EXTRACELLULAR CALCIUM INFUX

ADAM10 Mediates Ectodomain Shedding of the Betacellulin Precursor Activated by p-Aminophenylmercuric Acetate and Extracellular Calcium Influx

Received for publication, August 2, 2004, and in revised form, October 1, 2004
Published, JBC Papers in Press, October 26, 2004, DOI 10.1074/jbc.M408804200

Michael P. Sanderson‡§, Sarah N. Erickson‡, Peter J. Gough‡, Kyle J. Garton‡, Paul T. Wille‡, Elaine W. Raines‡, Andrew J. Dunbar‡¶, and Peter J. Dempsey‡ $$$

From the ‡Pacific Northwest Research Institute, Seattle, Washington 98122, the ¶Cooperative Research Centre for Tissue Growth and Repair, School of Biological Sciences, Flinders University, Adelaide 5001, Australia, the §Department of Pathology, University of Washington, Harborview Medical Center, Seattle, Washington 98104, GroPep Limited, Thebarton, South Australia 5031, Australia, and the $$$Department of Medicine, University of Washington, Seattle, Washington 98195

Betacellulin belongs to the family of epidermal growth factor-like growth factors that are expressed as transmembrane precursors and undergo proteolytic ectodomain shedding to release a soluble mature growth factor. In this study, we investigated the ectodomain shedding of the betacellulin precursor (pro-BTC) in conditionally immortalized wild-type (WT) and ADAM-deficient cell lines. Sequential ectodomain cleavage of the predominant cell-surface 40-kDa form of pro-BTC generated a major (26–28 kDa) and two minor (20 and 15 kDa) soluble forms and a cellular remnant lacking the ectodomain (12 kDa). Pro-BTC shedding was activated by calcium ionophore (A23187) and by the metalloprotease activator p-aminophenylmercuric acetate (APMA), but not by phorbol esters. Culturing cells in calcium-free medium or with the protein kinase C inhibitor rottlerin, but not with broad-based protein kinase C inhibitors, blocked A23187-activated pro-BTC shedding. These same treatments were without effect for constitutive and APMA-induced cleavage events. All pro-BTC shedding was blocked by treatment with a broad-spectrum metalloprotease inhibitor (GM6001). In addition, constitutive and activated pro-BTC shedding was differentially blocked by TIMP-1 or TIMP-3, but was insensitive to treatment with TIMP-2. Pro-BTC shedding was functional in cells from ADAM17- and ADAM9-deficient mice and in cells overexpressing WT or catalytically inactive ADAM17. In contrast, overexpression of WT ADAM10 enhanced constitutive and activated shedding of pro-BTC, whereas overexpression of catalytically inactive ADAM10 reduced shedding. These results demonstrate, for the first time, activated pro-BTC shedding in response to extracellular calcium influx and APMA and provide evidence that ADAM10 mediates constitutive and activated pro-BTC shedding.

Betacellulin (BTC) belongs to the epidermal growth factor (EGF)-like family of cytokines that are expressed as membrane-anchored precursor proteins containing an extracellular N-terminal ectodomain, a transmembrane domain, and a cytoplasmic domain. The extracellular N-terminal ectodomain can be proteolytically cleaved to release the mature active growth factor, which can then bind ErbB receptors in an autocrine or paracrine fashion, resulting in the activation of multiple signal transduction cascades affecting cell proliferation, differentiation, migration, and survival (1, 2). Although a number of in vitro studies have indicated that the membrane-anchored precursors may also activate ErbB receptor signaling in a juxta-crine manner (3–7), there is now convincing evidence that ectodomain shedding of EGF-like growth factors from the cell surface is fundamental to ErbB receptor signaling during normal development (8–10) and tumorigenesis (11–13). Thus, knowledge of the molecular mechanisms by which EGF-like growth factors are shed from the cell surface and the nature of the proteases that govern this process is crucial to understanding ErbB receptor signaling and potentially the development of novel cancer therapeutics.

Recent studies have highlighted a clear role for members of the ADAM (a disintegrin and metalloproteinase) family, in particular, the tumor necrosis factor-α-converting enzyme (ADAM17), as EGF-like factor “sheddases.” For example, mice that produce catalytically inactive ADAM17 protein cannot bind Zn²⁺ (ADAM17[Zn²⁺/Zn⁰] (8) display a severe phenotype similar to the embryonic and perinatal lethality observed in ErbB1⁻/⁻ mice (14–16). Moreover, the eye, hair, and skin phenotypes of newborn ADAM17[Zn⁰/3Zn²⁺] mice are identical to those reported previously for transforming growth factor-α (TGFα)⁻/⁻ mice (17, 18). The involvement of ADAM17 in TGFα shedding and, additionally, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), and neuregulin-α2c has been confirmed by the reduced shedding of these growth factors from ras- and myc-transformed embryonic fibroblasts derived

* This work was supported in part by the Australian Government Cooperative Research Centres Programme (to A. J. D.) and National Institutes of Health Grants DK59778 and DK63363 (to P. J. D.) and Grants HL18645 and HL67267 (to E. W. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence may be addressed: GroPep Ltd., 28 Dalgleish St., Thebarton, SA 5031, Australia. Tel.: 61-8-8354-7786; Fax: 61-8-8354-7788; E-mail: andrew.dunbar@gropep.com.au.

† To whom correspondence may be addressed: Pacific Northwest Research Inst., 720 Broadway, Seattle, WA 98122. Tel.: 206-568-1462; Fax: 206-726-1217; E-mail: pdempsey@pnri.org.
from ADAM17Δ22/Δ22 mice that can be rescued by ectopic ADAM17 expression (9, 19, 20). The role for ADAM17 in shedding of HB-EGF has been strengthened by the recent observation that ADAM17Δ22/Δ22 mice display similar heart and lung defects compared with HB-EGF−/− mice (21, 22), although there is some evidence to suggest that, under certain situations, HB-EGF may also be cleaved by other ADAMs such as ADAM9, ADAM10 (23, 24), ADAM12 (25), and ADAM15 (26), as well as by some matrix metalloproteases (MMPs), including MMP3 (27, 28) and MMP7/matrixins (29). In contrast to other members of the EGF-like growth factor family, recent reports have shown that ADAM17 does not appear to be the primary sheddase responsible for shedding of the BTC precursor (pro-BTC). Rather, a role for ADAM10 at least in the basal shedding of pro-BTC has been proposed (30, 31).

The ectodomain shedding of pro-BTC has not been extensively characterized. Analysis of the pro-BTC cDNA suggests that the primary translation product encodes a signal sequence, N- and O-linked glycosylation sites, and a hydrophobic transmembrane region followed by a hydrophilic C terminus (32). The ectodomain shedding of pro-BTC has been demonstrated to involve a single C-terminal proteolytic cleavage between Tyr111 and Leu112 proximal to the transmembrane region (30) and multiple N-terminal cleavage events, resulting in variable N-terminal ectodomain forms (33).

In this study, we describe a detailed map of the molecular sizes and identity of BTC protein fragments generated by ectodomain shedding and demonstrate for the first time that the metalloprotease-dependent shedding of pro-BTC is activated by extracellular calcium influx and ectodomain shedding and demonstrate for the first time that sizes and identity of BTC protein fragments generated by constitutive pro-BTC shedding is sensitive to TIMP-1 (tissue curric acetate (APMA). We also demonstrate that activated and tagged wild-type murine ADAM10 and catalytically inactive ADAM17(E406A) (34) and wild-type murine ADAM10 and catalytically inactive, ADAM10(E385A) (37) were cloned into the psi-IRES-PURO retroviral vector. All cDNA constructs were verified by DNA sequencing. High titer retroviral supernatants were generated as described previously (36). For retroviral transduction, 4 × 10⁵ wild-type or ADAM9- or ADAM17-deficient cells were seeded into 25-cm² tissue culture flasks and cultured for 24 h prior to infection. Cells were incubated with 5 ml of virus stock for 12 h in the presence of 4 μg/ml Polybrene and then replated with fresh medium. For Puro-vector cells, cells were then grown for 48 h prior to passage into medium containing 2–5 μg/ml puromycin. Resistant cells were used in subsequent experiments. For ADAM coexpression experiments, cells were initially transduced with pBM-BTC-PAE-IRES-EGFP retrovirus, and a pooled population of enhanced green fluorescent protein-positive cells was isolated by fluorescence-activated cell sorting using a MoFlo cytometer (Cyto- mation, Inc.). Recombinant human BTC and mouse interleukin-2 (IL-2) were generous gifts from Chris Overall (University of British Columbia) and Steve Warren (MRC Laboratory of Molecular Biology, Cambridge, U.K.). Recombinant human BTC-PAE was generated by subcloning the unique SfiI restriction sites. For stable expression of BTC-HA alone, the cDNA encoding human BTC containing a C-terminal HA epitope tag (Jackson Immunoresearch Laboratories, Inc.). Recombinant human BTC containing a C-terminal HA epitope tag antibody, biotinylated goat anti-human BTC ectodomain (Asp32–Tyr111) antibody, biotinylated rat anti-mouse ADAM10 antibody, and rabbit anti-HA antibody (Zymed Laboratories, Inc.) was used. The isolation and characterization of conditionally immortalized wild-type, ADAM17Δ22/Δ22, and ADAM9Δ1/Δ1 dermal fibroblast and stomach epithelial cell lines have been described previously (34, 35). Cells were cultured at 33 °C in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum/penicillin/streptomycin/nonessential amino acids and 5 units/ml IFN-γ. Prior to experiments, cells were cultured for 36 h at 37 °C in medium lacking IFN-γ to arrest immunoreactivity by the SV40 large T antigen. All treatments were performed at 37 °C.

**Generation of Expression Constructs and Retroviral Transduction—** A cDNA encoding human BTC containing a C-terminal HA epitope tag was generated by PCR amplification using flanking primers (5'-GGCCATTCTGCGCGCCAMCATTTGAGGCGCCGGCGGTC and 3'-GGCGGCTGCGCGCCGGTGGTAACAAAACAGTCACCTG), containing unique SfiI restriction sites. For stable expression of BTC-PAE alone, the full-length pro-BTC cDNA fragment was directionally subcloned into the first-exon of the pBM-IRES-PURO retroviral vector, which contains an HA epitope tag cassette flanked by 5'-SfiI and 3'-NotI restriction sites (34). For coexpression with different ADAM cDNAs, BTC-PAE was cloned into the psi-IRES-PURO retroviral vector. All cDNA constructs were verified by DNA sequencing.

**Experimental Procedures**

**Antibodies and Reagents—** The following antibody reagents were used: goat anti-human BTC ectodomain antibody (Asp32–Tyr111) antibody, biotinylated goat anti-human BTC ectodomain antibody, mouse anti-human BTC ectodomain antibody, rat anti-mouse ADAM10 antibody, and rabbit anti-human ADAM17 cytoplasmic domain antibody (R&D Systems); rabbit anti-hemagglutinin (HA) epitope tag antibody (Zymed Laboratories Inc.); horseradish peroxidase-conjugated donkey anti-goat IgG (Jackson Immunoresearch Laboratories, Inc.). Recombinant human BTC and mouse interferon-γ (IFN-γ) were purchased from R&D Systems. Calcinium ionophore (A23187), phorbol 12-myristate 13-acetate (PMA), APMA, concanavalin A (ConA)-agarose, and protease inhibitor mixture were purchased from Sigma. Sulfosuccinimidyl 6-(biotinamido)hexanoate, SuperSignal West Pico substrate, heparin-agarose, streptavidin-agarose, and protein G-agarose were obtained from Pierce. Horseradish peroxidase-conjugated streptavidin was purchased from Jackson Immunoresearch Laboratories, Inc. SP600125 was obtained from BIOMOL Research Laboratories, Inc. SP600123 was obtained from BIORAD. Sulfosuccinimidyl 6-(biotinamido)hexanoate for 30 min at 4 °C. To evaluate stimulated pro-BTC shedding, cells were cultured for 1 h in serum-free DMEM with or without APMA, A23187, or PMA. Preinubcation of cells with GM6001, kinase inhibitors, calcium-free DMEM, or TIMPs was performed for 30 min prior to addition of shedding activators. For TIMP inhibition experiments, cells were incubated with 4 μg/ml TIMPs prior to addition of shedding activators. For TIMP inhibition experiments, cells were cultured at 33 °C in DMEM plus 10% fetal bovine serum/penicillin/streptomycin/interleukin-2, and ADAM9- or ADAM17-deficient cells were seeded into 25-cm² tissue culture flasks and cultured for 24 h prior to infection. Cells were incubated with 5 ml of virus stock for 12 h in the presence of 4 μg/ml Polybrene and then replated with fresh medium. For Puro-vector cells, cells were then grown for 48 h prior to passage into medium containing 2–5 μg/ml puromycin. Resistant cells were used in subsequent experiments. For ADAM coexpression experiments, cells were initially transduced with pBM-BTC-PAE-IRES-EGFP retrovirus, and a pooled population of enhanced green fluorescent protein-positive cells was isolated by fluorescence-activated cell sorting using a MoFlo cytometer (Cytomation, Inc.). Recombinant human BTC and mouse interleukin-2 (IL-2) were generous gifts from Chris Overall (University of British Columbia) and Steve Warren (MRC Laboratory of Molecular Biology, Cambridge, U.K.). Recombinant human BTC containing a C-terminal HA epitope tag antibody, biotinylated goat anti-human BTC ectodomain (Asp32–Tyr111) antibody, biotinylated rat anti-mouse ADAM10 antibody, and rabbit anti-HA antibody (Zymed Laboratories, Inc.) was used. The isolation and characterization of conditionally immortalized wild-type, ADAM17Δ22/Δ22, and ADAM9Δ1/Δ1 dermal fibroblast and stomach epithelial cell lines have been described previously (34, 35). Cells were cultured at 33 °C in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum/penicillin/streptomycin/nonessential amino acids and 5 units/ml IFN-γ. Prior to experiments,
transferred to Hybond nitrocellulose (Amersham Biosciences), and subsequently immunoblotted with specific antibodies. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, blots were developed using SuperSignal West Pico substrate. Western blots were developed using ECL chemiluminescence reagents (Amersham Biosciences) or ECL Western blotting substrate (GE Healthcare) following the manufacturer’s instructions. Membranes were reblocked and probed with another antibody.

For metabolic labeling, cells were washed twice and incubated for 30 min with RPMI 1640 medium lacking L-methionine supplemented with 5% dialyzed fetal bovine serum. Cells were then pulse-labeled with 250 μCi/ml 35S-methionine (Amersham Biosciences) for 20 min and chased in DMEM containing a 10-fold excess of unlabeled L-methionine. At different chase times, cells were washed twice with ice-cold PBS and lysed, and BTC was immunoprecipitated by overnight incubation with 2 μg of anti-HA antibody and 30 μl of a 50% slurry of protein A-agarose as described above. BTC immunoprecipitates were separated on 20% Tris/Tricine gels under reducing conditions and visualized by autoradiography.

BTC ELISA—A specific human BTC sandwich ELISA (R&D Systems) was used to quantify BTC levels in CM and cell lysates according to the manufacturer’s instructions. The recombinant human BTC ectodomain (R&D Systems) was used as a standard.

ADAM Western Blotting—For detection of ADAM17 and ADAM10 in coexpression experiments, cells were lysed with lysis buffer containing 5 mM o-phenanthroline. ConA-agarose precipitates were prepared from cell lysates, separated by 10% SDS-PAGE, and then transferred to nitrocellulose as described above.

Data Analysis—All experiments were repeated at least three times with similar results, and a representative figure is presented. Values for each experiment are expressed as the means ± S.D. of quadruplicate determinations.

RESULTS

Identification of Multiple Cellular and Shed Forms of BTC—For several EGF-like growth factors, including TGFα, HB-EGF, and amphiregulin, cell-surface ectodomain shedding and generation of soluble ligand are thought to be critical steps for functional ErbB receptor signaling. However, only limited knowledge about the sequential and regulated processes of EGF-like growth factors and the molecular mechanisms that control these shedding events is currently available. Soluble BTC forms have been detected in CM from BTC-expressing cell lines (7, 33, 38), indicating that these cells possess the appropriate proteolytic machinery to shed BTC from the cell surface. In this study, we have further characterized the shedding of BTC using mouse dermal fibroblasts and stomach epithelial cells transduced with a retroviral BTC-HA expression construct (Fig. 1A).

Immunoprecipitation and Western blotting using the anti-HA antibody detected five cellular BTC fragments of 40, 30, 25, 19, and 12 kDa in cell lysates from dermal fibroblasts transduced with BTC-HA (Fig. 1B, left panel). Immunoprecipitation with the anti-BTC ectodomain (Asp32–Tyr111) antibody (referred to as the anti-BTC antibody) or affinity purification with heparin-agarose and Western blotting with the anti-HA antibody, identified the 40-kDa BTC fragment as the major cell-surface form (data not shown). In addition, the molecular masses and the low levels of the 19- and 25-kDa BTC species were precipitated with ConA-agarose (data not shown). The appearance of the 40-kDa pro-BTC form, the 30-kDa form, were also biotinylated (Fig. 1C). At chase time = 0, one major form of 30 kDa was observed in cell lysates. Between 0.5 and 1 h of chase, a 40-kDa form and two minor forms of 25 and 19 kDa appeared with a corresponding decrease in the 30-kDa form. The rapid appearance of the BTC cytoplasmic remnant (12 kDa), generated following ectodomain shedding, coincided with the appearance of the 40-kDa pro-BTC form. The 30-kDa form was precipitated with ConA-agarose and likely represents an immature precursor of the 40-kDa form (also precipitated by ConA-agarose) (data not shown). The appearance of the 25- and 19-kDa forms may represent sequential N-terminal proteolysis of the larger pro-BTC forms. However, the relatively slow generation of these two minor forms compared with the cytoplasmic remnant (12 kDa) indicates that stalk cleavage of pro-BTC is precluded over N-terminal processing.

The BTC forms at the cell surface were characterized by labeling cells with plasma membrane-impermeable sulfooccsinimidyl 6-(biotinamido)hexanoate, followed by streptavidin precipitation and Western blotting using the anti-HA antibody. The 40-kDa form represents the major BTC species at the cell surface, yet all other cellular forms, with the exception of the 30-kDa form, were also biotinylated (Fig. 1D), supporting our hypothesis that the 30-kDa form represents an immature precursor form. We also found that binding of the anti-BTC antibody to the surface of live cells, followed by cell lysis, immunoprecipitation, and Western blotting with the anti-HA antibody, identified the 40-kDa BTC fragment as the major cell-surface form (data not shown). In addition, the molecular masses and the low levels of the 19- and 25-kDa pro-BTC forms at the cell surface correlate with these two species being shed to generate the minor 15- and 20-kDa soluble forms, respectively (Fig. 2A).

A Hydroxamic Acid inhibitor of Zinc Metalloproteases Blocks Constitutive and Activated Pro-BTC Shedding—Ectodomain shedding of a variety of transmembrane molecules is mediated by zinc-binding metalloproteases. To determine whether constitutive shedding of BTC is metalloprotease-dependent, dermal fibroblasts transduced with BTC-HA were cultured for 24 h in serum-free medium in the presence or absence of the broad-spectrum
FIG. 1. Identification of multiple cellular and shed forms of BTC. A, shown is a schematic of the C-terminally HA-tagged human pro-BTC (BTC-HA) used in this study. TM, transmembrane domain. B, wild-type dermal fibroblasts expressing BTC-HA were cultured in serum-free DMEM for 24 h. CM and cell lysates were precipitated (PPT) with either the anti-BTC ectodomain or anti-HA antibody (Ab) or with heparin-agarose and then analyzed by Western blotting (WB) using either the anti-HA antibody (cell lysates) or the anti-BTC ectodomain antibody (CM) as described under “Experimental Procedures.” C, wild-type dermal fibroblasts transduced with BTC-HA were pulsed-labeled with 250 μCi/ml L-[35S]methionine for 20 min and then chased in DMEM containing excess L-methionine. At each time point, cell lysates were prepared, and BTC was immunoprecipitated (IP) with the anti-HA antibody and then analyzed by Tris/Tricine gel electrophoresis and autoradiography. D, wild-type dermal fibroblasts transduced with BTC-HA were cell surface-biotinylated with sulfosuccinimidyl 6-(biotinamido)hexanoate, and cell lysates were prepared. Equivalent amounts of cell lysate were precipitated with streptavidin (SA)-agarose (cell-surface BTC) or immunoprecipitated with the anti-HA antibody (total cellular BTC) and then analyzed by Western blotting using the anti-HA antibody. In B–D, the molecular masses of different BTC forms are represented in kilodaltons. The asterisks indicate the position of the immunoglobulin band.
metalloprotease inhibitor GM6001. CM from cells treated with GM6001 had reduced levels of detectable shed BTC ectodomains (Fig. 2A, left panel). Consistent with this, analysis of the corresponding cell lysates indicated that GM6001 treatment led to a time-dependent (data not shown) decrease in the level of the BTC cytoplasmic remnant and all other cellular pro-BTC forms relative to the level of the major 40-kDa form (Fig. 2A, right panel). Taken together, these results demonstrate that stalk cleavage (and possibly N-terminal processing of pro-BTC) is mediated by a metalloprotease activity.

The shedding of many transmembrane molecules, including EGF-like growth factors, has been shown to be activated over short time periods (1–4 h) by a variety of chemical stimuli. These stimuli include, for example, PMA (30, 39–44), APMA (an organomercurial compound that activates latent metalloproteases by inducing removal of the enzyme propeptide inhibitory region) (20), and A23187 (39, 45–47). We therefore tested the ability of these agents to activate pro-BTC shedding in dermal fibroblasts. Treatment with A23187 (5 μM), APMA (50 μM) added directly to the culture medium. CM and cell lysates were collected and analyzed by Western blotting as described for A.
The effects of various inhibitors on constitutive and activated pro-BTC shedding were investigated. For constitutive shedding, wild-type stomach epithelial cells expressing BTC-HA were cultured for 24 h in the presence or absence of each inhibitor. For activated shedding, cells were preincubated in DMEM with or without inhibitors for 30 min, followed by direct addition of A23187 (5 μM) or APMA (100 μM) to the culture medium and incubation for 1 h. The level of pro-BTC shedding was determined by ELISA and is expressed as the percentage of BTC for each treatment compared with the level of BTC detected in the presence of vehicle alone. ND, not determined.

### Table I

| Treatment          | Constitutive BTC shedding | A23187 BTC shedding | APMA BTC shedding |
|--------------------|---------------------------|---------------------|-------------------|
| Vehicle            | 100                       | 100                 | 100               |
| GM6001 (50 μM)     | 21                        | 16                  | 18                |
| Calcium-free       | ND                        | 4                   | 107               |
| Rottlerin (10 μM)  | 127                       | 23                  | 97                |

The effects of the various kinase inhibitors on pro-BTC shedding are consistent with an activation of pro-BTC shedding. In contrast, PMA (0.1 μM) did not activate pro-BTC shedding in dermal fibroblasts. We also failed to detect PMA induction of pro-BTC shedding in other BTC-HA-expressing cells (see Fig. 4, A and B), even when used at concentrations far higher (up to 10 μM) than normally required for activation of shedding of other transmembrane proteins (data not shown). Similar to constitutive shedding, A23187- and APMA-induced pro-BTC shedding was blocked by GM6001 (Fig. 2B), indicating the involvement of a metalloprotease activity.

**A23187 Activation of Pro-BTC Shedding Is Blocked by Treatment with the Protein Kinase C6 (PKC6) Inhibitor Rottlerin and Calcium-free Medium** — The mechanism by which chemical stimuli activate shedding of transmembrane proteins remains largely uncharacterized. Several researchers have shown that PMA activation of shedding operates through a PKC-dependent mechanism (24, 39, 45, 48). Calcium ionophore (A23187) leads to calcium influx into the cell, which may then lead to the activation of a variety of cell signaling pathways that could be involved in shedding activation (49–51). We found that preincubation of stomach epithelial cells expressing BTC-HA in calcium-free medium or with the PKC inhibitor rottlerin led to a dramatic decrease in the activation of pro-BTC shedding by A23187 (Table I). Whereas constitutive and APMA-activated pro-BTC shedding was blocked by GM6001, rottlerin was without effect. The effect of calcium-free medium on constitutive shedding could not be determined due to toxicity over 24 h of culture, whereas APMA activation of pro-BTC shedding was unaffected by preincubation in calcium-free medium. These results indicate that constitutive and APMA-activated pro-BTC shedding events operate by distinct mechanisms, potentially involving the activation of multiple cell signals.

Rottlerin has generally been characterized as an inhibitor of PKC6, yet we found that other broad-spectrum PKC inhibitors such as GF109203X and G66983, as well as down-regulation of PKC by overnight incubation in PMA (47), were without effect on A23187-activated shedding (Table II). In addition, several other kinase inhibitors did not block A23187-activated pro-BTC shedding. These results suggest that the inhibitory effect of rottlerin is independent of PKC and possibly of several other kinase signaling pathways.

**Constitutive and Activated Pro-BTC Shedding Is Inhibited by TIMP-1 and TIMP-3, but Not by TIMP-2** — Wild-type stomach epithelial cells expressing BTC-HA were grown in 24-well plates and cultured for 24 h in DMEM with or without protease inhibitor at the indicated concentration. For activated shedding, cells were preincubated in DMEM with or without inhibitor for 30 min; A23187 (5 μM) or APMA (100 μM) was added directly to the culture medium; and cells were incubated for 1 h. Pro-BTC shedding was evaluated by ELISA and is expressed as the percentage of BTC shedding as defined in Table I.

**Table II**

| Inhibitor            | Primary specificity | BTC shedding |
|----------------------|---------------------|--------------|
| Vehicle              |                     | 100          |
| Calcium-free         | Extracellular Ca2+  | 4            |
| Rottlerin (10 μM)    | PKC6, CaM kinase III | 23          |
| GF109203X (5 μM)     | PKCαβ/δ/ε           | 100          |
| G66983 (2 μM)        | PKCαβ/δ             | 104          |
| G66983 (2 μM)        | PKCβδ               | 99           |
| PMA o/n (0.1 μM)     | Down-regulates PKC  | 105          |
| PD98059 (50 μM)      | MEK1                 | 102          |
| U0126 (10 μM)        | MEK1/2               | 101          |
| SB203580 (5 μM)      | p38 MAPK             | 106          |
| SP600125 (5 μM)      | JNK1/2               | 99           |
| AG1478 (5 μM)        | EGFR kinase          | 105          |
| PP2 (5 μM)           | Src kinases          | 105          |
| LY294002 (10 μM)     | PI3K                 | 98           |

**Table III**

| Protease inhibitor | BTC shedding |
|--------------------|--------------|
| Vehicle            | 100          |
| GM6001 (50 μM)     | 21           |
| TIMP-1 (0.5 μM)    | 48           |
| TIMP-2 (0.5 μM)    | 102          |
| TIMP-3 (0.5 μM)    | 84           |

TIMP-1 and TIMP-3 have been shown to inhibit the in vitro proteolytic activity of ADAM10 (52) and MMPs (53), whereas ADAM17 is blocked by TIMP-3 alone (54). TIMP-2 has been demonstrated to inhibit the activity of some MMPs (53, 55, 56), but not ADAMs (52, 54, 56).

Culture of stomach epithelial cells expressing BTC-HA in the presence of TIMP-1 significantly blocked the constitutive and APMA-activated shedding of pro-BTC, whereas TIMP-2 and TIMP-3 were without significant effect (Table III). A23187-activated shedding was blocked by TIMP-3, but not by TIMP-1 or TIMP-2. An explanation for the differential sensitivity of each BTC shedding event to TIMP-1 and TIMP-3 is still not known, but collectively, these results raise the possibility that constitutive and activated pro-BTC shedding may be associated with ADAM metalloproteases such as ADAM17 and ADAM10 and that MMPs are less likely to be involved.

**ADAM17 and ADAM9 Are Not Essential for Constitutive or Activated Pro-BTC Shedding** — Shedding of other EGF-like proteins has been linked to one or more metalloproteases of the ADAM family, especially ADAM17 and ADAM9 (9, 23, 24). To investigate the role of ADAM17 and ADAM9 in pro-BTC shedding, BTC-HA was expressed in dermal fibroblast and stomach epithelial cells derived from wild-type (WT) and
ADAM17 and ADAM9 mice (35) and from ADAM9−/− mice (57), respectively. The level of BTC in the CM and cell lysates was determined by ELISA, and the ratio of BTC shed into CM to the level of cell-associated pro-BTC in cell lysates was determined to give an indication of the relative level of pro-BTC shedding in each cell line. No defect in constitutive pro-BTC shedding was detected in ADAM17+/−/− mice (57) and ADAM9+/−/− mice (Fig. 3, A–D) even though constitutive shedding in all cell lines was blocked by GM6001, indicating the involvement of distinct metalloprotease activities. However, the level of pro-BTC detectable in cell lysates did not change significantly with GM6001, indicating that these cell lines do not appear to continue to accumulate pro-BTC despite inhibition of shedding.

Comparable pro-BTC shedding was observed in WT, ADAM17+/−/−, and ADAM9−/− cells following stimulation with A23187 and APMA (Fig. 4, A and B) and was blocked by GM6001 in all cell lines. In contrast, PMA did not activate pro-BTC shedding, although the shedding of several other transmembrane proteins (34, 35), including the EGF-like growth factor amphiregulin,2 was induced by PMA in these cell lines in an ADAM17-dependent manner.

Overexpression of ADAM10, but Not ADAM17, Affects Constitutive and Activated Pro-BTC Shedding—The finding that WT, ADAM17+/−/−, and ADAM9−/− cells shed equivalent levels of BTC does not rule out the possibility that BTC may be a substrate for these enzymes. However, it does indicate that, in this system, these two ADAMs are not the primary metalloproteases responsible for pro-BTC shedding. Considerable focus has recently turned to ADAM10 as a mediator of ectodomain shedding of EGF-like factors (26, 58) and other transmembrane proteins (37, 59). To investigate the role of ADAM10 in pro-BTC shedding, we overexpressed ADAM10 and a catalytically inactive form of the enzyme in wild-type dermal fibroblasts also overexpressing BTC-HA (Fig. 5A). Overexpression of WT ADAM10 led to an increase in constitutive pro-BTC shedding over a 24-h period in serum-free DMEM (Fig. 5, B and C, left panel) that was associated with an increase in the level of the BTC cytoplasmic remnant in cell lysates (Fig. 5C, right panel). In contrast, overexpression of catalytically inactive ADAM10(E385A) led to a decrease in the level of constitutive pro-BTC shedding (Fig. 5, B and C, left panel) and a decrease in the level of the cytoplasmic remnant (Fig. 5C, right panel). In line with previous results (see Fig. 3), we also found that overexpression of WT ADAM17 or ADAM17(E406A) (Fig. 5B) or WT ADAM8, ADAM9, ADAM15, or ADAM9(E348A) (data not shown) was without effect on constitutive pro-BTC shedding.

To further evaluate the role of ADAM10 in activated pro-BTC shedding, the same panel of cell lines was treated with shedding activators A23187, APMA, and PMA. A23187 and

---

2 M. P. Sanderson, S. N. Erickson, P. J. Gough, K. J. Garton, P. T. Wille, E. W. Raines, S. Al-Noori, A. J. Dunbar, and P. J. Dempsey, unpublished data.
APMA activation of pro-BTC shedding was reduced by overexpression of ADAM10(E385A), but not by ADAM17(E406A) (Fig. 6A). Overexpression of WT ADAM10 did not consistently enhance A23187- and APMA-induced shedding compared with control cells as judged by ELISA (Fig. 6A), whereas the biochemical data (Fig. 6, B and C) suggest a minor enhancement of pro-BTC shedding. This result is in contrast with the significant enhancement of stimulus-induced shedding of ADAM17 substrates in these same cells by ADAM17 overexpression (34, 35). In addition, overexpression of either ADAM10 or ADAM17 did not result in activation of pro-BTC shedding by PMA.

**DISCUSSION**

BTC is a novel member of the EGF-like growth factor family with dual specificity for ErbB1 and ErbB4. In several different cell systems, BTC can activate distinct downstream signaling events that lead to unique biological responses (60–62). For example, BTC can induce differentiation and promote regeneration of pancreatic β-cells in models of experimental diabetes (63–67). BTC is also a potent growth factor for vascular smooth muscle cells and its enhanced expression in vascular lesions may be associated with the progression of cardiovascular disease (68–71). Differences in biological activity between BTC and other EGF-like growth factors may also be defined by the unique cell and environmental signals that regulate the release and presentation of soluble ligands to specific receptor populations. However, the mechanisms involved in the regulated shedding of the BTC precursor to generate soluble biologically relevant BTC are poorly defined (8, 11, 13, 38). In this study, we have demonstrated that pro-BTC shedding is activated by a unique set of stimuli. Pro-BTC cleavage and shedding could be activated by APMA, calcium influx, and extracellular calcium influx, but PMA, which is a common shedding activator for other EGF-like growth factors, failed to stimulate pro-BTC shedding. Furthermore, using ADAM-deficient cells and overexpression of wild-type and catalytically inactive ADAMs, we have shown that ADAM10 mediates constitutive and activated pro-BTC shedding.

Three BTC ectodomain isoforms identified in CM of dermal fibroblasts have molecular masses similar to those of soluble BTC forms, which have been shown previously to vary in their
N-terminal sequence (33). This raises the possibility that the three soluble BTC ectodomains are generated via sequential N-terminal processing of alternative pro-BTC transmembrane molecules, but utilizing a common juxtamembrane site (Tyr111-Leu112) (30). Five cellular BTC forms were identified in cell lysates, with the lowest molecular mass form detected only with the anti-HA antibody, indicating that it is the membrane-anchored cytoplasmic remnant fragment generated following ectodomain shedding. The high molecular mass cellular BTC forms represent transmembrane pro-BTCs that may undergo shedding to generate the alternative soluble ectodomains. In addition, these forms were detected by cell-surface biotinyla-

Fig. 5. Overexpression of ADAM10, but not ADAM17, increases constitutive pro-BTC shedding. The effect of ADAM10 and ADAM17 overexpression on constitutive pro-BTC shedding was examined in dermal fibroblasts. A, Western blot (WB) analysis of ADAM10 and ADAM10 overexpression in wild-type dermal fibroblasts transduced with BTC-HA. Cells infected with the pBM-IRE-PURO or pBM-ADAM-IRE-PURO retroviral vector were grown for 2 days in 6-well plates prior to concentration by ConA-agarose precipitation of cell lysates as described under “Experimental Procedures.” The extent of overexpression of ADAM10 and ADAM17 was determined by Western blotting using the anti-mouse ADAM10 ectodomain antibody (Ab; 1 μg/ml) and the anti-human ADAM17 cytoplasmic domain antibody (1:1000 dilution), respectively, as described under “Experimental Procedures.” E>A ADAM10, ADAM10(E385A); E>A ADAM17, ADAM17(E406A). B, wild-type dermal fibroblasts transduced with BTC-HA and coexpressing ADAM vectors were grown in 6-well plates and cultured in serum-free DMEM for 24 h. CM and cell lysates were collected and evaluated for BTC content by ELISA. Results are expressed as the means ± S.D. of the ratio of soluble (Sol) to cellular BTC from quadruplicate determinations. C, wild-type dermal fibroblasts transduced with BTC-HA and coexpressing ADAM vectors were cultured in serum-free DMEM for 24 h in the absence or presence of GM6001 (50 μM). CM and cell lysates were analyzed by Western blotting using the anti-BTC ectodomain and anti-HA antibodies, respectively.
tion, indicating that they are present at the plasma membrane, where they are susceptible to shedding. Pulse-chase radiolabeling indicated that the cellular form of pro-BTC undergoes sequential modification events, which may include glycosylation and N-terminal proteolysis. Similar sequential modification events have been observed with other EGF-like growth factors (39, 41, 46, 47, 72, 73). Release of the BTC ectodomain by C-terminal processing appears to be preferred over N-terminal cleavage events, a preference also observed with amphiregulin and EGF processing (41, 43, 72).

Ectodomain shedding of EGF-like growth factors has been linked to metalloproteases of the ADAM and MMP families. ADAM17 (tumor necrosis factor-α-converting enzyme) has been implicated in the shedding of TGFα (8), neuregulin-1 (19), and HB-EGF and amphiregulin (9). In addition, HB-EGF processing has been linked to ADAM9 (24), ADAM10 (58), ADAM12 (25), ADAM15 (26), MMP3 (27), and MMP7 (29), whereas ADAM19 has been implicated in the processing of neuregulin-1 (74). Here, we provide several lines of evidence for the role of metalloproteases in constitutive and stimulus-induced shedding of BTC.

TIMP-1 and the broad-spectrum metalloprotease inhibitor GM6001 blocked constitutive shedding of pro-BTC. These findings, together with the inability of TIMP-2 to block constitutive pro-BTC shedding, suggest the possible involvement of an ADAM metalloprotease. Constitutive pro-BTC shedding was functional in ADAM17 and ADAM9 null cells. A previous study reported that ADAM17 is capable of cleaving a peptide corre-
Regulation of Betacellulin Ectodomain Shedding

In summary, the work presented here provides the first detailed map of the protein fragments generated by pro-BTC processing and the first evidence of the regulation of pro-BTC shedding by APMA and A23187. We have shown that constitutive and activated pro-BTC shedding occurs by an ADAM10-mediated process that is independent of ADAM17, ADAM9, and probably MMPs. Elucidation of the signaling pathways that are involved in pro-BTC shedding will identify possible targets for the manipulation of BTC bioavailability in vivo and hence regulation of the function of this potent growth factor.

Acknowledgments—We thank GroPep Ltd. for the use of research facilities, Roy Black and Jacques Peschon (Amgen) for providing ADAM17 reagents, Carl Blobel (Memorial Sloan-Kettering Institute) for providing ADAM9-/- mice for cell line generation, Garry Nolan (Stanford University) for providing the pB/M retroviral vector, and Cathy Abbott (Flinders University) for continued support of the project. We also thank Sarah Fitzgerald (Pacific Northwest Research Institute) for excellent technical assistance.

REFERENCES
1. Holbro, T., and Hynes, N. E. (2004) Annu. Rev. Pharmacol. Toxicol. 44, 195–217
2. Yarden, Y., and Sliwkowski, M. X. (2001) Nat. Rev. Mol. Cell. Biol. 2, 127–137
3. Wong, S. T., Winchell, L. F., McCune, B. K., Earp, H. S., Teixido, J., Massague, J., Herman, B., and Lee, D. C. (1989) Nat. Rev. Mol. Cell. Biol. 2, 127–137
4. Brachmann, R., Linquist, P. B., Nagashima, M., Kohr, W., Lapi, T., Napior, M., and Derynck, R. (1989) Cell 56, 691–700
ADAM10 Mediates Ectodomain Shedding of the Betacellulin Precursor Activated by p-Aminophenylmercuric Acetate and Extracellular Calcium Influx

Michael P. Sanderson, Sarah N. Erickson, Peter J. Gough, Kyle J. Garton, Paul T. Wille, Elaine W. Raines, Andrew J. Dunbar and Peter J. Dempsey

J. Biol. Chem. 2005, 280:1826-1837.
doi: 10.1074/jbc.M408804200 originally published online October 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408804200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 84 references, 52 of which can be accessed free at
http://www.jbc.org/content/280/3/1826.full.html#ref-list-1