary translation product that corresponds to the bBTC precursor. Overall the primary translation product is highly similar to hBTC and mBTC. Pro-bBTC is 88 % identical at the amino acid level with pro-hBTC and 79 % identical with pro-mBTC. Within the mature sequence, bBTC is 90 % identical with hBTC and 72.5 % identical with mBTC. Similarly to mBTC and hBTC, mature bBTC contains the six-cysteine consensus motif (CX₇CX₄CX₁₀CX₁CX₆C) characteristic of other members of the EGF family. The 'hinge' residue between the fourth and fifth cysteine residues, which defines the EGF receptor contact point [41], was identified as a valine residue in bBTC, which is similar to hBTC and TGF- α . Other residues conserved between members of the EGF family were also conserved in bBTC, such as Gly⁴⁹, Gly⁷⁰, Arg⁷² and Leu⁷⁸. Leu⁷⁸ (Leu⁴⁷ and Leu⁴⁸ in EGF and TGF- α respectively) is particularly important because it has been identified as a key residue of EGF and TGF- α for mitogenic and receptor binding activities [42]. Interestingly, BTC contains an additional two cysteine residues in the N-terminal portion of the mature molecule; these are likely to form a fourth disulphide bond [22]. It is unknown whether this disulphide bond imparts an additional structural configuration required for erbB-receptor binding and mitogenic activity. Alanine-scanning mutagenesis of these additional cysteine residues might give insight into the structure-function relationships of this fourth disulphide bond.

The N- and C-terminal residues of purified bBTC are Asp and Tyr respectively, as in hBTC and mBTC. Given this, it is highly likely that mature BTC is released from the cell surface after proteolytic cleavage between Ala³¹-Asp³² and Tyr¹¹¹-Leu¹¹². However, little is currently known about the identity and the mode of regulation of the proteolytic enzymes responsible for the cleavage of soluble mature BTC from the membrane-anchored precursor. However, unlike other members of the EGF family, this process does not seem to be regulated by a protein-kinase-C-

dependent mechanism [43]. A number of recent reports suggest that ectodomain processing of members of the EGF family might be mediated by TACE (tumour-necrosis-factor- α -converting enzyme), a member of the ADAM ('a disintegrin and metalloproteinase') family of metalloproteinases [44,45]. An additional feature of the membrane-anchored precursor of BTC is an extracellularly positioned RGD integrin-binding motif. The presence of an RGD sequence in BTC raises the possibility that BTC, in addition to its role as a soluble growth factor, could act as a membrane-anchored integrin ligand mediating direct cell-cell interactions.

The biological significance of BTC in bovine milk is currently unclear. One possible function for milk-borne BTC might be to stimulate the proliferation of the gastrointestinal epithelia in the newborn, as has been widely proposed for both milk-borne EGF and TGF- α in other species. Moreover, if BTC is absorbed through the small intestine from maternal milk, it might also contribute to the growth of other organs in the newborn, such as the pancreas and kidney. In this regard, BTC has been shown to stimulate the proliferation of a fetal pancreatic epithelial cell line *in vitro* [46]; members of the EGF family including BTC are able to induce epithelial branching tubulogenesis in embryonic kidney [47]. In addition, BTC might also be important in the growth and differentiation of the maternal mammary gland; in the presence of BTC, MDA-MB453 mammary epithelial cells undergo phenotypic differentiation leading to the formation of neutral-lipid-containing droplets [21] characteristic of terminal mammary epithelial differentiation and milk secretion. Apart from the unknown function of BTC in milk, the site(s) and regulation of BTC are also unknown. Although macrophages, lymphocytes, neutrophils and epithelial cells present in milk might synthesize BTC, milk-borne BTC might also be produced from secretory epithelial cells of the mammary gland or secreted from plasma. Clearly, further studies are required to define the biological role of milk-borne BTC.

In summary, we have identified BTC, a member of the EGF family of growth factors, as a novel growth factor in bovine milk. The isolation of BTC from bovine milk suggests that it is likely to be important in neonatal development and possibly mammary growth and differentiation.

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