Purification and molecular characterization of recombinant rat betacellulin

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ABSTRACT

A method for the large scale expression and purification of rat betacellulin (BTC) from *Escherichia coli* has been developed using a cleavable fusion protein strategy. Insoluble fusion protein collected as inclusion bodies was dissolved in urea under reducing conditions, re-folded, and purified by gel filtration chromatography and C₄ RP-HPLC. Authentic rat BTC was obtained after proteolytic cleavage of the fusion protein with Factor Xa.

Factor Xa cleaved an additional site within the BTC protein, generating a truncated isoform separable from full-length BTC by heparin-affinity chromatography. Recombinant rat BTC stimulated the proliferation of mouse Balb/c 3T3 fibroblasts and competed for binding to the ErbB1 receptor in a dose-dependent manner analogous to that of BTC purified from natural sources.

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INTRODUCTION

Betacellulin (BTC) belongs to a class of growth factors characterized by a six-cysteine consensus motif that forms three intramolecular disulphide bonds critical for binding to the ErbB receptor tyrosine kinase family (Dunbar & Goddard 2000). Collectively, this group of proteins are referred to as the epidermal growth factor (EGF) family. Mammalian members of this family identified to date include EGF, amphiregulin, and transforming growth factor- α (TGF- α), which bind specifically to ErbB1, and heparin-binding EGF (HB-EGF), epiregulin and BTC, which exhibit dual receptor specificity in that they bind both ErbB1 and ErbB4. The third group comprises the neuregulins (NRGs) which encompass the products of four genes (*NRG1–NRG4*). Members of this sub-family bind ErbB4 but not ErbB1, and can bind ErbB3 in the context of an ErbB2–ErbB3 heterodimer (Harari & Yarden 2000). Recently, a new member of the EGF family has been described (Strachan *et al.* 2001). This molecule, termed epigen, has been shown to activate ErbB1. Characterization of the full ErbB receptor specificity of epigen, however, awaits further investigation. Binding and activation of the ErbB receptors by the EGF family results in the

initiation and transmission of highly conserved signal transduction cascades (such as the mitogen-activated protein kinase and phosphatidyl inositol 3-kinase pathways), culminating in diverse cellular effects, including growth, differentiation, migration and survival.

BTC was initially purified and characterized from the conditioned medium of a mouse pancreatic β -cell carcinoma cell line (Shing *et al.* 1993) and subsequently has been identified and characterized in human (Sasada *et al.* 1993), bovine (Dunbar *et al.* 1999) and rat (Tada *et al.* 2000). Increased expression of BTC mRNA in the pancreas compared with other tissues, and an increasing number of *in vitro* studies with cultured cell lines have supported the hypothesis that BTC signaling through ErbB receptors plays an important part in islet growth and development in the pancreas. For example BTC, together with activin-A, can convert populations of AR42J rat pancreatic tumour cells into insulin-secreting cells (Mashima *et al.* 1996); BTC is required for the induction of insulin and glucokinase gene expression in PDX-1-expressing glucagonoma cells (Watada *et al.* 1996) and BTC is able to mediate the proliferation and differentiation of the rat insulinoma cell line, INS-1 (Huotari *et al.* 1998). Significantly, in these studies, neither EGF

or TGF- α could mimic these effects. In addition BTC has been shown to exert a mitogenic effect on human undifferentiated pancreatic epithelial cells (Demeterco *et al.* 2000) and ductal epithelial cells derived from developing pancreatic buds of rat embryos (Sundaresan *et al.* 1998). Furthermore, immunohistochemical analysis has localized BTC to primitive duct cells in fetal pancreas and to some islet cell populations closely associated with insulin producing cells (Miyagawa *et al.* 1999, Tada *et al.* 1999). More recently, detailed analysis of ErbB1 deficient (-/-) mice has revealed that disruption of ErbB1 signaling leads to defects in pancreatic epithelial proliferation and an associated delay in β -cell development (Miettinen *et al.* 2000).

In all cases described above, the evidence linking a role for BTC signaling to pancreas development and function through ErbB1 and possibly ErbB4 is based almost solely on the use of cultured cell lines *in vitro*. Clearly, analysis of animal models in which the *BTC* gene has been either selectively eliminated or overexpressed in the pancreas *in vivo* will be important, together with detailed investigation into the effects of short- and long-term infusion of BTC into animal disease models. Indeed, a recent study by Yamamoto *et al.* (2000) revealed that administration of recombinant human BTC significantly improved glucose tolerance in mice with diabetes induced by selective perfusion of alloxan. This effect was considered to be the result of promoting β -differentiation and regeneration from ductal or acinar cells, or both.

In this paper we report a method for the large-scale production and purification of authentic recombinant rat BTC from *Escherichia coli* and the biological analysis of the purified protein. The ability to produce rat BTC in high yield and purity will be particularly useful in examining the physiological effects of the administration of BTC to various rodent models of human disease (including diabetes). Furthermore, these animal studies can be performed without the complication of potential immunological 'interference' after reaction to a heterologous protein (i.e. human BTC), particularly as we have recently demonstrated that human BTC is highly immunogenic in the rabbit (Bastian *et al.* 2000).

MATERIALS AND METHODS

Cell culture

Balb/c 3T3 and IEC-6 cells were from the American Type Tissue Culture Collection (Manassas, VA, USA) and AG2804 human lung fibroblasts were kindly donated by Dr J M Gunn (Texas A and M

University, College Station, TX, USA). Both 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 (FBS), and propagated at 37 °C in a humidified atmosphere of 5% CO₂.

Cloning and expression

The cDNA encoding the mature form of rat BTC (Asp³²-Tyr¹¹¹) was generated by RT-PCR. Total RNA was isolated from 80–90% confluent rat IEC-6 cells (ATCC CRL-1592) and cDNA synthesized from 1 μ g total RNA using oligo dT primer and Superscript II (Life Technologies, Melbourne, Australia). The subsequent cDNA was used as a template for PCR with oligonucleotide primers, 5' ATC TAG GTT AAC ATC GAA GGT CGT GAT GGG AAC ACG ACC AGA ACC 3' (single underline, HpaI restriction site; double underline, nucleotide sequence encoding the Ile-Glu-Gly-Arg Factor Xa recognition site) and 5' CTA GAT AAG CTT TCA TCA GTA AAA CAG GTC CAC CTG 3' (single underline, HindIII restriction site, double underline stop codons). The resultant 282 bp PCR product was purified, digested with HpaI/HindIII and cloned into HpaI/HindIII-digested 46-amino acid porcine growth hormone (pGH(1–46)) expression vector (King *et al.* 1992) to generate pGH(1–46)-Ile-Glu-Gly-Arg-BTC (Fig. 1). This vector was subsequently transformed into *E. coli* JM101. Large scale IPTG-induced protein expression was performed in 4 × 1 litre fermenters (Applicon, Schiedam, The Netherlands).

Fusion protein purification and re-folding

After fermentation, cells were disrupted by homogenization, and insoluble inclusion bodies collected by centrifugation (10 000 *g*, 25 min, 4 °C). Inclusion bodies were washed twice with 30 mM NaCl, 10 mM KH₂PO₄, 0.5 mM ZnCl₂ and resuspended at 10% (w/v) in 100 mM Tris (pH 9.0), 8 M urea, 40 mM glycine, 40 mM dithiothreitol (DTT) and 0.5 mM ZnCl₂ and stirred for 30 min at room temperature. Solubilized inclusion bodies were centrifuged (14 000 r.p.m., 20 min) to remove particulate matter and the supernatant was applied to a Cellufine GCL-1000 column (5 × 100 cm) (Chisso Corp., Tokyo, Japan) equilibrated with 100 mM Tris (pH 9.0), 8 M urea, 40 mM glycine, 40 mM DTT and 0.5 mM ZnCl₂, at 2 ml/min. Fractions containing fusion protein (pGH46-rat BTC) were pooled and subject to oxidative re-folding by diluting the mixture to a final protein

concentration of 0.1 mg/ml in buffer containing 100 mM Tris (pH 9.0), 4 M urea, 40 mM glycine, 5 mM EDTA, 0.4 mM DTT and 1 mM 2-hydroxyethylsulphoxide. After being stirred for 3 h at room temperature, the re-fold reaction was terminated by pH adjustment to 4.0 with trifluoroacetic acid (TFA), filtered against a 1 µm membrane to remove insoluble material and the mixture applied to a C₄ Prep-Pak RP-HPLC column (40 mm × 100 mm; 300 Å, 15 µm; Millipore-Waters, North Ryde, NSW, Australia) at 50 ml/min. After extensive washing in 0.1% TFA the fusion protein was eluted with a gradient of 15–50% acetonitrile over 70 min (in the presence of 0.08% TFA) at a flow rate of 25 ml/min. Fractions were collected and those containing pure pGH46–rat BTC fusion protein (as determined by SDS-PAGE) were pooled and lyophilized.

Factor Xa cleavage and purification

pGH46–rat BTC fusion protein was dissolved in a minimal volume of 10 mM HCl and resuspended in 50 mM Tris chloride (pH 8.0), 100 mM NaCl, 1 mM CaCl₂ to give a final protein concentration of 0.05 mg/ml. One hundred units of Factor Xa (Amersham-Pharmacia Biotech., Castle Hill, NSW, Australia) were then added per 20 mg fusion protein and the reaction carried out for 20 h at room temperature before terminating by pH adjustment to 3.0 with 1.5% TFA. Authentic rat BTC was separated from the pGH46 fusion partner by RP-HPLC. The cleavage reaction was diluted 1:4 (v/v) with 0.1% TFA and applied to a C₄ RP-HPLC column (2.5 × 10 cm, 300 Å, Millipore-Waters) at 20 ml/min. The column was then washed with 0.1% TFA and BTC eluted from the column with a linear gradient of 0–50% acetonitrile (in the presence of 0.08% TFA) at 25 ml/min over 80 min. Fractions containing rat BTC were pooled and lyophilized. To separate full-length recombinant rat BTC from a shorter 'mis-cleaved' contaminant (see Results), the protein pool was resuspended in 50 mM Tris chloride (pH 7.5) and applied to a Progel-TSK Heparin-5PW column (7.5 mm × 7.5 mm, Supelco, Castle Hill, NSW, Australia) attached to the HPLC at a flow rate of 0.5 ml/min. The column was washed with 50 mM Tris chloride (pH 7.5) and then eluted with a three-step gradient of 50 mM Tris chloride (pH 7.5)/0.2 M NaCl for 10 min, followed by 50 mM Tris chloride (pH 7.5)/0.6 M NaCl for 15 min, followed by 50 mM Tris chloride (pH 7.5)/0.2 M NaCl for 10 min. Fractions containing authentic full-length rat BTC were pooled, desalted and the purity of the final preparation analysed by microbore C₄ RP-HPLC, electrospray

ionization mass spectroscopy (Perkin-Elmer API 300, Shelton, CT, USA) and N-terminal sequence analysis (Hewlett-Packard G1000A, Palo Alto, CA, USA).

Mitogenic and ErbB1 receptor binding assay

Mitogenic activity of recombinant rat BTC (rrBTC) on Balb/c 3T3 fibroblasts was determined by colourimetric assay using methylene blue as previously described (Dunbar *et al.* 1999). The ability of rrBTC to bind ErbB1 specifically was determined by competitive displacement of ¹²⁵I-recombinant human EGF (rhEGF) from ErbB1 receptors present on AG2804 human lung fibroblasts. Briefly, AG2804 cells were grown to 70–80% confluence in DMEM/10% FBS 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, 8 mM glucose, 0.1% BSA) and then incubated with ¹²⁵I-rhEGF (labeled with Na[¹²⁵I] using chloramine-T to a specific activity of approximately 20 µCi/µg) and increasing concentrations of unlabelled rrBTC (0–44 nM) in binding buffer at 4 °C for 18 h. Cells were then washed three times in Hanks's buffered salt solution and lysed with 1 ml 0.5 M NaOH/0.1% (v/v) Triton X-100 for 30 min. Radioactivities of cell lysates were then determined with a γ-counter (Wallac 1470). Non-specific binding was determined by the addition of excess unlabelled rhEGF (100 nM) and was typically about 5% of total binding. Experimental data for both the mitogenesis assay and ErbB1 receptor binding assay were fitted to a logistic four-parameter dose–response model with variable slope (SigmaPlot v.4.0).

ErbB-1 receptor tyrosine phosphorylation assay

AG2804 cells were grown to confluence in 10 cm dishes and subsequently incubated for 12 h in serum-free medium. Cells were then stimulated with 10 nM rrBTC for 10 min at room temperature, washed twice in PBS and then suspended in lysis buffer (0.5 ml) (50 mM Tris chloride pH 7.4, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 5 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM EGTA and complete protease inhibitors (Roche Biochemicals, Castle Hill, NSW, Australia). Cell lysates were cleared by centrifugation (20 min, 15 000 g at 4 °C) and ErbB1 immunoprecipitated by incubating the lysate with 1 µg anti-ErbB1 (Upstate Biotech., New York, NY, USA) and protein-G sepharose (Roche Biochemicals) for 2 h at 4 °C. Immune complexes were

FIGURE 1. Description of the pGH(1-46)-rat BTC expression construct. The downward arrow indicates the Factor Xa cleavage point. Amino acid and nucleotide sequences of the various parts of the fusion protein are shown. The boxed amino acid sequence corresponds to the complete amino sequence of rat BTC (Asp³²-Tyr¹¹¹). Note: amino acid numbering is with respect to the full 177 amino acids of the precursor rat BTC sequence. *Stop codon.

collected by centrifugation, washed three times in lysis buffer and heated (3 min, 95 °C) in SDS-PAGE sample buffer. Proteins were separated on 6% SDS-PAGE gels and transferred to nitrocellulose filters (Hybond C, Amersham Pharmacia Biotech.). Blots were probed with anti-phosphotyrosine monoclonal antibody (PY20, Santa Cruz Biotech., Santa Cruz, CA, USA) and then with HRP-conjugated sheep anti-mouse IgG (Silenus Laboratories, Boronia, Australia). HRP-labeled proteins were visualized using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech.). To confirm equal loading, blots were stripped and re-probed with the anti-ErbB1 antibody and HRP-conjugated rabbit anti-sheep IgG (Zymed Laboratories Inc., San Francisco, CA, USA).

Production of anti-rat BTC antibody

Antibody production was approved by the Animal Ethics Committee of the Women's and Children's Hospital, Adelaide, South Australia and the procedure followed the Australian Code of Practice for the care and use of animals for scientific purposes. Briefly, three female semi-lop rabbits (obtained from the Institute of Medical and Veterinary Sciences, Gillies Plains, South Australia) were injected with 500 µg of a 14-residue peptide (G⁵⁰ENCTGTTTPRQKSK⁶³) conjugated to diphtheria toxoid (Chiron Technologies, Melbourne, Victoria, Australia) in Freund's complete adjuvant, following standard procedures (Cooper & Petterson 1999). After 6 weeks, animals were given a first booster injection of 100 µg peptide in Freund's incomplete adjuvant; they received a similar, second boost, 4 weeks later. Blood samples were obtained 2 weeks after each booster injection for determination of antibody titre. Animals were killed 4 weeks after the second booster and blood collected by cardiac

puncture. Rat BTC antisera was affinity-purified using a High Trap protein-G sepharose column (Amersham-Pharmacia Biotech.), following the manufacturer's instructions.

RESULTS

Production and molecular characterization of rat BTC

The mature form of rat BTC (Asp³²-Tyr¹¹¹) was expressed as a recombinant fusion protein containing the first 46 amino-terminal amino acids of porcine growth hormone (pGH) (King *et al.* 1992). A proteolytic cleavage site (Ile-Glu-Gly-Arg) was engineered downstream of the pGH fusion partner to allow for 'release' of rat BTC after digestion with Factor Xa (Fig. 1).

Insoluble pGH46-rat BTC within inclusion bodies was dissolved in urea/DTT-containing buffer and partially purified by cellulose gel filtration chromatography to remove endogenous proteases. The solubilised fusion protein was then subject to oxidative re-folding in the presence of 2-hydroxyethyl disulphide. Fusion protein containing the correctly folded six-cysteine consensus EGF motif was then separated from mis-folded isomers by C₄ RP-HPLC. pGH46-rat BTC fusion protein from this two-step procedure was considered to be essentially homogeneous as judged by analytical HPLC and SDS-PAGE (Fig. 2).

Separation of the pGH fusion partner from authentic rat BTC

Incubation of the fusion protein with Factor Xa for 20 h at 22 °C generated a complex profile of protein peaks (P1-5) that were resolvable by C₄ RP-HPLC (Fig. 3A, B). P3 was identified as containing rat

FIGURE 2. Analytical RP-HPLC analysis of purified pGH46-rat BTC fusion protein. An aliquot of the purified pGH46-rat BTC fusion protein preparation was analysed on a microbore C_4 RP-HPLC column (2.1 mm $\times$ 100 mm, Brownlee Laboratories, Santa Clara, CA, USA). Protein was eluted with an increasing gradient of acetonitrile to 80% in the presence of 0.08% TFA over 37 min. *Inset*: SDS-PAGE analysis of the purified preparation. Lane 1, molecular weight markers; lane 2, pGH46-rat BTC fusion protein.

BTC by monitoring mitogenic activity (data not shown). The identity and composition of the other peaks were not investigated. The purity of the recovered rat BTC (P3) was assessed by analytical RP-HPLC (Fig. 3C), SDS-PAGE and electrospray ionization mass spectrometry (Fig. 4A, B). A protein of 9036.1 ± 0.8 Da corresponding to full-length rat BTC (theoretical M_r 9034.2 Da) was identified. In addition however, a second protein species of M_r 5992.0 ± 0.4 was present in approximately equivalent amounts. N-Terminal sequence analysis (five cycles) of the purified P3 preparation identified two protein sequences: Asp-Gly-Asn-Thr-Thr and Ser-Lys-Thr-His-Phe. Therefore, Factor Xa, in addition to cleaving pGH46-rat BTC fusion protein after Ile-Glu-Gly-Arg also cleaved an additional cryptic Factor Xa site within the BTC protein ($^{27}\text{Pro-Arg-Gln-Lys}\downarrow\text{Ser-Lys-Thr}^{33}$) (Fig. 4B). The theoretical molecular mass of the 'clipped' BTC isoform was predicted to be 5989.6 Da which is consistent with the observed molecular mass of 5992.0 ± 0.4 Da. As expected, a polyclonal antibody raised against a synthetic peptide corresponding to the amino acid sequence Gly-Glu-Asn-Cys-Thr-Gly-Thr-Thr-Pro-Arg-Gln-Lys-Ser-Lys only recognized the full-length BTC isoform and not the clipped form (Fig. 4A).

FIGURE 3. Analytical RP-HPLC of the pGH46-rat BTC fusion protein cleavage reaction. Pure pGH46-rat BTC fusion protein was incubated for 20 h with Factor Xa at 22 °C and an aliquot of the reaction analysed by microbore C_4 RP-HPLC as described in Fig. 2. (A) RP-HPLC analysis of the pGH46-rat BTC fusion protein at baseline ($T=0$ h). (B) Analysis of an aliquot of the reaction after 20 h ($T=20$ h). (C) Analysis of an aliquot of pooled fractions containing P3 after large-scale resolution of the factor Xa peptides.

FIGURE 4. (A) SDS-PAGE and western blot analysis of the purified P3 preparation. An aliquot of the P3 preparation was resolved by SDS-PAGE and either stained with Coomassie blue (left panel) or immunoblotted and probed with a polyclonal anti-rat BTC antibody to the peptide Glu-Asn-Cys-Thr-Gly-Thr-Thr-Pro-Arg-Gln-Lys (right panel). (B) N-terminal sequence analysis (five cycles) (double underline) and electrospray ionization mass spectrometry of the P3 preparation. Single underline indicates the amino acid sequence used to generate a polyclonal anti-rat BTC antibody. ↓ indicates the cryptic Factor Xa cleavage site within the BTC protein.

Heparin-affinity chromatography effectively separated the full-length and clipped BTC isoforms (Fig. 5A, B). Full-length BTC eluted slightly earlier than the clipped form (0.32 M and 0.4 M NaCl respectively). Fractions collected from the heparin-affinity column containing full-length rat BTC were pooled, desalted and analysed by analytical RP-HPLC, electrospray ionization mass spectrometry and N-terminal sequence analysis. The purified peptide eluted as a single symmetrical peak (Fig. 5C) with a molecular weight of 9036 Da. N-Terminal sequence analysis confirmed the presence of only a single protein sequence, corresponding to rat BTC.

Biological activity of rrBTC

rrBTC competed with ^{125}I -rhEGF for binding to ErbB1 receptors present on AG2804 cells in a dose-dependent fashion; 50% inhibition of binding (IC_{50}) of ^{125}I -rhEGF was observed at 0.68 ± 0.05 nM (mean $\pm$ S.D.) (Fig. 6A). To determine whether binding to the extracellular domain of ErbB1 activated the receptor, AG2804 cells were incubated with or without rrBTC. After immunoprecipitation with an anti-ErbB1 antibody, tyrosine

phosphorylation of the ErbB1 receptor was analysed by western blotting using anti-phosphotyrosine antibodies. rrBTC clearly induced ErbB1 phosphorylation compared with control (Fig. 6A, *inset*). Furthermore, binding of rrBTC to ErbB1 receptors also stimulated the proliferation of Balb/c 3T3 cells in a dose-dependent fashion (Fig. 6B).

DISCUSSION

Human BTC has previously been produced in *E. coli* as a recombinant protein encompassing the mature peptide (Asp³²-Tyr¹¹¹). To initiate translation of this protein, a methionine residue was appended to the amino-terminus (Asp³²) (Seno *et al.* 1996). Here we have sought to produce, in large yields, a rat BTC of which the authenticity would not be compromised by the presence of additional amino acids at the amino-terminus. To do this, the mature form of rat BTC (Asp³²-Tyr¹¹¹) was expressed in *E. coli* as a fusion protein in which a site-specific proteolytic cleavage site was engineered downstream of the fusion partner. This allowed for the generation of authentic BTC

FIGURE 5. Heparin-affinity chromatography. The P3 preparation was further resolved on a Progel-TSK Heparin-5PW HPLC affinity column (A). Fractions were analysed by SDS-PAGE (B) and those containing pure rat BTC were pooled. Pure rat BTC ran as a single symmetrical peak when analysed by microbore C_4 RP-HPLC with a gradient of 0–50% acetonitrile over 20 min (C).

without the need to insert an amino-terminal methionine residue for translation initiation.

Like many other proteins that are overproduced in *E. coli*, pGH46–rat BTC fusion protein aggregated in the cell to form insoluble inclusion bodies. This insolubility made it possible to partially purify the protein from lysed cells by a simple low-speed

FIGURE 6. Biological activity of rrBTC. (A) Binding of rrBTC to ErbB1 receptors present on AG2804 cells. 125 I-rhEGF was added to AG2804 cells with increasing amounts of rrBTC. *Inset*: ErbB1 receptor phosphorylation. AG2804 cells were treated with or without rrBTC and ErbB1 receptor phosphorylation examined by immunoprecipitation and western blotting with anti-phosphotyrosine (p-Tyr) antibody. (B) Promotion of Balb/c 3T3 cell proliferation in the presence of rrBTC: cell proliferation is expressed as the % increase in absorbance (655 nm) above control lacking growth factor. Data in (A) and (B) are means $\pm$ s.d. for triplicate determinations and are fitted to a logistic four-parameter dose-response model with variable slope (SigmaPlot v.4.0).

centrifugation in which the inclusion bodies segregated to the pellet phase. From 4 litres of induced culture, we typically obtained about

35 g (wet weight) of inclusion bodies, containing approximately 1 g of fusion protein. After dissolution of inclusion bodies and gel filtration to remove endogenous proteases, pGH46-rat BTC fusion protein was re-folded in the presence of 2-hydroxyethyl disulphide, and correctly folded protein containing the six-cysteine consensus EGF motif (C₁-C₃, C₂-C₄ and C₅-C₆) separated from mis-folded 1-, 2- and 3-disulphide scrambled isomers by C₄ RP-HPLC.

The blood coagulation Factor Xa was then used to separate the pGH fusion partner from authentic rat BTC. In addition to cleaving the fusion protein at the canonical tetrapeptide sequence Ile-Glu-Gly-Arg, Factor Xa also efficiently cleaved a non-specific peptide sequence (²⁷Pro-Arg-Gln-Lys↓Ser-Lys-Thr³³) within the N-terminal region of rat BTC. The non-specific Factor Xa cleavage event did not appear to be a secondary consequence of extended incubation, as a time course analysis demonstrated that the generation of both peptides occurred simultaneously (data not shown). Interestingly, recombinant human BTC produced in mouse A9 cells is sensitive to endogenous proteolytic cleavage in an identical position (²⁷Thr-Gln-Ser-Lys↓Arg-Lys-Gly³³), suggesting that this region within both human and rat BTC is overly susceptible to proteolytic cleavage (Watanabe *et al.* 1994).

Factor Xa is primarily specific for the Ile-Glu-Gly-Arg recognition sequence, but it is also known to cleave additional sites. Only a few of these additional sites have been characterized, and they have little in common with the Ile-Glu-Gly-Arg sequence: for example, Cys-Asn-Gly-Arg↓Trp-Val (Nambiar *et al.* 1987), Ser-Leu-Ser-Arg↓Met-Thr (Quinlan *et al.* 1989), and Ala-Leu-Ala-Arg↓Lys-Tyr (Nagai & Thøgersen 1987). In some cases, where cleavage occurs immediately after lysine residues, as is the case here, reversible acylation by incubation with 3,4,5,6-tetrahydrophthalic anhydride is effective in blocking non-specific cleavage (Wearne 1990).

To separate full-length rat BTC from the clipped shorter form, we initially used RP-HPLC. However, even using very shallow gradients we were unable to achieve suitable separation of the two forms (data not shown). In contrast, heparin-affinity chromatography effectively separated the two forms (Fig. 5A, B). Full-length rat BTC eluted as a single peak from an analytical C₄ RP-HPLC and was shown to be greater than 99% pure as judged by electrospray ionization mass spectrometry and N-terminal sequence analysis. Using this procedure we were typically able to generate approximately 10–15 mg pure rat BTC from 4 litres of starting culture.

Biological activity of rrBTC

The biological activity of the rrBTC preparation was evaluated by both ErbB1 receptor binding and phosphorylation, and mitogenic stimulation of mouse fibroblasts. Recombinant rat BTC competed with [¹²⁵I]-rhEGF for binding to ErbB1 receptors present on AG2804 cells in a dose-dependent fashion; 50% inhibition of binding (IC₅₀) of ¹²⁵I-rhEGF was observed at 0.68 ± 0.05 nM, consistent with the findings of previous studies examining human BTC binding to CHO cells expressing ErbB1 (IC₅₀=1 nM; Pinkas-Kramarski *et al.* 1998). Binding of rrBTC to ErbB1 also induced receptor phosphorylation in AG2804 cells and stimulated proliferation of Balb/c 3T3 cells in a dose-dependent fashion (Fig. 6B). Interestingly, the clipped BTC also stimulated the proliferation of Balb/c 3T3 cells with a similar dose-dependency (data not shown), confirming previous reports that the amino-terminal 30 amino acids are dispensable for ErbB1 receptor binding and activation (Watanabe *et al.* 1994).

The expression system that we describe provides an efficient and consistent means of producing highly purified and biologically active full-length recombinant rat BTC. Authentic rat BTC will be useful in examining the effect(s) of the administration of this growth factor on various tissues (such as the pancreas) in normal rodents and various rodent models of human disease.

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