Journal of Cellular Biochemistry 72:423–434 (1999)

Processing and Juxtacrine Activity of Membrane-Anchored Betacellulin

Hiroko Tada,[1]* Reiko Sasada,[2] Yasuko Kawaguchi,[1] Itaru Kojima,[3] William J. Gullick,[4] David S. Salomon,[5] Koichi Igarashi,[2] Masaharu Seno,[1] and Hidenori Yamada[1]

[1]Department of Bioengineering Science, Faculty of Engineering, Okayama University, Okayama 700-8530, Japan
[2]Discovery Research Laboratories II, Discovery Research Division, Takeda Chemical Ind. Ltd., Ibaragi 300-42, Japan
[3]Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371, Japan
[4]ICRF Oncology Unit, Hammersmith Hospital, London, United Kingdom, WI2 ONN
[5]Tumor Growth Factor Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract Betacellulin (BTC) was originally isolated as a secreted growth factor from a mouse pancreatic β-tumor cell line, whereas the cDNA sequence predicts that BTC is synthesized as a larger transmembrane protein. In the present study, we have characterized the membrane-anchored forms of BTC, using Chinese hamster ovary (CHO) cells, mouse fibroblast A9 cells, and a human breast cancer cell line MCF-7, all of which were stably transfected with human BTC cDNA. A9 and MCF-7 transfectants produced membrane-anchored BTC isoforms of 21, 25, 29, and 40 kDa on the cell surface, as well as a secreted BTC isoform. CHO transfectants secreted little BTC but accumulated the membrane-anchored isoforms. The cleavage of the membrane-anchored forms to release a secreted from of BTC was not enhanced by biological mediators such as a phorbol ester, which stimulates the cleavage of other membrane-anchored growth factors. The membrane-anchored forms of BTC expressed on the transfected cells induced the insulin production and/or promoted the growth in subclones of AR42J rat pancreatic cells. These results suggest that the membrane-anchored BTC can function as a juxtacrine factor in regulating the growth and differentiation of pancreatic endocrine cells. J. Cell. Biochem. 72:423–434, 1999.

Key words: betacellulin; membrane-anchored growth factor; pancreatic cell differentiation; isoforms; juxtacrine stimulation

Abbreviations used: BTC, betacellulin; rhBTC, recombinant human BTC; ALP, placental alkaline phosphatase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; CSF-1, colony-stimulating factor-1; HB-EGF, heparin-binding EGF-like growth factor; PKC, protein kinase C; KL, c-*kit* ligands; PMA, phorbol 12-myristate 13-acetate; RA, all-*trans*-retinoic acid; CHO, Chinese hamster ovary; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; CHAPS, 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethylsulfoxide; FITC, fluorescein-5-isothiocyanate; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-di-phenyltetrazolium bromide; PKC, protein kinase C.

Contract grant sponsor: Ministry of Education, Science, Sports, and Culture of Japan.

*Correspondence to: Hiroko Tada, Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, 3-1-1 Tsushima-Naka, Okayama 700-8530, Japan. E-mail: tadahrk@biotech.okayama-u.ac.jp

Received 3 February 1998; Accepted 24 September 1998

Betacellulin (BTC) is a member of epidermal growth factor (EGF) family, which was originally isolated from the conditioned medium of a mouse pancreatic β-tumor cell line [Shing et al., 1993]. The secreted BTC is composed of 80 amino acid residues with extensive glycosylation. BTC promotes the proliferation of fibroblasts and epithelial and vascular smooth muscle cells through binding to the epidermal growth factor receptor (EGFR) [Shing et al., 1993]. Human BTC cDNA was also isolated from a cDNA library prepared from a human breast cancer cell line, MCF-7 [Sasada et al., 1993]. Recombinant human BTC (rhBTC) has been produced in the secreted form both in mammalian cells and in bacterial cells and has been well characterized [Seno et al., 1996; Watanabe et al., 1994]. BTC, but not EGF and transforming growth factor-α (TGF-α), induces

rat pancreatic amylase-secreting cells (AR42J) to secrete insulin [Mashima et al., 1996]. BTC can also stimulate the growth of a rodent insulinoma cell line INS-1 [Huotari et al., 1998]. Since expression of BTC has been observed in the pancreas and small intestine [Seno et al., 1996], involvement of BTC in the growth and differentiation of pancreatic endocrine cells has been suggested. BTC is shown to be a ligand for ErbB-4, as well as for EGFR [Beerli and Hynes, 1996; Riese et al., 1996a,b], however, it is not sufficient to explain the unique activity of BTC on these pancreatic cell lines. Ishiyama et al. [1998] suggested the involvement of another unknown receptor in signal transmission of BTC.

Analysis on human BTC cDNA predicts that BTC is initially synthesized as a precursor composed of 178 amino acids. The precursor contains a secretion signal, transmembrane, and cytoplasmic domains in addition to the sequence containing the EGF domain with an N-terminal extension [Sasada et al., 1993]. The transmembrane precursor is thought to be proteolytically cleaved to generate a soluble secreted form of BTC as occurs in other members of the EGF family [Bell et al., 1986; Burgess et al., 1995; Derynck et al., 1984; Higashiyama et al., 1991; Holmes et al., 1992; Plowman et al., 1990]. This mechanism for processing membrane-anchored proteins is known for other growth factors and cytokines such as tumor necrosis factor-α (TNF-α) and colony-stimulating factor-1 (CSF-1) [reviewed by Massague and Pandiella, 1993]. The cleavage may be physiologically important, because the cleavage of membrane-anchored factors such as TGF-α, heparin-binding EGF-like growth factor (HB-EGF), and neuregulin is regulated by similar mechanisms that are dependent on protein kinase C (PKC). It is also known that the membrane-anchored precursors often remain uncleaved on the cell surface and communicate with adjacent cells through juxtacrine stimulation [Anklesaria et al., 1990; Brachmann et al., 1989; Higashiyama et al., 1995; Nakagawa et al., 1996; Pandiella and Massague, 1991a]. The juxtacrine stimulation may play unique and significant roles in tissue development. For example, the development of melanocytes, germ cells, and hematopoietic stem cells requires a transmembrane form of the c-*kit* ligands (KL). Soluble KL factors can not substitute for the transmembrane form in vivo in the *steel* (*Sl*) mutant mice that contain an aberrant secreted form of KL [Brannan et al., 1991; Flanagan et al., 1991]. However, little is known about the membrane-anchored forms for BTC. In this report, we describe the isoforms, biological activity, and regulation of the cleavage of membrane-anchored forms of BTC, using transfected cells that are expressing human BTC cDNA. Furthermore, transfectants that are expressing a reporter gene consisting of alkaline phosphatase and membrane-anchored BTC cDNA have also been generated to aid in the evaluation of secretion of BTC.

MATERIALS AND METHODS

Reagents and Antibodies

Phorbol-12-myristate 13-acetate (PMA) was purchased from Wako Pure Chemicals (Japan), and all-*trans*-retinoic acid (RA) and a calcium ionophore A23187 were from Sigma. rhBTC was prepared as previously described [Seno et al., 1996]. Antisera against the N-terminal, the EGF-like, and the cytoplasmic domains of human BTC were raised by immunizing rabbits with a 15-residue peptide (D^{32}GNSTRSPETNGLLC^{46}), a 17-residue peptide (C^{95}DEGYIGARCERVDLFY^{111}), or a 15-residue peptide (D^{164}ITPINEDIEETNIA^{178}) from BTC (Fig. 1A), respectively. Mouse monoclonal antibody recognizing BTC, 5E5, was prepared using purified human BTC [Watanabe et al., 1994] as an immunogen. Rabbit anti-human placental alkaline phosphatase serum and mouse anti-insulin monoclonal antibody were purchased from Biomeda (Long Beach, CA) and from Advanced ImmunoChemical (Foster, CA), respectively.

Expression Constructs and Transfection

An expression plasmid encoding human full-length BTC cDNA, pTB1515, was previously described [Watanabe et al., 1994]. The dihydrofolate reductase (*dhfr*) gene from pTB348 [Sasada et al., 1987] was linked with pTB1515 to construct another expression plasmid for BTC, pTB1507. A plasmid pTB1634 encodes a BTC mutant whose cleavage site (Y^{111}/LRGDR) is disrupted by replacement with T^{111}IIGDI. Two kinds of alkaline phosphatase and BTC fusion protein genes, designated ALP-BTC-L and ALP-BTC-S, were generated by ligating the cDNA encoding a signal sequence and ectodomain of human placental alkaline phosphatase (1–489) [Millan, 1986] with the cDNA

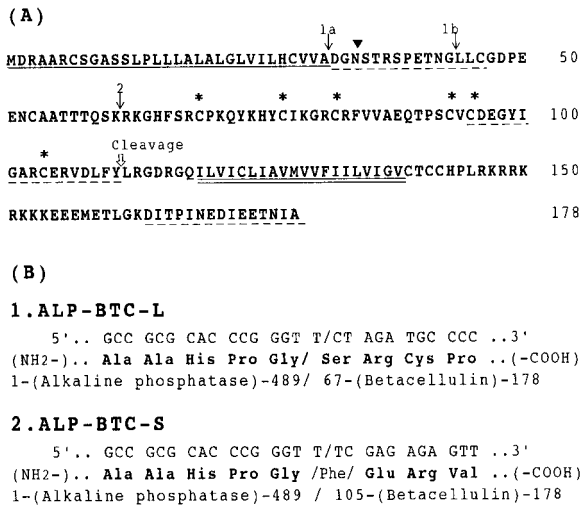

Fig. 1. Structures of betacellulin (BTC) and alkaline phosphatase-betacellulin (ALP-BTC) fusion proteins. **A:** Amino acid sequence of human BTC [Sasada et al., 1993]. Putative signal sequence and transmembrane domain are single- and double-underlined, respectively. ▼, N-glycosylation site; *, six cysteine residues conserved in EGF family. ↓, N-terminals of three isoforms of the secreted BTC [Watanabe et al., 1994], designated BTC-1a, BTC-1b, and BTC-2, respectively; open arrow, cleavage site for secreting ectodomain of BTC; --, regions used for immunizing rabbits to elicit anti-BTC peptide antibodies. **B:** Structures of ALP-BTC fusion proteins ALP-BTC-L (1) and ALP-BTC-S (2) for reporter assay. The nucleotide and amino acid sequences of the junction between alkaline phosphatase (1–489) [Millan, 1986] and BTC.

encoding the transmembrane and cytoplasmic domains of BTC(67–178) or BTC(105–178), respectively (Fig. 1B). Expression plasmids for ALP-BTC-L or ALP-BTC-S genes, pBO91 and pBO92, respectively, were constructed by inserting the ALP-BTC fused gene fragments downstream of the SRα′-promoter in pTB1458 [Tada et al., 1994]. These plasmids were introduced into mouse fibroblast A9 cells, a human breast cancer cell line MCF-7, and Chinese hamster ovary (CHO) cells deficient in *dhfr*. Stable transfectants containing the BTC-expression vector (pTB1515 or pTB1507) were screened for mitogenic activity in the culture media. The highest BTC producer clones, A9/1515-14 [Watanabe et al., 1994], MB52, and CHO1507-3510, were isolated. A low producer clone A9/1515-11 was also obtained from A9 transfectants. A stable transfectant clone expressing the cleavage site-disrupted BTC, A9/1634-3, was established by introducing pTB1634 into A9 cells. Stable transfectants producing ALP-BTC-L fused protein were obtained by introducing pBO91 into A9, CHO, and MCF-7 cells, followed by screening for alkaline phosphatase activity. The transfectants obtained were designated AABL, CABL, and MABL, respectively. A9 and CHO cells were also transfected with pBO92 to obtain the ALP-BTC-S-producing cells. The obtained transfectants were designated AABS and CABS, respectively.

Assays of Secreted BTC

Mitogenic assays for BTC on mouse BALB/c 3T3 clone A31-714-4 cells using Alamer Blue™ (Biosource International, Camarillo, CA) were performed to measure the secreted BTC in the culture medium as described previously [Seno et al., 1996]. Purified rhBTC produced by *Escherichia coli* [Seno et al., 1996], was used as the standard.

Detection of Membrane-Anchored BTC by Enzyme Immunoassay

The BTC-transfected cells which were grown in a 96-well plate for 1 day were cultured in 100 µl of fresh medium containing 0.5% fetal bovine serum (FBS) for 24 h. The culture medium was assayed as described above. The cells were washed with Dulbecco's phosphate-buffered saline (PBS) supplemented with 0.5 M NaCl and 0.5% FBS for 5 min, and fixed with 0.1% glutaraldehyde in PBS for 15 min. After several washes with PBS, BTC on the fixed cells was detected with a mouse monoclonal antibody against BTC (5E5) and alkaline phosphatase-labeled anti-mouse IgG antibody (Promega, Madison, WI). Alkaline phosphatase activity of each well was measured as described below and indicated as an increase in absorbance at 415 nm.

Western Blot Analysis

Subconfluent monolayers of cells grown in 35-mm dishes were cultured in 0.5 ml of serum-free medium for 24 h. The cells were dissolved by boiling with sample buffer (50 mM Tris-HCl, 1% sodium dodecyl sulfate [SDS], 20% glycerol, 20 mg/ml bromophenol blue, 0.14 M 2-mercaptomethanol, pH 6.8) for 5 min. Proteins in the culture medium were precipitated with final 8% of trichloroacetic acid and dissolved in sample buffer. The cell lysates and the proteins from culture medium were subjected to electrophoresis in a SDS-polyacrylamide gel (SDS-PAGE) and proteins were transferred to a nitrocellulose membrane. After blocking with 5%

nonfat milk, the membrane was incubated sequentially with the antibody indicated above and with horseradish peroxidase (HRP)-protein A conjugate (Prozyme, Richmond, CA). Blots were visualized with ECL Western blotting detection reagents (Amersham, Buckinghamshire, UK).

Glycanase Digestion

Cell lysates from A9/1515-14 cells were prepared by boiling approximately 5×10^5 cells in 50 mM sodium phosphate buffer containing 0.1% SDS, pH 7.5, for 5 min. After Nonidet P-40 (NP-40) was added to a final concentration of 0.7%, the cell lysate was incubated with N-glycanase (Genzyme, Cambridge, MA) overnight at 37°C. The reactions were analyzed by Western blotting with anti-BTC (D^{164}-A^{178}) antibody.

Immunofluorecence Microscopy

Cells were grown on Labtek chamber slides (No. 177437, Nunc, Naperville, IL). After washing the cells with PBS, the cells were fixed with 10% neutralized formaldehyde at 5°C for 16 h and treated with 0.1% Triton X-100 in PBS for 5 min. The fixed cells were incubated sequentially with Block Ace™ (Snow Brand, Sappro, Japan), rabbit anti-BTC(D^{164}-A^{178}), and rhodamine-conjugated anti-rabbit IgG (Biosource International) antibodies. The cells were examined under a laser scanning confocal microscope (model MRC-1024; Bio-Rad, Richmond, VA).

Assays for Juxtacrine Activity

To assess insulin-inducing activity, monolayers of A9/1515-14 cells were grown on Labtek chamber slides. After washing the cells with PBS, the cells were fixed with buffered formalin (5%) for 5 min, followed by several washes with PBS. AR42J-216 cells (5×10^3/well), a subclone of rat pancreatic AR42J cells [Mashima et al., 1996], were cultured on fixed BTC-transfected cells in DMEM containing 10% FBS for 5 days. Cells were then fixed and treated with 0.1% Triton X-100 as described above. The fixed cells were incubated with Block Ace™ and were doubly stained with rabbit anti-BTC(D^{164}-A^{178}), mouse anti-insulin antibodies, followed by rhodamine-conjugated anti-rabbit IgG, and fluorescein-5-isothiocyanate (FITC)-conjugated anti-mouse IgG (Biosource International) antibodies.

For mitogenic assays, BTC-transfected cells were precultured in 96-well plates, washed with PBS to eliminate the secreted isoform and fixed with buffered formalin (5%) for 5 min. The fixed cells were washed several times with PBS. AR42J-71-12 cells, which were another subclone of AR42J cells, were cultured on the fixed BTC-transfected cells at a density of 2×10^3 cells/well in 5% FBS containing Dulbecco's modified Eagle's medium; (DMEM) for 4 days. Proliferation of AR42J-71-12 cells was measured using a tetrazolium salt, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described [Tada et al., 1986] and was indicated as an increase in absorbance at 570 nm.

ALP-BTC Reporter Assay

Subconfluent monolayers of transfectant cells were grown in 35-mm dishes, rinsed with serum-free medium and cultured in 0.5 ml of medium containing either 10% or 0.25% FBS for 24 h. The culture media were collected and the cells were dissolved in 0.5 ml of lysis buffer on ice for 30 min. After the culture media or cell lysates were diluted serially with PBS containing 0.02% Triton X-100, 30-µl aliquots were incubated with 270 µl of substrate buffer for alkaline phosphatase (3 mM p-nitrophenyl phosphate, 10 mM $MgCl_2$, 2 mM Na_2CO_3) at 37°C. After 2 h, absorbance at 415 nm was measured. For 10% FBS-containing media, endogenous alkaline phosphatase activity derived from serum was inactivated by incubation for 30 min at 65°C [Feehan et al., 1996]. To assess the effect of biological mediators, 4×10^3 cells/well of transfectants were pre-cultured in 96-well plates for 2 days, washed with PBS, and then incubated with 100 µl of samples diluted in fresh medium containing 0.25% FBS for 2 h. The culture media and cell lysates were assayed for alkaline phosphatase activity as described above.

RESULTS

Establishment of Transfectants Expressing Human BTC cDNA

Transfectants producing BTC were established by introducing expression vectors encoding a full length human BTC cDNA into A9 and CHO cells in order to assess for membrane-anchored BTC. Using A9 transfectant clone, A9/1515-14, and CHO transfectant clone, CHO/1507-3510, the amounts of membrane-anchored BTC associated with the cells and the secreted BTC released into the culture media were as-

sessed by an enzyme-linked immunoassay and for mitogenic activity, respectively. As shown in Figure 2B, immunoreactive BTC was detected on both transfectant cells. The amount of BTC retained on CHO/1507-3510 cells was evaluated to be more than one-half of the amount on A9/1515-14. By contrast, the amount of BTC in the culture medium of CHO/1507-3510 was 100-fold less than that of A9/1515-14 [Watanabe et al., 1994] (Fig. 2A). A human breast cancer cell line MCF-7 was also transfected with BTC cDNA to use as a representative of native BTC-producer, as this cell line expresses BTC gene endogenously [Sasada et al., 1993], while the basal level of BTC production is too low to be evaluated by immunological methods. An MCF-7 transfectant, MB52, secreted more than 100 ng/ml of BTC, as well as displayed BTC on the cells similar to A9/1515-14 (data not shown). These results indicated that BTC can accumulate in these transfectant cells, while BTC secretion is apparently lower in the CHO than in the A9 and MCF-7 transfectants.

Isoforms of Membrane-Anchored Forms of BTC

BTC associated with the cells was analyzed by Western blotting using antibodies raised against BTC(D^{32}-C^{46}), BTC(C^{95}-Y^{111}), and BTC(D^{164}-A^{178}), respectively. In the cell lysates of A9/1515-14, two major bands of 25 and 40 kDa, two slight bands of 29 and 21-kDa were detected by anti-BTC(D^{164}-A^{178}) antibody (Fig. 3A). The smallest isoform of 21-kDa was suggested to lack the ectodomain, since anti-BTC(C^{95}-Y^{111}) antibody did not react with it. In contrast, only the largest band of 40 kDa was detected by anti-BTC(D^{32}-C^{46}) antibody. MB52 cells also produced these four isoforms with the 21-kDa as the main band, whereas in CHO1507-3510 cells the 21-kDa isoform was not detected. The amount of the 21 kDa was well correlated with BTC-releasing ability of these transfectants. Culture of A9/1515-14 cells with tunicamicin reduced the amount of the 40-kDa isoform but generated an additional band of 32 kDa (Fig. 3B). N-glycanase treatment of A9/1515-14 cell lysates also generated the 32-kDa band (Fig. 3B). These data suggest that the 40-kDa form of membrane-anchored BTC has an N-linked sugar moiety at Asn^{34}. Immunofluorescence microscopy using anti-BTC(D^{164}-A^{178}) antibody showed that the membrane-anchored BTC localized at the cell surface in these transfectants (Fig. 4A,B,F).

Membrane-Anchored BTC Exhibits Juxtacrine Activity

An important biological property of BTC is the induction of insulin production in rat pancreatic AR42J cells in the presence of activin [Mashima et al., 1996]. This activity of membrane-anchored BTC was assessed using a subclone of AR42J cells designated as AR42J-216, about 10% of which can differentiate into insulin-producing cells in response to the secreted form of recombinant human BTC (rhBTC) alone (data not shown). When AR42J-216 cells were cultured on the fixed A9/1515-14 monolayer displaying the membrane-anchored BTC, insulin-positive AR42J-216 cells appeared (Fig. 4F), whereas no insulin-positive AR42J-216 cells was

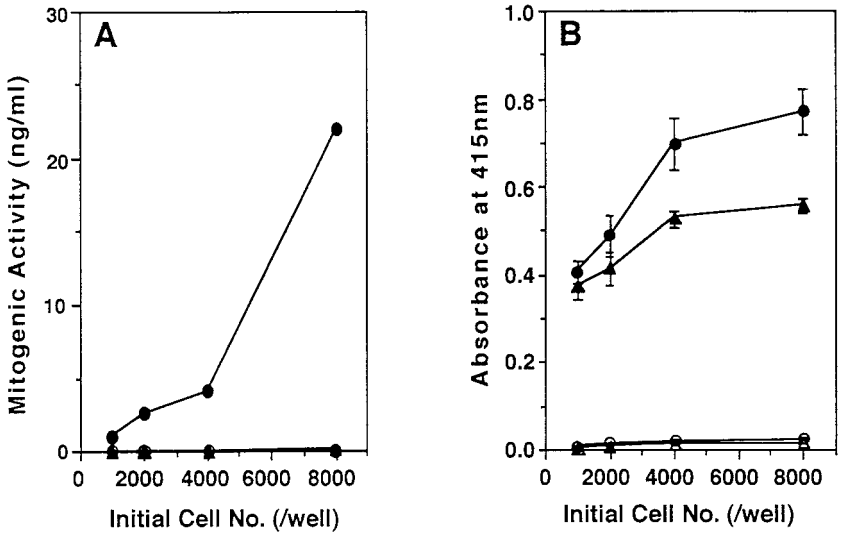

Fig. 2. Detection of secreted (**A**) and membrane-anchored (**B**) BTC prepared from transfectants expressing BTC cDNA (A9/1515-14 and CHO1507-3510). BTC transfected cells and their parental cells were precultured at the initial densities indicated for 1 day, and cultured in 100 µl of fresh medium containing 0.25% FBS for 2 days. **A:** Cell free-supernatants were assayed for secreted and mitogenically active BTC using Balb/c 3T3 cells. **B:** Membrane-anchored BTC associated with the cells was assessed by an enzyme-linked immunoassay using mouse anti-BTC monoclonal antibody (5E5). ●, A9/1515-14; ▲, CHO1507-3510; ○, parental A9; △, parental CHO cells.

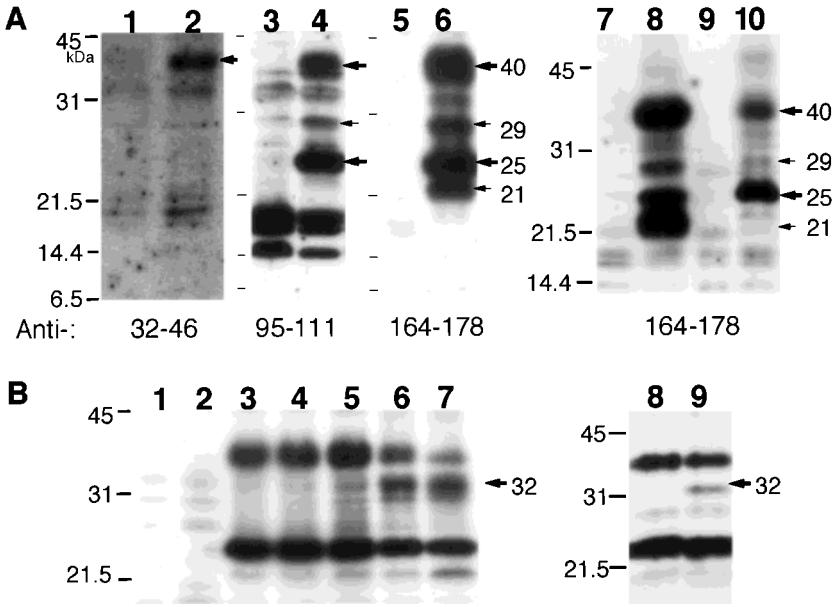

Fig. 3. Western blot analysis of the membrane-anchored BTC. **A:** Cell lysates prepared from A9 (**lanes 1, 3, 5**) and A9/1515-14 (**lanes 2, 4, 6**) were subjected to SDS-PAGE (15% gel) and analyzed by Western blotting with anti-BTC(D^{32}-C^{46}) antibody (lanes 1, 2), anti-BTC(C^{95}-Y^{111}) antibody (lanes 3, 4), and anti-BTC(D^{164}-A^{178}) antibody (lanes 5, 6), respectively. Cell lysates prepared from MCF7 (**lane 7**), MB52 (**lane 8**), CHO (**lane 9**), and CHO1507–3510 (**lane 10**) were also analyzed by Western blotting with anti-BTC(D^{164}-A^{178}) antibody. Arrows, BTC isoforms. **B:** Analysis of glycosylation of the membrane-anchored BTC. A9 (**lanes 1, 2**) and A9/1515-14 cells (**lanes 3–7**) were cultured in the presence of tunicamicin (lanes 1, 3, 0 μg/ml; lane 4, 0.25 μg/ml; lane 5, 0.5 μg/ml; lane 6, 0.75 μg/ml; lanes 2, 7, 1.0 μg/ml) for 2 days, and the cell lysates were detected with anti-BTC(D^{164}-A^{178}) antibody. **Lane 9** A9/1515-14 cell lysate digested with N-glycanase; **lane 8,** mock digestion. Arrows, a new 32-kDa band generated by tunicamicin treatment or glycanase digestion. Numbers at the left side are positions of molecular-weight standards (broad range, Bio-Rad).

detected in the whole area on the fixed A9 monolayer (Fig. 4G). rhBTC is also capable of stimulating the growth of AR42J-71-12 cells that are another subclone of AR42J cells (Fig. 5). The growth of AR42J-71-12 cells was also enhanced when these cells were grown on formalin-fixed monolayers of BTC transfectants of A9/1515-14 or A9/1515-11. The magnitude of growth stimulation generally exceeded the growth induced by 10 ng/ml of rhBTC. An A9 transfectant displaying a cleavage site-disrupted BTC mutant (A9/1634-3) also enhanced the proliferation of AR42J-71-12 cells. Little enhancement was observed for parental A9 cells. Thus membrane-anchored BTC can provide both an insulin-inducing and a growth-promoting signal in a juxtacrine manner.

Proteolytic Secretion of BTC

To evaluate the cleavage of the membrane-anchored BTC more quantitatively, we have constructed expression vectors for two kinds of ALP-BTC fusion proteins—ALP-BTC-L and ALP-BTC-S—and transfected them into CHO, A9, and MCF7 cells. ALP-BTC-L contains the EGF-domain of BTC but ALP-BTC-S does not, as depicted in Figure 1B. As for ALP-BTC-L transfected cells, A9 transfectants and MCF-7 transfectants showed apparent secretion of approximately 40% of total ALP activity, whereas CHO transfectants secreted only approximately 8% (Table I), consistent with the results of transfectants expressing BTC cDNA itself. However, secretion of ALP-BTC-S fused protein was quite different from those of BTC and ALP-BTC-L, since more than 50% was secreted in both CHO and A9 transfectants. The difference between ALP-BTC-L and ALP-BTC-S was more evident when the effects of PMA, all-*trans*-retinoic acid (RA), and A23187 on modifying the cleavage and secretion of BTC were compared (Fig. 6). The secretion of ALP-BTC-S was stimulated drastically within 2 h by 10 μM RA in both A9- and CHO transfectants without any effect on the viability. Treatment with PMA (1 μM) also clearly increased the secretion in CHO transfectants, but not obviously in A9 transfectants. By contrast, such a clear stimulation was not observed in ALP-BTC-L transfected cells,

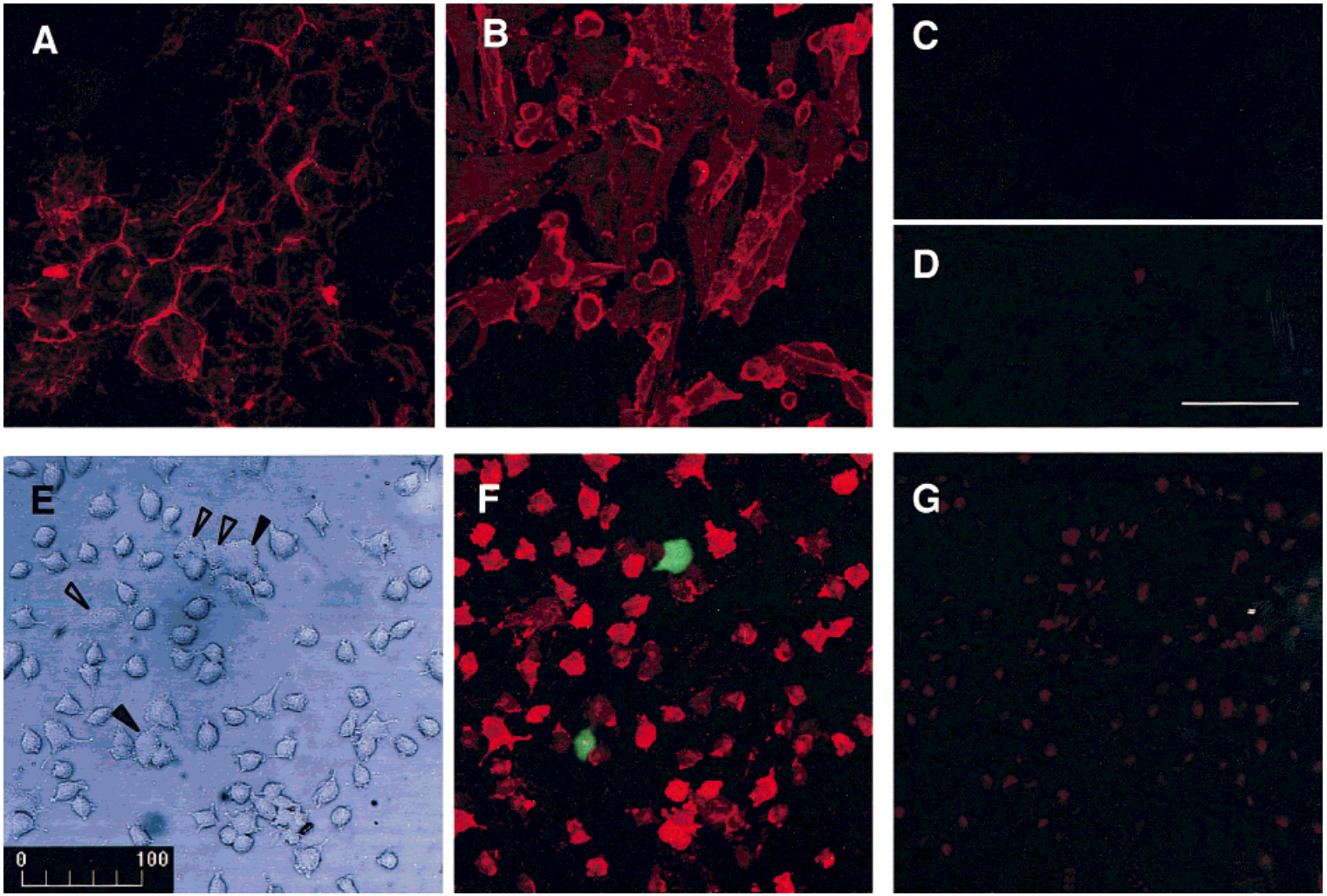

Fig. 4. Immunofluorescence microscopy of membrane-anchored BTC. MB52 (**A**), CHO1507-3510 (**B**), MCF7 (**C**), and CHO (**D**) cells were stained with rabbit anti-BTC antibody, followed by rhodamine conjugated anti-rabbit IgG. Membrane-anchored BTC was localized at cell membrane of transfectant cells. AR42J-216 cells cultured on fixed A9/1515-14 cells (**F**) and cultured on fixed A9 cells (**G**) were doubly stained with rabbit anti-BTC antibody and with mouse anti-insulin antibody, followed by rhodamine conjugated anti-rabbit IgG and FITC conjugated anti-mouse IgG. **E:** Transmission image of the same area of F. Open and solid arrowheads (E), insulin-negative and insulin-positive AR42J-216 cells, respectively. AR42J-216 cells are BTC-negative; they are larger and rounder in shape than BTC-positive A9/1515-14 cells. No insulin-positive cell was observed in the whole area of the culture of AR42J-216 cells on fixed A9 cells. Scale bars = 100 μm.

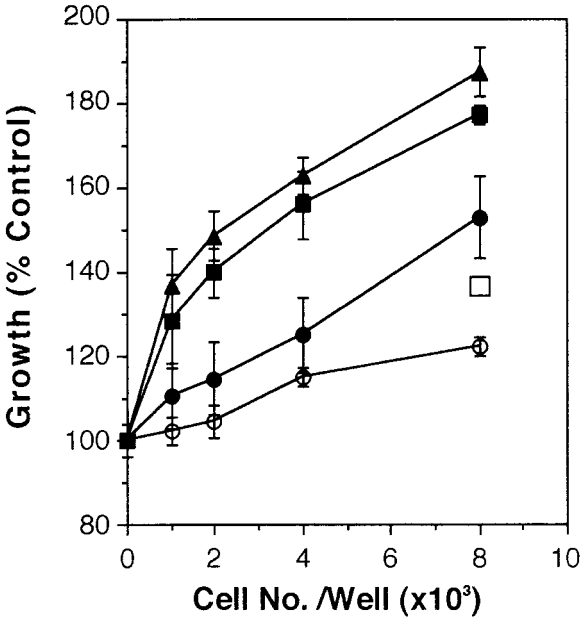

Fig. 5. Growth promotion of AR42J-71-12 cells by membrane-anchored BTC. Various transfectant cells were pre-cultured at the initial densities indicated for 1 day, and fixed. Proliferation of AR42J-71-12 cells on the various fixed cells was evaluated after 4-day cultivation. ○, fixed A9 cells; ▲, fixed A9/1515-14 cells; ■, fixed A9/1515-11 cells; ●, fixed A9/1634-3 cells, respectively. □, growth in the presence of 10 ng/ml secreted hBTC produced by *Escherichia coli.* Values represent the means ± SD of quadruplicate assays.

although only slight increase of secretion was induced by RA and A23187 in A9 transfectants but not in CHO- and MCF7-transfectants. The effects of RA and PMA on the cleavage of BTC itself were evaluated by Western blotting using anti-BTC(C^{95}-Y^{111}) antibody (Fig. 7). In A9 transfectants, the secreted BTC of 8 kDa was slightly increased by RA treatment, but not by PMA. In CHO transfectants, secretion of BTC was not increased by both reagents. These results were consistent with those obtained from the reporter assay with ALP-BTC-L-transfected cells.

DISCUSSION

The secreted forms of human recombinant BTC have been produced in mammalian cells and in *E. coli* and well characterized [Seno et al., 1996; Watanabe et al., 1994], whereas analysis of the BTC cDNA predicts that BTC is produced as a membrane-anchored molecule [Sasada et al., 1993] (Fig. 1A). This is the first report describing the detection and characteristics of membrane-anchored BTC. Membrane-anchored BTC with the EGF domain was clearly detected in all BTC-transfected cell lines (Fig. 3). By Western blot analysis, at least four isoforms of 21, 25, 29, and 40 kDa were detected. Three of these—40, 29, and 25 kDa—contained the C-terminus of the cytoplasmic tail as well as the EGF domain, indicating that they are transmembrane forms. Only the largest form of 40 kDa possesses the N-terminal domain and a sugar moiety at Asn^{34} N-glycosylation site. As for the secreted forms, three isoforms also have been purified from the conditioned medium of A9/1515-14 cells by Watanabe et al. [1994] (Fig. 1A). These include an intact 80-amino acid BTC (termed BTC-1a) of 30 kDa, corresponding to Asp^{32}-Tyr^{111} with N-linked and O-linked sugar moieties, a BTC truncated by 12 amino acids from the N-terminus (BTC-1b) of 17-kDa, and the smallest BTC further N-terminally truncated by 18 amino acids (BTC-2) of 8 kDa. Therefore, the 25, 29, and 40 kDa of the membrane-anchored BTC in this study might correspond to membrane-anchored counterparts of BTC-2, BTC-1b and BTC-1a, respectively. The 21-kDa isoform, which was detected only by anti-BTC(D^{164}-A^{178}) antibody is suggested to be BTC(L^{112}-A^{178}), because the amount of this isoform was correlated to BTC releasing ability of these transfectants. In our systems, only the 8-kDa form corresponding to BTC-2 was detected as a secreted BTC from A9/1515-14 (Fig. 7) and MB52 cells (data not shown), while BTC-1a had been reported to be the major form. Furthermore the 25-kDa BTC, which lacks the N-terminal domain, was detected as the major membrane-anchored isoform. Basic amino acid residues of Lys^{61}/$Arg^{62}Lys^{63}$ within the N-terminal region of the EGF-domain may susceptible to cleavage by trypsin-like proteases. In HB-EGF, the N-terminal domain modulates its biological activity by its capacity to bind heparin [Nakagawa et al., 1996]. However, the N-terminal domain of BTC shows little heparin affinity [Watanabe et al., 1994] and BTC-2 lacking the N-terminal domain exhibits same biological activity as BTC-1a [Shin et al., 1995; Watanabe et al., 1994]. Therefore, the membrane-anchored isoforms of 25, 29, and 40 kDa are thought to have equivalent biological activity.

BTC belongs to a growing family of membrane-anchored growth factors that are proteolytically processed extracellularly to release soluble factors [reviewed in Massague and Pandiella, 1993]. The transmembrane forms of some members of this group have been shown to bind

TABLE I. Secretion of ALP-BTC Fusion Proteins from Various Transfectants

Cell (clone no.)	Alkaline phosphatase activity[a]					
	10% Serum			0.25% Serum		
	Cell	Medium	% Secretion	Cell	Medium	% Secretion
MCF-7	0.02	−0.02	—	0.03	0.02	—
MABL-24	3.40	2.17	38.9	2.30	2.44	51.5
MABL-25	1.84	0.78	29.8	1.69	0.86	33.6
MABL-35	1.58	0.72	31.2	1.21	0.99	45.1
A9	0.08	0.09	—	0.05	0.02	—
AABL-1	4.56	2.72	37.4	3.92	2.89	42.4
AABL-6	1.12	0.69	38.1	1.39	0.75	34.9
AABS-6	—	—	—	0.99	0.95	51.0
CHO	0.05	−0.05	—	0.22	0.08	—
CABL-15	4.83	0.40	7.6	3.14	0.70	18.2
CABL-21	56.00	5.12	7.8	55.92	4.16	6.9
CABS-11	—	—	—	4.93	2.56	65.9
CABS-13	—	—	—	24.38	20.09	54.8

[a]Transfectants expressing ALP-BTC-L gene (MABLs, AABLs, and CABLs) and ALP-BTC-S gene (AABSs and CABSs), and their host cells (MCF-7, A9, and CHO, respectively) were cultured in 35-mm dishes in the presence of 10% or 0.25% fetal bovine serum for 1 day. Alkaline phosphatase activity in the culture medium and cell lysates were assessed using p-nitrophenyl phosphate as a substrate and were shown as increases in absorbance at 415 nm. Values are the means of duplicate assay.

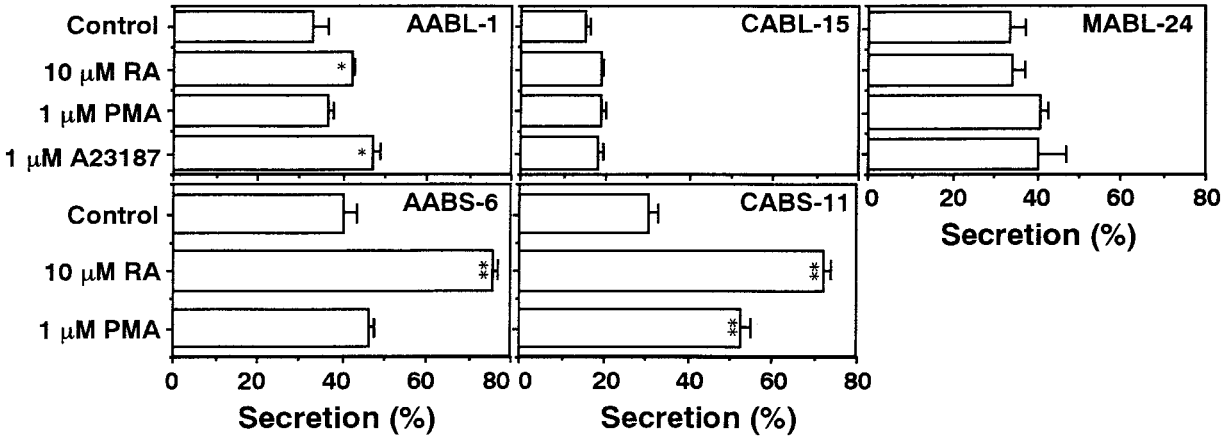

Fig. 6. Effects of reagents on the secretion of ALP-BTC fused proteins. Cells grown in 96-well plates for 2 days were cultured in 100 μl of samples in 0.25% FBS-containing medium for 2 h and alkaline phosphatase activity in the culture media and cell lysates were assayed. Percentage of alkaline phosphatase activity secreted into the culture media are shown as bars. Data represent the means ± SD of triplicate cultures. AABL-1, CABL-15, and MABL-24 are A9, CHO, and MCF7 transfectant clones expressing ALP-BTC-L. AABS-6 and CABS-11 are A9 and CHO transfectant clones expressing ALP-BTC-S, respectively. $*P < 0.025$ and $**P < 0.001$ compared with the control culture, respectively.

to and signal through their cognate receptors by direct cell–cell contacts [Anklesaria et al., 1990; Brachmann et al., 1989; Higashiyama et al., 1995; Wong et al., 1989]. On the other hand, a Spitz protein, a *Drosophila* TGF-α homologue, is active as a secreted form but inactive as a membrane-anchored form [Schweitzer et al., 1995]. In the present study we have demonstrated that membrane-anchored BTC exhibits biological activity with respect to inducing insulin production and/or promoting cell growth in subclones of rat pancreatic AR42J cells (Figs. 4, 5). The activity of BTC toward pancreatic cells has been suggested to be unique [Mashima et al., 1996] and to be important in vivo because high levels of BTC mRNA expression are observed in the pancreas relative to in other tissues [Seno et al., 1996]. Furthermore, immuno-

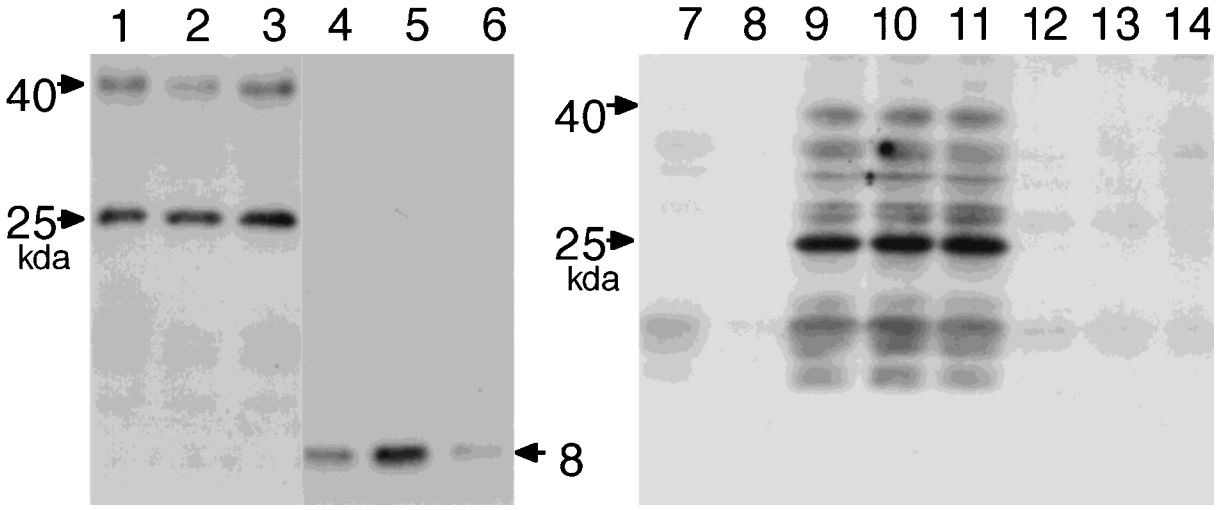

Fig. 7. Western blot analysis on the cleavage of the membrane-anchored BTC. Cell lysates and cell-free culture medium of BTC-transfected cells were subjected to SDS-PAGE (15% gel) and analyzed by Western blotting with anti-BTC (C^{95}-Y^{111}) serum. Left: Cell lysates (**lanes 1–3**) and culture medium (**lanes 4–6**) of A9/1515-14 prepared from untreated (lanes 1, 4), 10 μM RA-treated (lanes 2, 5), and 1 μM PMA- (lanes 3, 6) treated culture, respectively. Right: Cell lysates (**lane 7**) and culture medium (**lane 8**) of untreated CHO cells, and cell lysates (**lanes 9–11**) and culture medium (**lanes 12–14**) of CHO1507-3510 cells prepared from untreated (lanes 9, 12), 10 μM RA treated (lanes 10, 13), and 1 μM PMA (lanes 11, 14) treated culture, respectively. The positions of membrane-anchored forms (left side) and secreted forms (right side) of BTC are indicated.

staining for BTC demonstrates that BTC is localized in primitive duct cells in the fetal pancreas and in some populations of islet cells closely associated with insulin-producing cells in adult pancreas (J. Miyagawa et al., personal communication). These results suggest that membrane-anchored forms of BTC may also function to facilitate the development of insulin-producing cells in a juxtacrine manner in vivo.

The feature that the extracellular domain of membrane-anchored proteins can be proteolytically cleaved and released is shared with not only growth factors but also with growth factor receptors, cell adhesion molecules, ectoenzymes, and other proteins such as the β-amyloid precursor protein [reviewed in Ehlers and Riordan, 1991]. Proteolytic secretion of these proteins is often regulated in a similar process [Arribas and Massague, 1995; Arribas et al., 1996]; the cleavage is low in quiescent CHO cells but is activated immediately by agents that stimulate PKC, such as a phorbol ester. In the case of BTC, the degree of the cleavage was different among host cell types in that constitutionally high in A9 and MCF7 transfectants but very low in CHO transfectants (Figs. 2, 7). PMA failed to increase the secretion of BTC in CHO and A9 transfectants (Fig. 7), whereas it is well known to stimulate the cleavage of diverse membrane-anchored biological proteins in CHO cells [Goishi et al., 1995; Pandiella and Massague, 1991b; Raab et al., 1994]. RA activated BTC secretion mildly in only A9 cells; this effect might be mediated not through its nuclear receptor, but possibly by its ability to disrupt the Golgi apparatus [Wu et al., 1994], because very high concentrations (10 μM) of RA were required for increasing the cleavage. These data suggest that regulation of BTC cleavage is mainly dependent upon the cell types but independent on the PMA-responsive proteases that are responsible for the cleavage of diverse membrane-anchored proteins. Since MCF-7 is a native BTC-expressing cell line, cleavage of endogenous BTC is thought to occur as in the MCF-7 transfectants that produced the secreted BTC comparable to the membrane-anchored forms. When cleavage was evaluated using ALP-BTC fusion proteins, ALP-BTC-L secreted just as BTC itself, while ALP-BTC-S secreted differently because its cleavage was equally high in CHO cells and clearly stimulated by PMA (Fig. 6). This finding suggests that PMA-responsive proteases in CHO cells cannot cleave BTC and the ALP-BTC-L fusion protein, whereas ALP-BTC-S fusion protein is susceptible to these proteases. Some of the proteases involved in the cleavage/secretion of membrane-anchored proteins have been identified to be members of metalloproteinases [Moss et al., 1997; Suzuki

et al., 1997]. However, reports on the substrate requirement for this class of protease are sometimes conflicting, and no common determinant of primary sequence and length from transmembrane region has been found.

Several reasons for the susceptibility of ALP-BTC-S are to be considered. First, the longer juxtamembrane domain of ALP-BTC-S (30 residues) than that of BTC (14 residues) renders PMA-responsive proteases accessible by giving an enough stalk for them, as proposed by Ehler et al. [1997] and/or an additional cleavage site within ALP domain. According to this point of view, it should be noted that the length in BTC from transmembrane domain to the cleavage site (seven residues) is shorter than those in other membrane-anchored proteins susceptible to PMA-responsive cleavage. Second, a specific secondary structure around the cleavage site is disrupted by the fusion of juxtamembrane domains to be a recognizable structure (perhaps a looser structure) to the proteases as proposed by Arribus et al. [1997]. Third, the proximal ectodomain may be involved in regulating the cleavage by functioning as a recognition domain [Sadhukhan et al., 1998] for the proteases and/or for a third factor protecting the cleavage site. Thus, two possibilities are now suggested for the low BTC-cleavage activity in CHO cells. One is lack of BTC-processing protease, and the other is existing of an unknown third factor protecting BTC from the cleavage. As for the regulation of cleavage, we tested several other biological reagents including A23187, BTC, EGF, fibroblast growth factor, 8-Br-cAMP, 8-Br-cGMP, and dexamethasone using the reporter system with ALP-BTC-L. Only RA and A23187 slightly increased the secretion in A9 transfectants but not in CHO and MCF7 transfectants (Fig. 6). The other reagents failed to show any significant effect on the secretion in any transfectants (data not shown). Further studies are required to clarify the proteases responsible for BTC cleavage and its regulation.

ACKNOWLEDGMENTS

The authors thank Mr. T. Watanabe and Ms. A. Shintani for excellent technical assistance. We also thank Mrs. M. Koyama, M. Kita, and N. Azuma, and Ms. M. Kosaka for continuous encouragement.

REFERENCES

Anklesaria P, Teixido J, Laiho M, Pierce JH, Greenberger JS, Massague J. 1990. Cell–cell adhesion mediated by binding of membrane-anchored transforming growth factor alpha to epidermal growth factor receptors promotes cell proliferation. Proc Natl Acad Sci USA 87:3289–3293.

Arribas J, Massague J. 1995. Transforming growth factor-alpha and beta-amyloid precursor protein share a secretory mechanism. J Cell Biol 128:433–441.

Arribas J, Coodly L, Vollmer P, Kishimoto TK, Rose-John S, Massague J. 1996. Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. J Biol Chem 271:11376–11382.

Arribas J, Lopez-Casillas F, Massague J. 1997. Role of the juxtamembrane domains of the transforming growth factor-alpha precursor and the beta-amyloid precursor protein in regulated ectodomain shedding. J Biol Chem 272:17160–17165.

Beerli RR, Hynes NE. 1996. Epidermal growth factor-related peptides activate distinct subsets of ErbB receptors and differ in their biological activities. J Biol Chem 271:6071–6076.

Bell GI, Fong NM, Stempien MM, Wormsted MA, Caput D, Ku LL, Urdea MS, Rall LB, Sanchez-Pescador R. 1986. Human epidermal growth factor precursor: cDNA sequence, expression in vitro and gene organization. Nucleic Acids Res 14:8427–8446.

Brachmann R, Lindquist PB, Nagashima M, Kohr W, Lipari T, Napier M, Derynck R. 1989. Transmembrane TGF-alpha precursors activate EGF/TGF-alpha receptors. Cell 56:691–700.

Brannan CI, Lyman SD, Williams DE, Eisenman J, Anderson DM, Cosman D, Bedell MA, Jenkins NA, Copeland NG. 1991. Steel-Dickie mutation encodes a c-*kit* ligand lacking transmembrane and cytoplasmic domains. Proc Natl Acad Sci USA 88:4671–4674.

Burgess TL, Ross SL, Qian YX, Brankow D, Hu S. 1995. Biosynthetic processing of neu differentiation factor. Glycosylation trafficking, and regulated cleavage from the cell surface. J Biol Chem 270:19188–19196.

Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV. 1984. Human transforming growth factor-alpha: Precursor structure and expression in *E. coli*. Cell 38:287–297.

Ehlers MR, Riordan JF. 1991. Membrane proteins with soluble counterparts: Role of proteolysis in the release of transmembrane proteins. Biochemistry 30:10065–10074.

Ehlers MR, Schwager SL, Chubb AJ, Scholle RR, Brandt WF, Riordan JF. 1997. Proteolytic release of membrane proteins: Studies on a membrane-protein-solubilizing activity in CHO cells. Immunopharmacology 36:271–278.

Feehan C, Darlak K, Kahn J, Walcheck B, Spatola AF, Kishimoto TK. 1996. Shedding of the lymphocyte L-selectin adhesion molecule is inhibited by a hydroxamic acid-based protease inhibitor. Identification with an L-selectin-alkaline phosphatase reporter. J Biol Chem 271:7019–7024.

Flanagan JG, Chan DC, Leder P. 1991. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. Cell 64:1025–1035.

Goishi K, Higashiyama S, Klagsbrun M, Nakano N, Umata T, Ishikawa M, Mekada E, Taniguchi N. 1995. Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: Conversion from juxtacrine to paracrine growth factor activity. Mol Biol Cell 6:967–980.

Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. 1991. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 251:936–939.

Higashiyama S, Iwamoto R, Goishi K, Raab G, Taniguchi N, Klagsbrun M, Mekada E. 1995. The membrane protein CD9/DRAP 27 potentiates the juxtacrine growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. J Cell Biol 128:929–938.

Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, Yansura D, Abadi N, Raab H, Lewis GD, Shepard HM, Kuang W, Wood WI, Goeddel DV, Vandren R. 1992. Identification of heregulin, a specific activator of p185erbB2. Science 256:1205–1210.

Huotari MA, Palgi J, Otonkoski T. 1998. Growth factor-mediated proliferation and differentiation of insulin-producing INS-1 and RINm5F cells: Identification of betacellulin as a novel beta-cell mitogen. Endocrinology 139:1494–1499.

Ishiyama N, Kanzaki M, Seno M, Yamada H, Kobayashi I, Kojima I. 1998. Studies on the betacellulin receptor in pancreatic AR42J cells. Diabetologia 41:623–628.

Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I. 1996. Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. J Clin Invest 97:1647–1654.

Massague J, Pandiella A. 1993. Membrane-anchored growth factors. Annu Rev Biochem 62:515–541.

Millan JL. 1986. Molecular cloning and sequence analysis of human placental alkaline phosphatase. J Biol Chem 261:3112–3115.

Moss ML, Jin SL, Milla ME, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton LK, Schoenen F, Seaton T, Su JL, Warner J, Willard D, Becherer JD. 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature 385:733–736.

Nakagawa T, Higashiyama S, Mitamura T, Mekada E, Taniguchi N. 1996. Amino-terminal processing of cell surface heparin-binding epidermal growth factor-like growth factor up-regulates its juxtacrine but not its paracrine growth factor activity. J Biol Chem 271:30858–30863.

Pandiella A, Massague J. 1991a. Cleavage of the membrane precursor for transforming growth factor alpha is a regulated process. Proc Natl Acad Sci USA 88:1726–1730.

Pandiella A, Massague J. 1991b. Multiple signals activate cleavage of the membrane transforming growth factor-alpha precursor. J Biol Chem 266:5769–5773.

Plowman GD, Green JM, McDonald VL, Neubauer MG, Disteche CM, Todaro GJ, Shoyab M. 1990. The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. Mol Cell Biol 10:1969–1981.

Raab G, Higashiyama S, Hetelekidis S, Abraham JA, Damm D, Ono M, Klagsbrun M. 1994. Biosynthesis and processing by phorbol ester of the cells surface-associated precursor form of heparin-binding EGF-like growth factor. Biochem Biophys Res Commun 204:592–597.

Riese DJ, II, Bermingham Y, van Raaij TM, Buckley S, Plowman GD, Stern DF. 1996a. Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-beta. Oncogene 12:345–353.

Riese DJ, II, Kim ED, Elenius K, Buckley S, Klagsbrun M, Plowman GD, Stern DF. 1996b. The epidermal growth factor receptor couples transforming growth factor-alpha, heparin-binding epidermal growth factor-like factor, and amphiregulin to Neu, ErbB-3, and ErbB-4. J Biol Chem 271:20047–20052.

Sadhukhan R, Sen GC, Ramchandran R, Sen I. 1998. The distal ectodomain of angiotensin-converting enzyme regulates its cleavage-secretion from the cell surface. Proc Natl Acad Sci USA 95:138–143.

Sasada R, Onda H, Igarashi K. 1987. The establishment of IL-2 producing cells by genetic engineering. Cell Struct Funct 12:205–217.

Sasada R, Ono Y, Taniyama Y, Shing Y, Folkman J, Igarashi K. 1993. Cloning and expression of cDNA encoding human betacellulin, a new member of the EGF family. Biochem Biophys Res Commun 190:1173–1179.

Seno M, Tada H, Kosaka M, Sasada R, Igarashi K, Shing Y, Folkman J, Ueda M, Yamada H. 1996. Human betacellulin, a member of the EGF family dominantly expressed in pancreas and small intestine, is fully active in a monomeric form. Growth Factors 13:181–191.

Shin SY, Kang SW, Ha J. 1995. Chemical synthesis and determination of biological activity of the epidermal growth factor-like domain of mouse betacellulin. J Biochem Mol Biol 28:87–93.

Shing Y, Christofori G, Hanahan D, Ono Y, Sasada R, Igarashi K, Folkman J. 1993. Betacellulin: A mitogen from pancreatic beta cell tumors. Science 259:1604–1607.

Suzuki M, Raab G, Moses MA, Fernandez CA, Klagsbrun M. 1997. Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. J Biol Chem 272:31730–31737.

Tada H, Shiho O, Kuroshima K, Koyama K, Tsukamoto K. 1986. An improved colorimetric assay for interleukin-2. J Immunol Methods 93:157–165.

Tada H, Kurokawa T, Seita T, Watanebe T, Iwasa S. 1994. Expression and characterization of a chimeric bispecific antibody against fibrin and urokinase-type plasminogen activator. J Biotechnology 33:137–174.

Watanabe T, Shintani A, Nakata M, Shing Y, Folkman J, Igarashi K, Sasada R. 1994. Recombinant human betacellulin. Molecular structure, biological activities, and receptor interaction. J Biol Chem 269:9966–9973.

Wong ST, Winchell LF, McCune BK, Earp HS, Teixido J, Massague J, Herman B, Lee DC. 1989. The TGF-alpha precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. Cell 56:495–506.

Wu YN, Gadina M, Tao-Cheng JH, Youle RJ. 1994. Retinoic acid disrupts the Golgi apparatus and increases the cytosolic routing of specific protein toxins. J Cell Biol 125:743–753.