INFECTION AND IMMUNITY, Sept. 2006, p. 5382–5390 Vol. 74, No. 9
0019-9567/06/$08.00+0 doi:10.1128/IAI.00060-06

The *Bacteroides fragilis* Toxin Binds to a Specific Intestinal Epithelial Cell Receptor

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Received 11 January 2006/Returned for modification 6 February 2006/Accepted 28 June 2006

The *Bacteroides fragilis* toxin (BFT) is the only known virulence factor of enterotoxigenic *B. fragilis*. BFT has previously been shown to act, at least in part, through cleavage of the intercellular adhesion protein E-cadherin. A specific cellular receptor for BFT has not been identified. The goal of this study was to determine if the initial interaction of BFT with intestinal epithelial cells was consistent with binding to a specific cellular receptor. Purified BFT was labeled with a fluorophore or iodide to assess specific cellular binding and the properties of BFT cellular binding. BFT binds specifically to intestinal epithelial cell lines in vitro in a polarized manner. However, specific binding occurs only at 37°C and requires BFT metalloprotease activity. The BFT receptor is predicted to be a membrane protein other than E-cadherin or a known protease-activated receptor (PAR1 to PAR4). BFT binding is resistant to acid washing, suggesting an irreversible interaction. Sugar or lipid residues do not appear to be involved in the mechanism of BFT cellular binding, but binding is sensitive to membrane cholesterol depletion. We conclude that intestinal epithelial cells in vitro possess a specific membrane BFT receptor that is distinct from E-cadherin. The data favor a model in which the metalloprotease domain of BFT processes its receptor protein, initiating cellular signal transduction that mediates the biological activity of BFT. However, activation of recognized protease-activated receptors does not mimic or block BFT biological activity or binding, suggesting that additional protease-activated receptors on intestinal epithelial cells remain to be identified.

Bacteroides fragilis strains are the leading anaerobes in human disease (18, 21). A subset of these genetically variable organisms has acquired a novel conjugative transposon (CTn86) containing the *B. fragilis* pathogenicity island (6). These strains, termed enterotoxigenic *B. fragilis* (ETBF), are associated with diarrheal disease of animals as well as of children and adults in both developing and industrialized countries (26, 34). Recent data further suggest an association between ETBF carriage and active inflammatory bowel disease (2, 20). The only recognized virulence factor of ETBF is the *B. fragilis* toxin (BFT), a zinc-dependent nonlethal metalloprotease toxin that exhibits a spectrum of biological activities detectable to date only for epithelial cell lines that polarize (forming monolayers with cell-to-cell adherens junctions) in vitro (26). Consistent with the association of ETBF with human diarrheal disease, BFT induces increased permeability and chloride secretion in intestinal epithelial cell (IEC) monolayers (3, 16). Treatment of IECs with BFT also induces the cleavage of the zonula adherens protein E-cadherin, triggering β-catenin-dependent nuclear signaling, induction of expression of the proto-oncogene protein c-Myc, and cellular proliferation (31, 32). In addition, BFT is a potent inducer of expression and secretion by human IECs of the proinflammatory chemokine interleukin-8 (IL-8), which may contribute to the colonic inflammatory response noted for infected animals, for active inflammatory bowel disease, and, in preliminary data, for ETBF-associated human diarrheal disease in Bangladesh (F. Qadri, R. B. Sack, and C. L. Sears, unpublished data) (11, 24, 33).

Receptors for enteric bacterial toxins include proteins (e.g., guanylate cyclase C for *Escherichia coli* heat-stable enterotoxin), carbohydrate epitopes (e.g., the ganglioside GM1 for cholera toxin) and lipid-containing molecules (e.g., the glycolipid Gb3 for Shiga toxin) (27). Based on the predicted structure of BFT, the toxin is a member of the matrix metalloprotease subfamily of the metzincin superfamily of zinc-dependent metalloprotease enzymes (7). Other related zinc-dependent metalloproteases known to be important in human disease include tetanus, botulinum, and anthrax toxins. Tetanus and botulinum toxins are neurotoxins that act at femtomolar concentrations and specifically bind to the presynaptic membrane of neurons. Although their mode of binding remains poorly defined, both toxins bind select polysialogangliosides with high affinity, and tetanus toxin additionally binds a glycosylphosphatidylinositol-anchored membrane protein, Thy-1 (13). In contrast, two distinct type I transmembrane proteins, the capillary morphogenesis protein and anthrax toxin receptor/tumor endothelial marker, serve as the anthrax toxin receptors (25). For all three enzyme toxins, receptor binding precedes modification of their specific cellular substrates. Based on these models of bacterial toxin action as well as the potency (0.5 pM BFT modifies IEC function [23]) and epithelial specificity of BFT bioactivity, we hypothesized that BFT activates IEC cel-

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lular signaling by binding to a specific cellular receptor. Our data demonstrate that BFT specifically binds to IECs but that, unexpectedly, binding requires the protease activity of BFT and possibly temperature-dependent cellular processes.

MATERIALS AND METHODS

Cell lines and cell culture. HT29/C1 cells (human colonic carcinoma cells, C1 clone), T84 cells (human colonic carcinoma cells), and Hep2 cells (human laryngeal epithelial cells) were grown subconfluently or as polarized monolayers on Transwell filters (Costar, Fisher Scientific) in Dulbecco's modified essential medium (DMEM) or minimal essential medium (MEM)-based growth medium with 5 to 10% fetal bovine serum, human transferrin (10 μg/ml), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Invitrogen Life Technologies, Carlsbad, CA) in 5% CO_2 (except for HT29/C1 cells, which were grown in 10% CO_2). Primary rat cortical cells (a mixture of neuronal and glial cells) were kindly provided by Jay Baraban (Johns Hopkins University School of Medicine). SW480-EC and SW480-mock (human colonic carcinoma cell lines that stably overexpress human E-cadherin and that are mock transfected as a control, respectively; obtained from Barry Gumbiner, University of Virginia School of Medicine) were grown in DMEM-based growth medium with G418 (400 μg/ml). Overexpression and membrane location of E-cadherin in SW480-EC cells compared to mock-transfected parent SW480 cells were confirmed by Western blotting and confocal immunofluorescence (data not shown).

BFT purification. The high-BFT-expression strain I-1345(pFD340::P-*bft*) (8) and the catalytic-domain BFT mutant I-1345(pFD340::P-*bft*ΔH352Y) (termed BFT-H352Y) (7) or the C-terminal deletion mutant I-1345(pFD340::P-*bft*ΔC-4) (termed BFT-C4; A. Franco and C. L. Sears, unpublished data) were grown in brain heart infusion broth supplied with clindamycin (6 μg/ml) to maintain the plasmid construct. Wild-type and mutant BFTs were purified to homogeneity from supernatants of overnight cultures using a urea-based purification method as previously reported (30).

BFT labeling. BFT was conjugated with Alexa Fluor-488 dye using a monoclonal antibody labeling kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions. Briefly, 100 μg of purified BFT in 100 μl of phosphate-buffered saline (PBS), 0.1 M sodium bicarbonate (pH 8.3) was incubated with Alexa dye for 1 h at room temperature. Nonconjugated dye was separated from labeled BFT by microdialysis using Slide-A-Lyzer (Pierce Biotechnology, Rockford, IL). At the concentrations studied (6 to 10 nM), the Alexa Fluor-488-conjugated BFT (Alexa 488-labeled BFT) exhibited a level of biological activity similar to that of unlabeled BFT based on the HT29/C1 cell assay (14). For use as a control in some experiments, bovine serum albumin (BSA) (Pierce, Rockford, IL) was similarly labeled with Alexa Fluor-488.

For labeling of the tyrosine residues in BFT by iodination, 10 μl (1.0 mCi) of $Na^{125}I$ (Amersham Biosciences, Piscataway, NJ) was added to 100 μl of Tris iodination buffer (25 mM Tris-HCl, pH 7.5, 0.4 M NaCl) in an IODO-Gen precoated iodination tube (Pierce Biotechnology, Rockford, IL) with activation of the iodide for 6 min at room temperature. The activated iodide was transferred to a BFT solution (0.3 nmol BFT in 100 μl of Tris iodination buffer). After an 8-min reaction at room temperature, 50 μl scavenging buffer (10 mg/ml tyrosine in PBS) was added with incubation for 5 min to quench the active iodide species. Iodinated BFT (^{125}I-BFT) was purified using a desalting column (Pierce Biotechnology, Rockford, IL), and radioactive fractions were combined. The BFT concentration was estimated by comparison of known concentrations of unlabeled BFT to those of iodinated BFT on silver-stained sodium dodecyl sulfate gels. The biological activity of ^{125}I-BFT was similar to that of unlabeled BFT by the HT29/C1 cell assay.

Detection of labeled BFT and other proteins. (i) Confocal microscopy. HT29/C1 cells grown on eight-well chamber slides were treated with Alexa 488-labeled BFT (6 to 10 nM, unless otherwise stated) at 4°C or 37°C for differing times as indicated in serum-free DMEM with or without preincubation for 30 min with a 100-fold excess of unlabeled BFT or other reagents (see Results for details). Cells were fixed in 4% paraformaldehyde, and the fluorescent BFT signal was examined either directly or, in some instances, after amplification of the bound BFT signal by incubation with anti-Alexa-488 antibody (Invitrogen Life Technologies, Carlsbad, CA). For E-cadherin and protease-activated receptor 2 (PAR2) costaining, cells were fixed and permeabilized (1% Triton X-100 [TX-100] for 10 min, room temperature) prior to incubation with a monoclonal E-cadherin antibody (C36; BD Biosciences, Palo Alto, CA) directed to the E-cadherin C-terminal domain or PAR2 antibody (SAM11; Zymed Laboratories, San Francisco, CA). An anti-mouse Alexa-568-conjugated secondary antibody was used to detect cell fluorescence signals by confocal microscopy (Zeiss LSM410).

(ii) Flow cytometry. HT29/C1 cells grown on 24-well plates were treated with Alexa 488-labeled BFT with or without preincubation for 30 min with a 100-fold excess of unlabeled BFT or other reagents (see Results). After a 1-h incubation at 37°C, the cells were washed with cold PBS and suspended using 0.05% trypsin (Invitrogen Life Technologies, Carlsbad, CA), and trypsin activity was subsequently neutralized with 30% fetal bovine serum in DMEM. Suspended cells were immediately analyzed by use of a fluorescence-activated cell sorter (FACS); results were analyzed using CELLQuest (version 3.3) at the Flow Cytometry Core Analytic Laboratory, Johns Hopkins University School of Medicine (instrument and software from Becton Dickinson Immunofluorescent Systems, San Jose, CA).

(iii) ^{125}I-BFT binding assay. Subconfluent HT29/C1 cells grown on 96-well plates or isolated HT29/C1 membrane preparations were preincubated with or without a 100-fold excess of unlabeled BFT for 30 min and treated with various concentrations of ^{125}I-BFT for various times (see Results) and subsequently washed in ice-cold PBS to remove unbound ^{125}I-BFT. For membrane preparations, HT29/C1 cells were disrupted by sonication in ice-cold HEPES-buffered saline. After a low-speed spin to remove intact cells, membranes were pelleted by centrifugation (45 min, 18,000 × *g*) and resuspended in 50 mM Tris-Cl, pH 7.4. For intact-cell ^{125}I binding assays, cells were lysed with 2 N NaOH, and bound ^{125}I-BFT in the cell lysates was quantitated by use of a gamma counter (1191 GamaTrac; TM Analytic, Brandon, FL).

Specific binding of labeled BFT (^{125}I-BFT or Alexa 488-labeled BFT) was calculated by subtracting the nonspecific labeled-BFT binding (in the presence of a 100-fold excess of unlabeled BFT) from the total level of binding of labeled BFT.

Inhibitors and other treatments of HT29/C1 cells. Unless otherwise stated, all treatments were tested by incubating HT29/C1 cells with or without the inhibitor or reagent for 30 min prior to Hanks' balanced salt solution washes and addition of unlabeled BFT (5 nM) or, in some instances, Alexa 488-labeled BFT (6 to 10 nM) for 60 min at 37°C. BFT biological activity on HT29/C1 cells was assessed semiquantitatively (14), and binding of Alexa 488-labeled BFT was assessed by FACS as described above. Tested lectins, enzymes, and other reagents are listed in Table 1; all Table 1 reagents and human thrombin were from Sigma (St. Louis, MO). Other reagents were as follows: (i) GM6001, a metalloprotease inhibitor, from Calbiochem (San Diego, CA); (ii) PAR agonists, including PAR1 (TFLLR-NH_2), PAR2 (2-furoyl-LIGRLO-amide), and PAR4 (GYPGQV-NH_2), from Peptides International (Louisville, KY); and (iii) a chimeric construct of the extracellular-domain human E-cadherin fused with the human immunoglobulin Fc domain (Cad-Fc), which was expressed and purified as previously reported (31).

For acid wash experiments, incubation of HT29/C1 cells with ^{125}I-labeled or Alexa 488-labeled BFT for 20 to 40 min was followed by a PBS wash and two sequential 5-min incubations with an acid wash (150 mM NaCl, 28 mM acetic acid, pH 3.4) or a PBS wash (as a control). For HT29/C1 cells incubated with ^{125}I-labeled BFT, the acid wash and 2 M NaOH cell lysates were counted with a gamma counter; HT29/C1 cells incubated with Alexa 488-labeled BFT were quantified by FACS analysis after acid washing.

For assessment of the cellular distribution of BFT, cell proteins were extracted with 1% Triton X-100 in phosphate-buffered saline for 10 min at 4°C. The supernatant (TX-100-soluble fraction) was collected, and the residual portion (TX-100-insoluble fraction) was solubilized with 1% sodium dodecyl sulfate lysis buffer (15). The proteins from both fractions were analyzed by Western blotting or by gamma counting for the presence of labeled BFT.

To test modifiers of membrane cholesterol, HT29/C1 cells were incubated with methyl-β-cyclodextrin (mβCD), filipin, or cholesterol oxidase (all from Sigma) in DMEM for 30 min at 37°C and subsequently washed and labeled with Alexa 488-labeled BFT for 60 min. To replete membrane cholesterol, mβCD-treated HT29/C1cells were washed with Hanks' balanced salt solution and incubated with 0.4 to 0.8 mM water-soluble cholesterol (Sigma) for 20 min at 37°C prior to additional washes before experimental use.

Statistical analysis. Data are expressed as means ± standard deviations. Student's *t* test (GraphPad Instat, version 3.05) was used to evaluate data for statistical significance; *P* values of ≤0.05 were considered statistically significant.

RESULTS

BFT binds specifically to a receptor on HT29/C1 cells. To examine if BFT binds specifically to an IEC receptor, subconfluent HT29/C1 cells were initially incubated with Alexa 488-labeled BFT (6 nM) for 1 h at 37°C, suspended by trypsin

TABLE 1. Reagents evaluated for inhibition of BFT biological activity on HT29/C1 cells

Reagent name (origin)	Biological specificity	Concn tested
ConA (*Canavalia ensiformis*)	Mannose/glucose	100 μg/ml
PNA (*Arachis hypogaea*)	Terminal galactose	100 μg/ml
DBA (*Dolichos biflorus*)	Terminal *N*-acetylgalactosamine	100 μg/ml
UEA I (*Ulex europaeus*)	Fucose	100 μg/ml
WGA (*Triticum vulgaris*)	Sialic acid	100 μg/ml
MAA (*Maackia amurensis*)	Sialic acid	100 μg/ml
LPA (*Limulus polyphemus*)	Sialic acid	100 μg/ml
SNA (*Sambucus nigra*)	Sialic acid	100 μg/ml
Neuraminidase	Cleaves terminal sialic acid residues	0.06 U/ml
Hyaluronidase V	Cleaves β-*N*-acetylhexosamine-[1→4] glycosidic bonds	160 U/ml
Galactose	Monosaccharide	100 mM
Galactosamine	Substituted monosaccharide	100 mM
Glucose	Monosaccharide	100 mM
Glucosamine	Substituted monosaccharide	100 mM
Lactose	Disaccharide	100 mM
Methyl-α-d-glucoside	Substituted monosaccharide	100 mM
Methyl-β-d-galactoside	Substituted monosaccharide	100 mM
Chitin	Polysaccharide (polymer of *N*-acetyl-D-glucosamine)	4 mg/ml
Fetuin	Glycoprotein	0.5 mM
Thyroglobulin	Glycoprotein	15 mM
Glycoamidase F	Releases N-linked oligosaccharides from glycoproteins	5 U/ml
Lysozyme	Cleaves *N*-acetylglucosamine-*N*-acetylmuramic acid linkages in polysaccharides	25,000 U/ml
Mixed gangliosides	From bovine brain	1 mM
Trypsin	Serine protease	0.005 mg/ml
Pepsin	Aspartic protease	400 U/ml
Proteinase K	Serine protease	400 U/ml
Pronase E	Protease mixture including a serine protease	0.046 U/ml
Thermolysin	Metallopeptidase	0.43 U/ml
Subtilisin A	Serine protease	0.78 U/ml

treatment, and analyzed by FACS. BFT is known to be trypsin resistant (30). An interval of 1 hour was selected because prior studies indicated that BFT exhibits unequivocal HT29/C1 biological activity by this time point (31). These data revealed that 78% ± 12% of HT29/C1 cells bound fluorescently labeled BFT and that BFT binding was >98% blocked when cells were preincubated with a 100-fold excess of unlabeled BFT for 30 min (Fig. 1A and B), consistent with a specific ligand-receptor interaction. BFT binding was not blocked by preincubation with media containing BSA (2 mg/ml) (data not shown). By confocal microscopy, Alexa 488-labeled-BFT binding to HT29/C1 cells was detected diffusely on the cell membrane of subconfluent HT29/C1 cells (Fig. 1B). As a control, the binding of Alexa 488-labeled BSA to HT29/C1 cells was examined; no specific binding was detected (Fig. 1B and data not shown).

The time course of specific BFT binding to HT29/C1 cells was analyzed using ^{125}I-labeled BFT (1 nM) in the presence or absence of a 100-fold excess of unlabeled BFT (Fig. 1C, left panel). The onset of specific binding was detected by 10 min, with saturation of binding being detected between 30 and 60 min. To characterize the concentration dependency of BFT binding, subconfluent HT29/C1 cells were incubated with differing concentrations of ^{125}I-BFT (0.25 nM to 4 nM) (Fig. 1C, right panel) in the presence or absence of a 100-fold excess of unlabeled BFT for 40 min to define specific binding. Saturation of ^{125}I-BFT binding occurred at 3 nM BFT. Examination of Alexa 488-labeled-BFT binding (6 nM) by confocal microscopy confirmed the onset of membrane-associated BFT binding at 15 min and a higher level of binding at 30 min that remained stable up to approximately 3 h. After 3 h, a speckled intracellular pattern of fluorescence was detected, suggesting internalization of Alexa 488-labeled BFT. By 24 h, only modest membrane and intracellular fluorescence remained, suggesting that the Alexa 488-labeled BFT was degraded or shed by HT29/C1 cells over time (data not shown).

BFT receptor binding was assessed at 4°C to determine the proportion of binding that is localized to the cell membrane versus that secondary to potential internalization of the receptor by cells at 37°C. Unexpectedly, there was no specific binding of either Alexa 488-labeled BFT or ^{125}I-BFT to HT29/C1 cells at 4°C (data not shown), suggesting that cellular activities (e.g., enzyme activities, membrane fluidity) and/or BFT-dependent proteolysis may contribute to the interaction of BFT with its cellular receptor. Similarly, experiments to evaluate specific ^{125}I-BFT binding to isolated HT29/C1 cell membranes or lysates did not demonstrate specific BFT binding at 37°C or 4°C (data not shown), further supporting the hypothesis that an active cell process and/or BFT metalloprotease activity is required for BFT binding.

Another approach to examine both the reversibility and cell surface association of a receptor-ligand interaction is to determine whether the ligand separates from its receptor with gentle acid washes. HT29/C1 cells were incubated with iodinated BFT for 20 to 30 min or with Alexa 488-labeled BFT for 40 min at 37°C prior to acid washing. Acid washing did not remove either iodinated BFT or Alexa 488-labeled BFT from the surface of HT29/C1 cells (data not shown), suggesting that the interaction of BFT with the cell surface does not involve a readily reversible ligand-receptor interaction. Similarly, cell-bound ^{125}I-labeled BFT at saturation was not displaced by up to a 400-fold excess of unlabeled BFT (data not shown). These

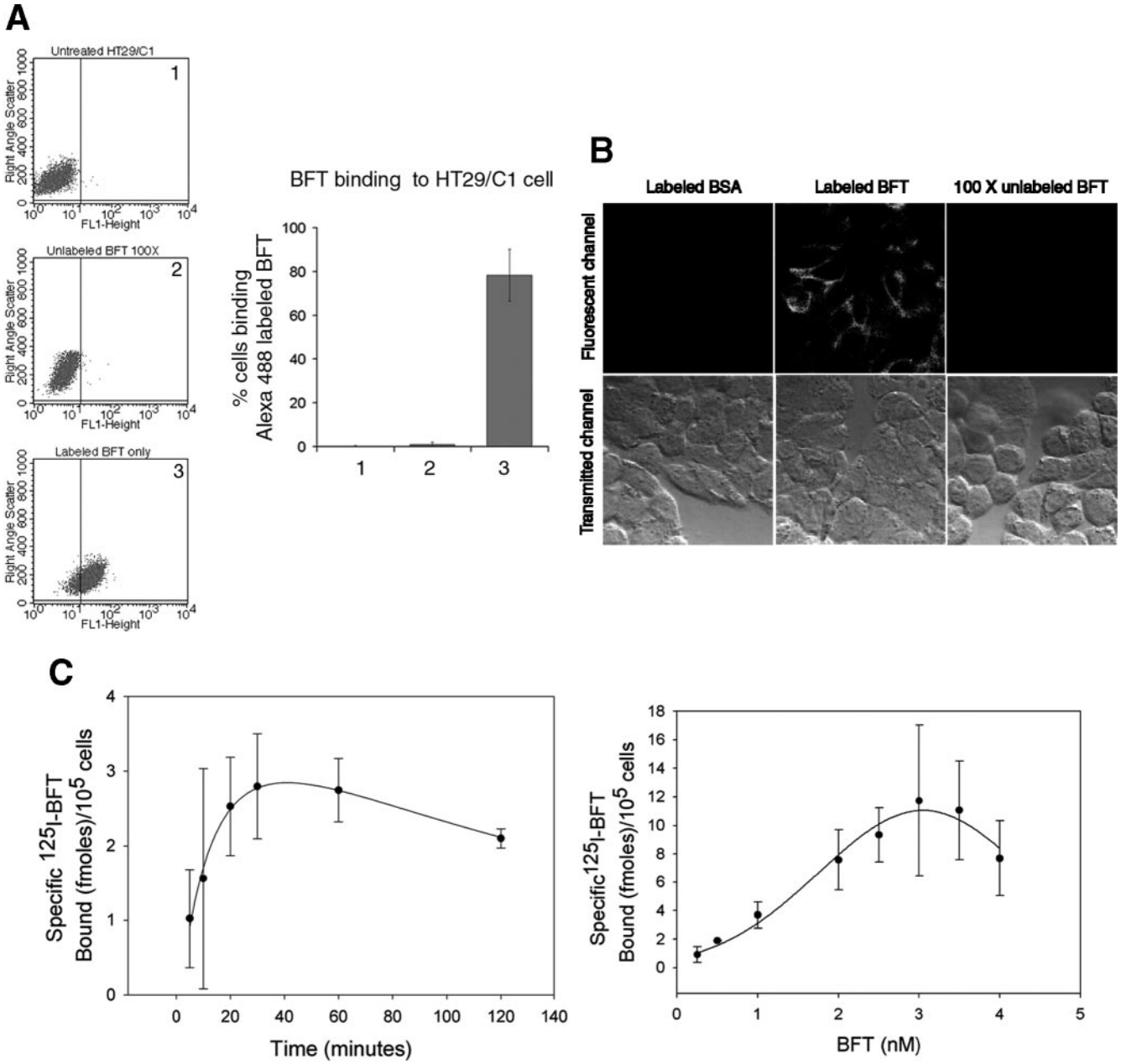

FIG. 1. BFT binds specifically to a HT29/C1 cell membrane-associated IEC receptor. (A) Subconfluent HT29/C1 cells incubated with Alexa 488-labeled BFT (6 nM) for 1 h at 37°C in the presence or absence of a 100-fold excess of unlabeled BFT and analyzed by FACS as described in Materials and Methods. The left panels demonstrate the shift in the fluorescence spectrum of HT29/C1 cells incubated with Alexa 488-labeled BFT (panel 3 versus control cells in panel 1) that is completely inhibited by excess unlabeled BFT (panel 2). The right panel shows the aggregate FACS data from five experiments. (B) Phase-contrast microscopy and fluorescent confocal microscopy demonstrate localization of Alexa 488-labeled BFT binding to the HT29/C1 cell membrane, which is abrogated by a 100-fold excess of unlabeled BFT. As a control, Alexa 488-labeled bovine serum albumen was tested and found not to bind to HT29/C1 cells. (C) Specific binding of ^{125}I-labeled BFT to subconfluent HT29/C1 cells. The left panel demonstrates time-dependent specific ^{125}I-labeled-BFT (1 nM) binding and the right panel demonstrates concentration-dependent specific ^{125}I-labeled-BFT binding to HT29/C1 cells. Significant specific BFT binding to HT29/C1 cells is detected at 0.25 nM BFT ($P < 0.04$) and saturates at ~3 nM BFT.

results prohibited calculation of the binding constants by Scatchard analysis.

BFT metalloprotease activity is required for HT29/C1 cell binding. To initially address the hypothesis that BFT biological activity is required for specific BFT binding, the effect of GM6001 (5 μM), a general metalloprotease inhibitor (12), on BFT binding was evaluated (Fig. 2). When HT29/C1 cells were treated with GM6001 for 30 min and washed prior to incubation with Alexa 488-labeled BFT (6 to 10 nM), the level of BFT binding was similar to that observed with HT29/C1 cells incubated only with Alexa 488-labeled BFT (the *P* value was not significant). In contrast, preincubation of Alexa 488-labeled

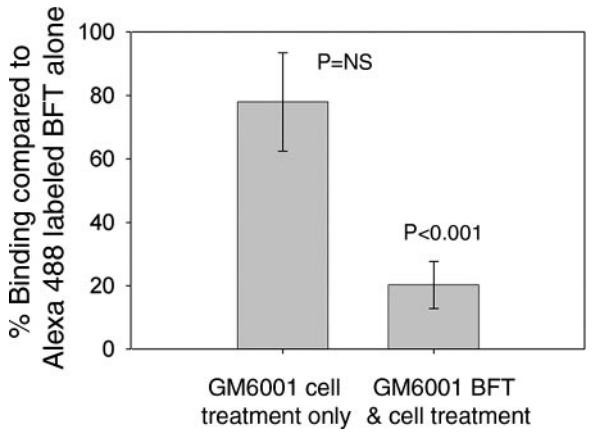

FIG. 2. BFT metalloprotease activity is necessary for binding to HT29/C1 cells. Preincubation of Alexa 488-labeled BFT (6 to 10 nM) in cell culture medium with the general metalloprotease inhibitor GM6001 (5 μM) for 30 min prior to addition to HT29/C1 cells significantly inhibits BFT binding compared to Alexa 488-labeled BFT alone (6 to 10 nM, 1 h, 37°C) ($P < 0.001$). In contrast, incubation of HT29/C1 cells for 30 min with GM6001 (5 μM) with washing prior to incubation with Alexa 488-labeled BFT as described above does not inhibit BFT binding compared to Alexa 488-labeled BFT alone (P was not significant [NS]). Four experiments were performed.

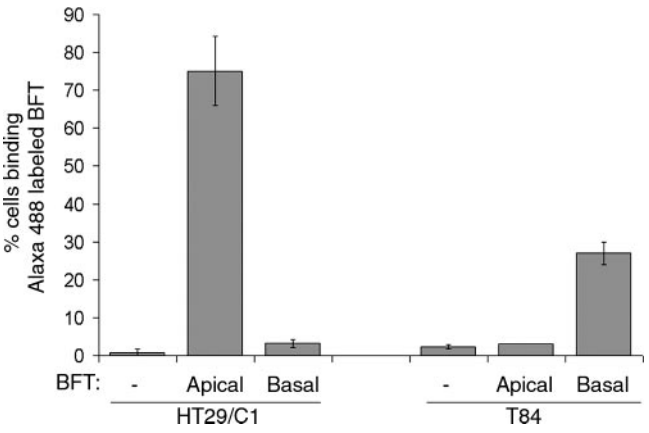

FIG. 3. The IEC receptor for BFT is differentially localized to the apical and basolateral membranes in HT29/C1 and T84 monolayers, respectively. The apical and basolateral membranes of HT29/C1 and T84 monolayers were incubated with Alexa 488-labeled BFT for 1 h prior to washing, trypsinization, and analysis by FACS. Three experiments were performed.

BFT with GM6001 for 30 min prior to the addition to HT29/C1 cells significantly reduced Alexa 488-labeled-BFT binding compared to that seen with HT29/C1 cells incubated only with Alexa 488-labeled BFT ($P < 0.001$). These data support the hypothesis that BFT-dependent proteolysis is required for binding of the toxin to HT29/C1 cells but, because GM6001 is a reversible metalloprotease inhibitor, do not rule out the involvement of a host cell protease in the initial interaction of BFT with HT29/C1 cells.

To further test the requirement of BFT biological activity for BFT binding, we utilized recombinant BFT mutants with either a single-amino-acid mutation in the extended BFT metalloprotease motif ($H_{348}EXXHXXGXXHX_7M$) or a 4-amino-acid deletion in the C terminus of BFT. We have previously reported that single-amino-acid mutations in the extended BFT metalloprotease motif result in diminished (BFT-G355R) or absent (BFT-H348D, BFT-E349A, BFT-H352Y, BFT-H358Y, BFT-M366R) BFT biological activity, as assessed by E-cadherin cleavage or stimulation of IL-8 secretion by HT29/C1 cells (7). We reasoned that if the protease domain of BFT is required for BFT binding, then an inactive catalytic domain mutant of BFT would not bind to the BFT receptor and hence would not inhibit wild-type BFT binding. We tested this hypothesis by using mutant BFT-H352Y, since its secondary protein structure is predicted to be identical to that of wild-type BFT (7). Consistent with our hypothesis, preincubation of HT29/C1 cells with a 100-fold excess of purified mutant BFT-H352Y did not block Alexa 488-labeled-BFT binding to HT29/C1 cells, whereas a 100-fold excess of wild-type BFT totally abolished Alexa 488-labeled-BFT binding analyzed by FACS (2.9% BFT binding in the presence of a 100-fold excess of wild-type unlabeled BFT versus 94.4% in the presence of a 100-fold excess of mutant BFT-H352Y; BFT binding in the positive control was 93.6%; $n = 2$). In contrast, the purified C-terminal truncation mutant of BFT with reduced BFT biological activity (BFT-C4) (1-hour endpoint HT29/C1 cell titer, 9.4 ng/ml for wild-type BFT versus 30 ng/ml for the BFT-C4 mutant) demonstrated a significant decrease in HT29/C1 cell binding (65% ± 11% cells bound wild-type BFT versus 31% ± 18% binding to the BFT-C4 mutant; $P < 0.01$; $n = 4$).

The BFT receptor is differentially associated with the apical and basolateral membranes of polarized HT29/C1 and T84 cell monolayers. We and others have previously demonstrated that BFT is more potent and modifies IEC monolayer function more rapidly (e.g., cell morphology changes, E-cadherin cleavage, barrier function and IL-8 secretion) when applied to the basolateral surfaces of IEC (especially T84 cell) monolayers (3, 16; S. Wu and C. L. Sears, unpublished data). Thus, we hypothesized that the BFT receptor is located predominantly on the basolateral membrane of polarized epithelial cell monolayers. To test this hypothesis, the binding of Alexa 488-labeled BFT applied to the apical and basolateral membranes of HT29/C1 and T84 monolayers, respectively, was assessed by FACS analysis. As predicted, Alexa 488-labeled BFT bound predominantly to the basolateral membranes of T84 monolayers. Unexpectedly, however, Alexa 488-labeled BFT bound predominantly to the apical membrane of HT29/C1 monolayers (Fig. 3). By confocal microscopy, Alexa 488-labeled BFT was also visualized differentially on the apical and basolateral membranes of HT29/C1 and T84 monolayers, respectively (data not shown). These results correlate with the known enhanced basolateral biological activity of BFT on T84 cell monolayers, whereas distinct differences in apical and basolateral BFT activity levels on HT29/C1 cell monolayers are harder to detect due to the high permeability and BFT sensitivity of these monolayers (3).

The BFT receptor is predicted to be a membrane protein other than E-cadherin or a presently known PAR. To investigate the molecular characteristics of the BFT receptor, we assessed the abilities of a panel of lectins (carbohydrate-containing proteins), sugars, and enzymes, including proteases, to inhibit the biological activity of BFT on HT29/C1 cells (Table 1) (10, 19). Except for wheat germ agglutinin, none of the

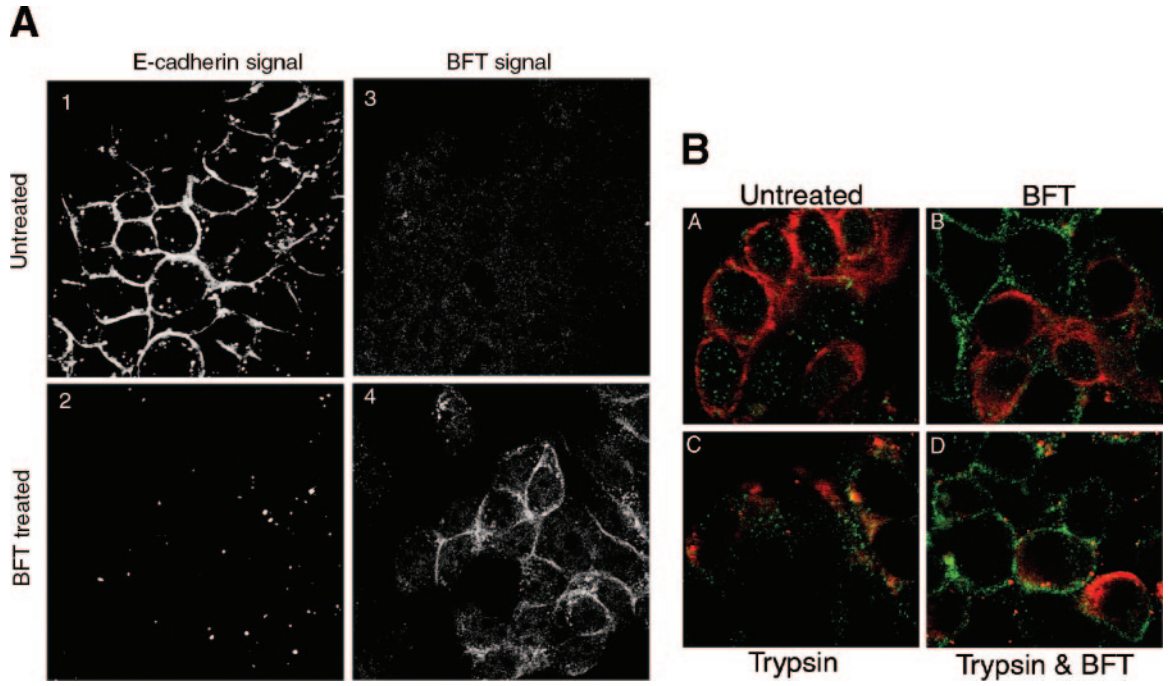

FIG. 4. The HT29/C1 cell receptor for BFT is a membrane protein other than E-cadherin. (A) Confocal microscopic examination of control subconfluent HT29/C1 cells stained with an E-cadherin antibody (C36) demonstrates diffuse membrane staining (panel 1). HT29/C1 cells treated with Alexa 488-labeled BFT (6 nM) for 1 hour at 37°C demonstrate the near absence of E-cadherin staining (panel 2) on cells binding Alexa 488-labeled BFT (panel 4). Panel 3 demonstrates low background fluorescence in untreated control cells in the channel detecting Alexa 488-labeled BFT. (B) PAR2 does not colocalize with Alexa 488-labeled-BFT cell binding. Panel A shows the distribution of PAR2 (red) in control HT29/C1 cells. Panel B shows no colocalization of PAR2 and Alexa 488-labeled-BFT (green) binding to HT29/C1 cells (10 nM, 1 h, 37°C). Panel C shows downregulation of PAR2 immunostaining by treatment of HT29/C1 cells with trypsin (10 nM, 15 min, 37°C). Panel D shows that Alexa 488-labeled-BFT staining remains intact when PAR2 is downregulated by trypsin treatment.

tested lectins, sugars, or mixed bovine brain gangliosides inhibited the HT29/C1 cell morphology changes induced by BFT (data not shown). However, wheat germ agglutinin did not block Alexa 488-labeled-BFT binding to HT29/C1 cells, as assessed by FACS or confocal microscopy analyses (data not shown). In contrast, of proteases tested, pretreatment of HT29/C1 cells with thermolysin (a metalloendopeptidase) or subtilisin A (a serine endopeptidase) followed by washes inhibited both BFT biological activity on HT29/C1 cells and nearly 90% of binding of Alexa 488-labeled BFT (Table 1 and data not shown). None of the tested proteases modified HT29/C1 cell morphology at the tested concentrations. Together, these results suggest that BFT binds to a HT29/C1 membrane protein but does not require sugar or lipid moieties for binding.

We have previously reported that BFT rapidly cleaves E-cadherin, with the initial cleavage site predicted to be in the E-cadherin extracellular domain near the cell membrane (31; Wu and Sears, submitted for publication). Thus, we hypothesized that BFT binds directly to the extracellular domain of E-cadherin. To test this hypothesis, E-cadherin staining was evaluated by confocal microscopy for control HT29/C1 cells and HT29/C1 cells treated with Alexa 488-labeled BFT for 3 h. Figure 4A, panel 2, demonstrates a marked decrease in membrane E-cadherin staining in BFT-treated HT29/C1 cells (6 nM, 3 h) compared to untreated control HT29/C1 cells (panel 1). Alexa 488-labeled-BFT membrane staining remains intact (panel 4) despite the loss of membrane E-cadherin (panel 2). These data suggest that E-cadherin is not the cellular receptor for BFT. To further confirm this observation, levels of specific BFT binding were compared using SW480-EC cells, a human colonic carcinoma cell line overexpressing E-cadherin (see Materials and Methods), and its parent cell line, SW480-mock. We reasoned that if E-cadherin is directly involved in BFT binding, SW480-EC cells would exhibit enhanced specific BFT binding. However, SW480-EC cells did not demonstrate an increase in specific Alexa 488-labeled-BFT binding but rather 42 to 58% less binding than the parent SW480 cell line (two experiments). Additionally, Hep2 cells and primary rat neuronal cells, both cell types that express E-cadherin, were tested for BFT binding and E-cadherin cleavage. Hep2 cells did not bind BFT or exhibit morphological changes stimulated by BFT, and E-cadherin cleavage was not observed by Western blot analysis (data not shown). Similarly, no specific binding of Alexa 488-labeled BFT to primary rat neuronal cells was detected by confocal microscopy (data not shown). In addition, Alexa 488-labeled BFT did not bind to the purified extracellular domain of E-cadherin (Cad-Fc) under cell-free conditions, nor did excess purified Cad-Fc inhibit binding of labeled wild-type BFT. These data indicate that neither human nor rat E-cadherin is likely to be the receptor for BFT.

PARs are transmembrane protein receptors activated physiologically by thrombin (PAR1, PAR3, and PAR4) or by multiple trypsin-like enzymes (PAR2) (29). In particular, PAR-2

activation stimulates HT29/C1 cell proliferation similarly to BFT (5, 17). Three approaches were used to test the hypothesis that PARs may mediate BFT biological activity and/or serve as the cellular receptor for BFT. First, HT29/C1 cells were treated with PAR1, PAR2, or PAR4 agonists, thrombin, or BFT (10 nM) and evaluated by light microscopy at intervals from 30 min to 24 h. In contrast to BFT, which induced rapid onset and persistent cell morphology changes, PAR agonists did not alter HT29/C1 cell morphology (data not shown; three experiments). Second, when HT29/C1 cells were treated with PAR agonists for 30 min followed by treatment with BFT (10 nM), no change in the HT29/C1 morphological response to BFT was observed, suggesting that PAR activation did not inhibit or prevent BFT biological activity. Third, reported data indicate that PAR2 activation on HT29/C1 cells induces rapid (within 15 min) internalization of PAR2 with a loss of cell membrane staining for PAR2 (5). Thus, HT29/C1 cells were treated with trypsin (10 nM) for 15 min, and binding of Alexa 488-labeled BFT (60 min, 10 nM) was subsequently assessed. This concentration of trypsin does not alter HT29/C1 cell morphology. Alexa 488-labeled BFT bound similarly to control and trypsin-treated HT29/C1 cells (Fig. 4B, compare panels B and D). However, trypsin diminished cell surface PAR2 immunostaining compared to what was seen for control cells (Fig. 4B, compare panel A to C or D). Together, these data suggest that PARs do not mediate the biological activity of BFT and are not the cell surface receptor for BFT.

BFT receptor binding is sensitive to membrane cholesterol depletion but is unlikely to be contained in a lipid raft. Treatment of HT29/C1 cells with BFT activates multiple cellular signaling pathways (26). Recent data suggest that membrane microdomains, commonly known as lipid rafts, that are rich in cholesterol or sphingolipids localize certain signal transduction receptors (4). Although definitions of membrane microdomains vary, lipid rafts typically are defined as membrane complexes that are insoluble in nonionic detergents such as Triton X-100 (4). To assess whether the BFT receptor may be associated with a cellular lipid raft, HT29/C1 cells were incubated with Alexa 488-labeled BFT after membrane cholesterol was depleted by treatment with mβCD (4 mM) and analyzed by FACS. BFT binding was nearly abrogated by mβCD treatment of HT29/C1 cells (Fig. 5). Inhibition of Alexa 488-labeled-BFT binding was reversed by repletion of HT29/C1 membrane cholesterol (Fig. 5). Confocal microscopy confirmed the absence of binding of Alexa 488-labeled BFT to cholesterol-depleted HT29/C1 cells (data not shown). To further assess whether the BFT receptor may be contained in a lipid raft, the ability of either cholesterol oxidase (16 U/ml) or filipin (which binds to and sequesters membrane cholesterol; 2.5 μg/ml) to inhibit BFT binding was tested. Neither reagent inhibited BFT binding (data not shown). Assessment of BFT binding in Triton X-100-soluble or -insoluble cell fractions identified BFT binding in both cell fractions, but the association of BFT was observed predominantly in the Triton X-100-soluble cell fraction (data not shown).

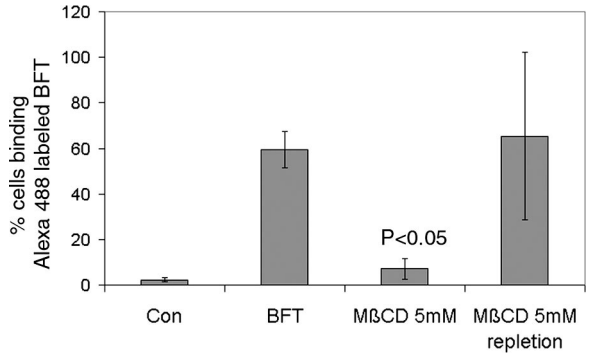

FIG. 5. Depletion of membrane cholesterol with mβCD (4 mM) for 30 min at 37°C nearly ablates binding of Alexa 488-labeled BFT (10 nM). Repletion of membrane cholesterol (see Materials and Methods) in cells treated with mβCD restores binding of Alexa 488-labeled BFT. Three experiments were performed.

DISCUSSION

These data indicate that BFT binds specifically to a receptor present on polarized IECs, a result consistent with prior observations indicating that the biological activities of BFT are restricted to epithelial cell lines that polarize in vitro (26). HT29/C1 and T84 cells represent more-differentiated IECs and crypt cells, respectively (1, 9). The predominant localizations of specific BFT binding to the apical membranes in polarized HT29/C1 cells and to basolateral membranes in polarized T84 cells suggest the hypothesis that the BFT receptor is a membrane protein that either migrates or is redirected from the basolateral to the apical membrane as IECs differentiate along the crypt-villus axis. Localization of the BFT receptor to the apical membrane of the HT29/C1 cells is consistent with a putative role of the receptor in the pathogenesis of ETBF disease. It remains possible, however, that basolateral BFT receptors may also contribute to disease pathogenesis, given that BFT markedly increases IEC monolayer and human colon permeability in vitro, potentially allowing access of BFT to receptors localized to the basolateral membranes of crypt cells (3, 16, 22). Identification of specific BFT binding in vivo to IECs with detailed time course studies will be necessary to define precisely the tissue distribution of the BFT receptor and its relationship to the onset of BFT-induced pathophysiology.

Genetic and biochemical analyses indicate that BFT is a zinc-dependent metalloprotease toxin similar to, for example, tetanus, botulinum, and anthrax toxins, for which distinct receptor and catalytic protein domains are critical to their molecular mechanisms of action (7, 13, 25, 28). Unexpectedly, specific binding of BFT could not be detected at 4°C, exposure of BFT to a metalloprotease inhibitor significantly reduced specific BFT binding, a catalytically inactive mutant of BFT did not inhibit binding of wild-type BFT, and a BFT mutant with reduced biological activity also exhibited reduced HT29/C1 cell binding. Together, these data suggest that the protease activity of BFT is necessary for specific receptor binding, although the data do not eliminate the possibility that temperature-dependent host cell processes or metalloprotease(s) acts cooperatively in facilitating BFT binding and bioactivity. Studies of the biological activity of BFT indicate that the toxin acts swiftly and irreversibly early on to modify host cell function, with initial cleavage of E-cadherin being detectable after only 1 min and the onset of both HT29/C1 cellular morphological changes and increases in T84 monolayer permeability being identified

within 15 min (3, 23, 31). Further, BFT acts within a narrow concentration range, with the threshold and maximum concentrations of BFT that yield HT29/C1 cell bioactivity at 3 h being 0.2 nM and ~2.5 nM, respectively (23). The rapid, irreversible onset of action with a near "on-off" concentration dependency that is very similar to the concentration dependency of BFT binding (Fig. 1C) is consistent with a critical role for the enzymatic activity of BFT in its initial interaction with epithelial cells. We attempted to further define the characteristics (number of cell receptors and binding affinity) of the BFT receptor via Scatchard analysis; however, Scatchard analysis is dependent on characterization of binding under fixed experimental conditions. Because BFT did not exhibit specific binding at 4°C and the action of BFT on HT29/C1 cells is dynamic, with rapid, ongoing changes in cellular morphology and stimulation of cell surface protein shedding (Wu and Sears, unpublished data), Scatchard analysis was not experimentally feasible.

Our data suggest that the BFT receptor is a membrane protein sensitive to depletion of membrane cholesterol but that the receptor is unlikely to be contained in a putative lipid raft structure, given that BFT binding is predominantly in the TX-100-soluble cell fraction but that localization to the TX-100-insoluble cell fraction is predicted for lipid raft-associated receptors (4). Our data do not provide support for the involvement of sugar or lipid residues in the interaction of BFT with this membrane protein. Previously, we postulated that BFT directly cleaves E-cadherin (31), and the data herein do not eliminate this possibility. However, our data do indicate that neither E-cadherin nor known PARs (PAR1 to -4) are likely to be the membrane receptor for BFT.

Based on the available data, three potential models of BFT receptor binding and onset of action can be considered. First, based on the data in this report and our prior data demonstrating that BFT rapidly stimulates several cellular signal transduction mechanisms, including some host cell signaling that occurs prior to the initiation of E-cadherin cleavage (26, 33; Wu and Sears, unpublished data), we favor a model in which the catalytic domain of BFT is required for processing of and binding to its receptor. We hypothesize that receptor processing by BFT activates a cellular signal transduction cascade in which a host enzyme, possibly a metalloprotease, cleaves E-cadherin. This model accommodates the possibility that BFT can bind and act at some distance from E-cadherin and also allows for signal transduction amplification from BFT receptor binding. Second, BFT may directly cleave E-cadherin, and BFT catalytic activity may also be necessary for binding to its receptor. This model suggests complex protein interactions in which BFT rapidly and sequentially processes two IEC membrane proteins. In this model, the BFT receptor would be predicted to be in close proximity to E-cadherin. However, our data show the BFT receptor to be located diffusely over the apical and basolateral membranes of HT29/C1 and T84 monolayers rather than focally at the plane of E-cadherin expression in these cell lines. A third potential model predicts biologically active and inactive receptors for BFT, with active receptors being located adjacent to E-cadherin and other distant membrane receptors that lack the capability of activating signal transduction serving to bind and neutralize BFT biological activity. Additional experimentation to identify and determine if one or more proteins serve alone or cooperatively as the BFT receptor(s) will be necessary to test these models of BFT action. Identification of the BFT receptor, predicted to be a novel protease-activated receptor, is expected to contribute to our fundamental understanding of the biology and physiology of IECs and to provide insight into new mechanisms contributing to diarrheal disease as well as IEC proliferation and inflammation.

ACKNOWLEDGMENTS

We thank Dwight Derr for assistance with tissue culturing.
This work was supported by grant RO1 DK 45496 (to C.L.S.).

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Editor: J. T. Barbieri