Intestinal trefoil factor binds to intestinal epithelial cells and induces nitric oxide production: priming and enhancing effects of mucin

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Intestinal trefoil factor (ITF or TFF3), NO and epithelium-associated mucin have important roles in sustaining mucosal integrity in the gastrointestinal tract. In the present study we examined ITF-binding molecules on IEC-18 cells (an intestinal epithelial cell line) with the use of flow cytometry and localized these molecules on the cell surface by confocal microscopy. Furthermore, we studied the interaction of mucin and ITF and their co-operative effect on NO production by the epithelium. Stimulation of cells with mucin (5 mg/ml) for 90 min resulted in a 5-fold increase in ITF binding. Treatment of IEC-18 cells with actinomycin D or cycloheximide attenuated mucin-enhanced ITF binding. Ligand blot analysis confirmed the induction of ITF-binding protein in IEC-18 cells by mucin. These results indicate that transcriptional and translational mechanisms are involved in the effect of mucin. Treatment with ITF overnight resulted in a low level of nitrite production by the cells, a 5-fold increase over control, in a concentration-dependent manner. ITF-induced NO production was attenuated by 1400W, a selective type II nitric oxide synthase (NOS2) inhibitor. By immunoblotting we found that NOS2 was up-regulated by ITF treatment. Priming IEC-18 cells with mucin for 90 min enhanced the effect of ITF on NO production, suggesting that the up-regulation of ITF-binding molecules by mucin might be physiologically relevant. Taken together, these observations indicate (1) that ITF-binding molecules that are up-regulated by mucin exist on the intestinal epithelial surface, and (2) that ITF modulates epithelial NO production via the NOS2 pathway, which is enhanced by mucin.

Key words: confocal microscopy, epithelial restitution, flow cytometry, intestinal-trefoil-factor-binding protein, nitric oxide synthase 2.

INTRODUCTION

The trefoil factor (TFF) family is a group of extracellular peptides highly expressed in the gastrointestinal tract. Their physiological role is not well understood [1]. These peptides contain distinctive cystine-rich 'trefoil' domains and are resistant to proteolytic degradation [1,2]. Three trefoil peptides have been identified in the gastrointestinal tract: pS2 (TFF1), expressed mainly in the stomach [3,4]; SP (TFF2), in the pancreas and stomach [5]; and intestinal TFF (ITF or TFF3) in the small and large intestine [6,7]. ITF, the most recently described member of the trefoil peptide family, is expressed in the intestine of humans [8], rats [6,7] and adult mice, but not in embryos or newborns [9]. It is secreted on the luminal mucosal surface by goblet cells [6,7]. Mature ITF is a peptide containing 59 residues [6]. Previous investigations indicate that: (1) the expression of trefoil peptides increases in the gastrointestinal tract in several pathological conditions of mucosal injury such as inflammatory bowel disease (IBD) and gastric and duodenal ulcers [10,11]; (2) ITF promotes mucosal epithelial restitution (a process involved in resealing superficial wounds of the mucosa) after injury [12]; (3) ITF knockout mice have impaired mucosal healing after the oral administration of dextran sulphate sodium [13]; (4) oral ITF protects against ethanol- and indomethacin-induced gastric injury [14]; and (5) mice overexpressing TFF are resistant to indomethacin-induced intestinal injury [15]. Taken together, these observations suggest that ITF has an important role in both sustaining mucosal integrity and promoting epithelial wound healing in the gastrointestinal tract.

Although ITF has been cloned, very little is known about the molecular mechanism of its action [1,16]. A previous investigation has shown that ITF promotes intestinal epithelial migration via a transforming growth factor β-independent pathway [17]. Recent studies have suggested that ITF also modulates the function of the E-cadherin/catenin complex (cell–cell adhesion molecules in the epithelium) and regulates the phosphorylation of extracellular signal-related protein kinase [2,18]. It is still unknown whether ITF also regulates the production of mediators involved in maintaining mucosal integrity, such as nitric oxide (NO) or cytokines [19–21].

Previous studies showed that: (1) specific ITF-binding sites exist in the gastric epithelium, crypt cells of small intestine and colon [22–24]; (2) ITF-binding protein (ITF-BP) is a 50 kDa glycosylated intestinal membrane protein [22]; (3) stimulation by ITF for 24 h (at concentrations as low as 1 nM) leads to cell detachment and to a decreased intracellular adhesion in human carcinoma cells [25]; (4) ITF (100 nM) leads to the inhibition of extracellular signal-related protein kinase and the mitogen-activated protein kinase pathways in IEC-6 cells through the activation of tyrosine or dual-specific phosphatase [2]; (5) ITF (10 nM) modulates EGF effects on intestinal epithelial ion transport [26]; and (6) ITF effect is mediated by signal transduction pathways linked to the protein tyrosine kinase/tyrosine phosphatase pathway [2,18,25]. These observations strongly suggest that a receptor-like membrane protein might mediate the action of ITF. TFFs are epithelial mucin-associated molecules [27]. Mucin glycoprotein has been shown to act in conjunction with ITF to promote wound healing in the mucosa [12,17].

However, it is not clear whether mucin regulates the interaction between ITF and intestinal epithelium.

In the present study we tested the hypotheses that: (1) ITF modulates the generation of mediators such as NO by intestinal epithelial cells; and (2) ITF-induced production of NO by the epithelium is enhanced by mucin. Furthermore, we localized ITF-binding molecules on the epithelial cell surface, examined whether mucin regulates ITF-binding molecules on the epithelial cell surface and studied the mechanism of ITF effect on epithelial NO production.

MATERIALS AND METHODS

Materials

IEC-18 cells, a non-transformed rat intestinal crypt cell line, were obtained from the American Type Tissue Culture Collection (Rockville, MD, U.S.A.). All cell culture media were purchased from Gibco/BRL (Grand Island, NY, U.S.A.). FITC-conjugated mouse anti-biotin monoclonal antibody was purchased from Accurate Chemical (Westbury, NY, U.S.A.). Phycoerythrin (PE)-conjugated goat anti-biotin polyclonal antibody was obtained from Rockland (Gilbertsville, PA, U.S.A.). Rabbit polyclonal antibody against type II nitric oxide synthase (NOS2) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-(E-cadherin) monoclonal antibody (mAb) was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Enhanced chemical luminescence (ECL) kit was obtained from Amersham (Arlington Heights, IL, U.S.A.). 1400W compound was purchased from Alexis Corporation (San Diego, CA, U.S.A.). 2,3-Diaminonaphthalene was supplied by Aldrich (Milwaukee, WI, U.S.A.). Mucin (from pig stomach), NADPH, nitrate reductase (from *Aspergillus niger*) and all other reagents were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Recombinant rat ITF (rITF), biotinylated ITF fusion protein (bITF) and biotinylated Tag control peptide (bTag) were prepared as described previously [22]. To avoid contamination of endotoxin, the rITF was further purified by chromatography through a detoxin gel (Pierce Chemical Co., Rockford, IL, U.S.A.).

Cell culture

IEC-18 cells were cultured in T75 flasks with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% (w/v) non-essential amino acids, 0.1 i.u./ml insulin, 100 i.u./ml penicillin and $100\ \mu g/ml$ streptomycin unless indicated otherwise. Cells were cultured at 37 °C in a water-saturated air/CO_2 (19:1) atmosphere and used from passages 15 to 20 at 85–100 % confluence.

In some experiments, cells were cultured in 24-well culture plates. After cells become confluent, the culture medium was changed to Phenol Red-free DMEM containing 0.1 % (v/v) fetal bovine serum. Cells were incubated for a further 18 h in DMEM or in DMEM containing rITF (2.5 μM). In addition a group of cells was pretreated with mucin (1 mg/ml in DMEM) for 90 min and then changed to DMEM with or without rITF (2.5 μM) and incubated for 18 h. Cell supernatant was collected and assayed for nitrite content. Cell viability was examined by Trypan Blue exclusion and cell counts were determined with a haemocytometer.

Nitrite determination

The nitrite content of the culture medium was determined by using a fluorimetic assay as described by Misko et al. [28]. This assay was done on 96-well plates. In brief, 100 μl of sample was mixed with 10 μl of freshly prepared diaminonaphthalene (0.05 mg/ml in 0.62 M HCl) and incubated for 10 min at room temperature. The reaction was terminated by adding 5 μl of 2.8 M NaOH to each well. The intensity of fluorescence in each well was measured with a Biolumin 960 system (Molecular Dynamics, CA, U.S.A.) with excitation at 360 ± 10 nm and emission at 405 ± 10 nm. In all assays, nitrite standards were routinely made freshly and the sample buffer (including culture medium) was used for the construction of the standard curve. In some experiments, nitrate was converted to nitrite with nitrate reductase by following the protocol of Misko et al. [28].

Labelling of cells by ITF

IEC-18 cells were detached at 37 °C in an enzyme-free cell-dissociation solution (Specialty Media, Lavallette, NJ, U.S.A.). After being washed with PBS (pH 7.2), 5×10^5 detached cells were incubated on ice in PBS/1 % BSA containing 10 % (v/v) fetal calf serum, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.25 μM bITF for 30 min. bITF-labelled cells were washed with cold PBS, pH 7.2, and incubated with FITC-conjugated mouse anti-biotin mAb or PE-conjugated goat anti-biotin polyclonal antibody for 30 min on ice. Fluorescent ITF-labelled cells were then washed with cold PBS. After measurement of cell viability by staining with Trypan Blue, labelled cells were fixed with PBS-buffered formaldehyde [pH 7.2, 2 % (v/v)] containing 0.5 % BSA for 15 min at 4 °C before being processed for flow cytometric assay. In each labelling, some cells were (1) incubated with bTag (0.25 μM) instead of bITF and then reacted with FITC-conjugated mouse anti-biotin mAb or PE-conjugated goat anti-biotin polyclonal antibody, or (2) incubated directly with the antibody. These samples were used as negative controls.

Flow cytometry

For flow cytometric analysis, 10^4 events were collected on a FACScan cytometer and analysed with CELLQUEST software (Becton Dickinson, Mountain View, CA, U.S.A.). Both fluorescence histograms and contour plots were displayed on a 4-decade logarithmic scale.

Quantification of fluorescence by flow cytometry [29]

After incubation with bITF, cells were reacted with PE-conjugated goat anti-biotin polyclonal antibody and read with a FACScan cytometer (50000 events per sample). By using the calibrated standardized PE-conjugated beads (Becton Dickinson, San José, CA, U.S.A.), a standard curve was established with the method provided by the manufacturer. The fluorescence (PE molecules per cell) of the samples was determined. Because bTag did not bind to IEC-18 cells, the intensity of fluorescence of cells treated with bTag should represent the non-specific binding, whereas the fluorescence intensity of cells treated with bITF represents total binding. The specific binding of each sample was calculated by subtracting the non-specific binding (fluorescence in the presence of bTag peptide) from total ITF binding (fluorescence in the presence of bITF probe).

Confocal fluorescence microscopy

To identify ITF binding to the cell surface, fluorescent ITF-labelled cells (see above) were examined with a confocal microscopic system (Model MRC600; Bio-Rad Laboratories, Hercules, CA, U.S.A.) attached to a microscope (Nikon, Tokyo, Japan) with the use of the filters for FITC (excitation wavelength

488 nm; emission wavelength 515 nm). Images were imported into Adobe Photoshop and processed.

Immunoblot with polyclonal antibody against NOS2 protein

IEC-18 cells were lysed in a buffer containing 2 mM Tris/HCl, pH 7.6, 30 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 1% (v/v) Nonidet P40. After centrifugation of cell lysate at 10000 $\textbf{\textit{g}}$ for 10 min at 4 °C, the supernatant was mixed with an equal volume of 2 × Laemmli buffer and boiled for 5 min. Protein (60 μg) was resolved by SDS/PAGE [10% (w/v) gel] together with molecular mass standards (Amersham). The protein was then transferred to a nitrocellulose membrane (Bio-Rad) by electrophoresis in transfer buffer containing 39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% (v/v) methanol. The membrane containing sample proteins was used for the immunodetection of NOS2 protein. In brief, after blocking of the residual protein sites on the membrane with 5% (v/v) skimmed milk (in PBS) for 60 min at room temperature, the membrane was reacted with 1 μg/ml rabbit anti-NOS2 polyclonal antibody for 2 h at room temperature. After incubation the blot was washed four times with PBS containing 0.05% Tween 20 (PBS-T), then incubated for 1 h at room temperature with the secondary antibody, a peroxidase-conjugated goat anti-(rabbit IgG) antibody (Amersham) diluted 1:1500 in PBS-T. After additional washing with PBS-T, the NOS2 protein on the blot was detected with an ECL system. The membrane was then reprobed with anti-(E-cadherin) antibody after removing the primary and secondary antibodies by following the protocol provided by Amersham.

Ligand blot analysis

Solubilized membrane protein (40 μg) from IEC-18 cells, prepared as described previously [22], was suspended in an equal volume of 2 × Laemmli buffer containing 2% (v/v) 2-mercapto-ethanol, then resolved by SDS/PAGE [10% (w/v) gel] and transferred to a nitrocellulose membrane. After being blocked with PBS containing 5% (v/v) skimmed milk and bacterial lysates (which expressed Tag protein), the membrane was reacted overnight with PBS containing bTag-ITF peptide (3.4 μg/ml) at 4 °C. After several washings with PBST, the blot was incubated for 1 h with HRP-conjugated streptavidin (1:1000 dilution; Amersham Life Science, Arlington Heights, IL, U.S.A.). Signals were detected with the ECL system (Amersham).

Statistical analysis

Data are reported as means $\pm$ S.E.M. Comparisons between multiple groups were made by a one-way analysis of variance followed by Fisher's protected least-significant-difference *post hoc* test. $P < 0.05$ was considered significant.

RESULTS

ITF stimulates IEC-18 cells to generate NO via the NOS2 pathway, which is enhanced by mucin

In preliminary experiments (results not shown) we found that more than 95% of the NO metabolites in IEC-18 cells are nitrite. We therefore measured only nitrite content in this study. As shown in Table 1, unstimulated IEC-18 cells constitutively generated nitrite (0.23 ± 0.02 nmol/ml during an 18 h period). Treating the cells overnight with rITF resulted in an increase in NO production in a concentration-dependent manner, compared with controls ($P < 0.01$). ITF-induced NO production was

Table 1   Effect of a selective NOS2 inhibitor on ITF-induced NO production in IEC-18 cells

After cells had been cultured in various media for 18 h, the nitrite concentration in cultured medium was measured (see the Materials and methods section). Results are means $\pm$ S.E.M. for four to eight different experiments. $^{**}P < 0.01$ compared with the DMEM group.

Treatment	Nitrite concentration (nmol/ml)
DMEM	0.23 ± 0.02
ITF (1 μM, 18 h)	0.30 ± 0.01
ITF (2.5 μM, 18 h)	$0.65 \pm 0.04^{**}$
ITF (6.25 μM, 18 h)	$1.04 \pm 0.08^{**}$
1400W (2 μM)	0.10 ± 0.02
ITF + 1400W[1]	0.18 ± 0.01

[1] IEC-18 cells were cultured in medium containing both ITF (2.5 μM) and 1400W (2 μM) for 18 h.
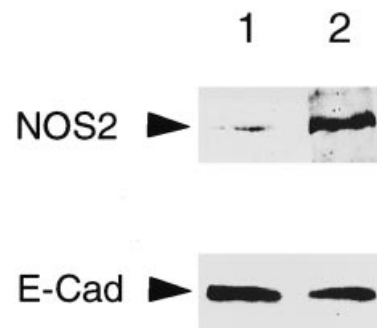

Figure 1   ITF induces NOS2 protein in IEC-18 cells

IEC-18 cells were cultured overnight in DMEM (lane 1) or DMEM containing 2.5 μM ITF (lane 2). Total cellular protein was isolated. Top panel: NOS2 protein was detected by Western blotting with an anti-NOS2 antibody. Bottom panel: the blot was probed with an anti-(E-cadherin) antibody.

Table 2   Effect of mucin on ITF-induced NO production in IEC-18 cells

Cells were cultured in various media for 18 h, after which the nitrite concentration in the cultured medium was measured. Results are means $\pm$ S.E.M., $n = 4$. $^{**}P < 0.01$ compared with the DMEM group.

Treatment	Nitrite concentration (nmol/ml)
DMEM	0.25 ± 0.01
ITF (2.5 μM, 18 h)	$0.58 \pm 0.03^{**}$
Mucin + ITF[1]	$1.06 \pm 0.06^{**}$
Mucin[2]	0.08 ± 0.01

[1] IEC-18 cells were cultured in medium containing 1 mg/ml mucin for 90 min, followed by washing three times with PBS. Cells were then cultured in medium containing ITF (2.5 μM) for 18 h.
[2] IEC-18 cells were cultured in medium containing 1 mg/ml mucin for 90 min, followed by washing three times with PBS. Cells were then cultured in DMEM for 18 h.

attenuated by 1400W (2 μM), a selective inhibitor for NOS2. By immunoblotting, we found that NOS2 protein was constitutively present in IEC-18 cells, and treatment overnight with ITF (2.5 μM) resulted in the induction of NOS2 protein (Figure 1). Table 2 shows that priming IEC-18 cells with mucin (1 mg/ml) for 90 min enhanced the effect of rITF: nitrite generation was
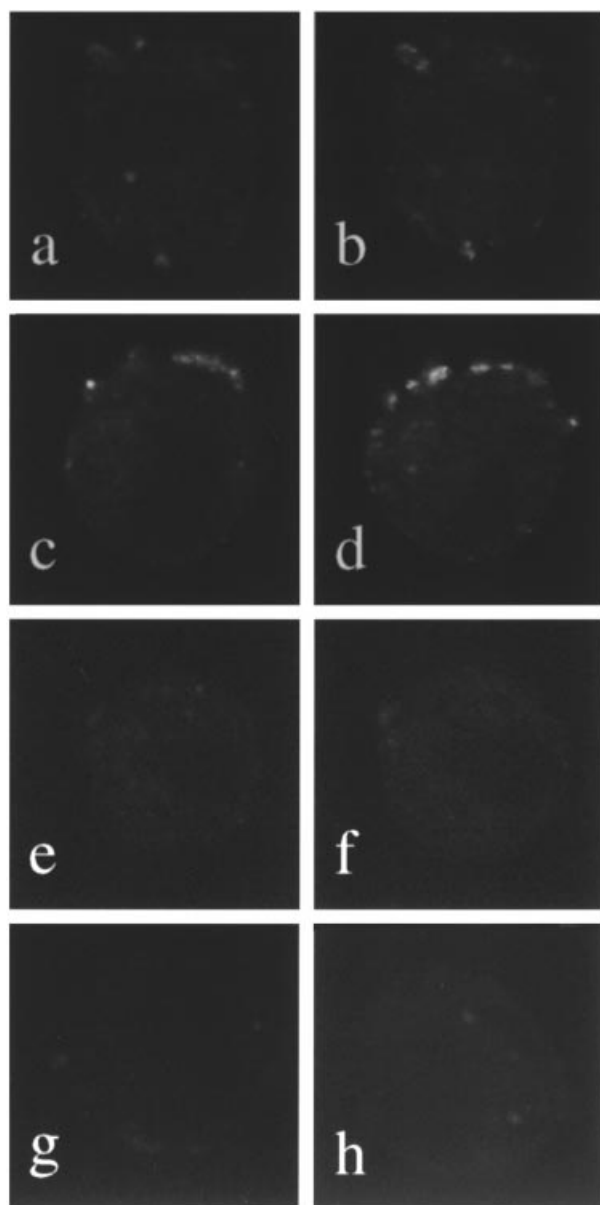
Figure 2   Localization of ITF-binding molecules on an IEC-18 cell surface after stimulation with mucin

IEC-18 cells were cultured in DMEM (**a**, **b**, **e** and **g**) or DMEM containing 5 mg/ml mucin (**c**, **d**, **f** and **h**) at 37 °C for 90 min, detached with enzyme-free cell dissociation solution, incubated at 4 °C for 30 min with 0.25 μM bITF (**a–d**), 0.25 μM of bTag (**e** and **f**), or 0.25 μM bITF together with 25 μM unlabelled ITF (**g** and **h**), and reacted with FITC-conjugated anti-biotin mAb (see the Materials and methods section). The cells were examined with a confocal microscope. Images of (**a**) and (**b**) (the same cell) or (**c**) and (**d**) (the same cell) are scanned through a 2.5 μm plane. Magnification ×1800.

increased to 1.06 ± 0.06 nmol/ml in 18 h. Mucin itself (1 mg/ml, 90 min) paradoxically decreased the production of nitrite to 0.08 ± 0.01 nmol/ml. The cell viability in each treatment group was more than 97 %.

ITF-binding molecules on IEC-18 cell surface are regulated by mucin

The mechanism by which the priming of intestinal epithelial cells by mucin enhances the ITF response is unclear. Confocal
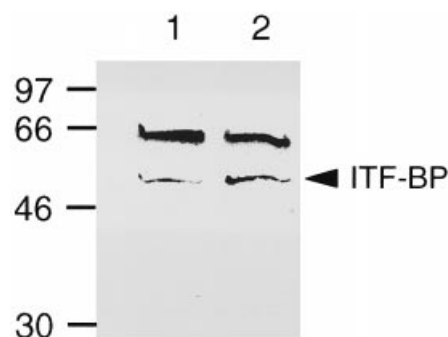

Figure 3   Identification of ITF-BP in IEC-18 cells by ligand blot analysis

Solubilized membrane proteins were resolved by SDS/PAGE [10 % (w/v) gel] and transferred to a nitrocellulose membrane. The blot was probed with bITF and the signal was detected with the ECL system. Lane 1, protein sample isolated from DMEM-treated cells; lane 2, protein sample isolated from cells treated with mucin (5 mg/ml, 90 min). ITF-BP (50 kDa) is marked with an arrow. The positions of molecular mass markers are indicated (in kDa) at the left.
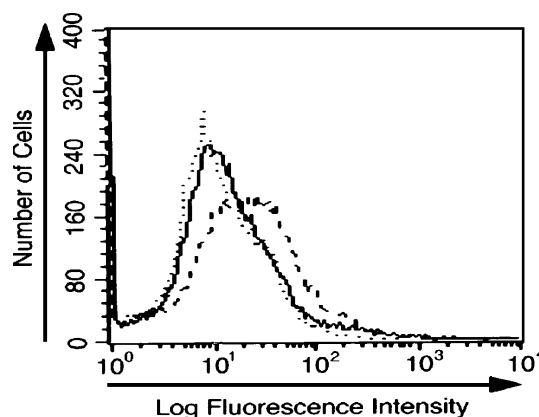

Figure 4   Characterization of ITF-binding molecules on the IEC-18 cell surface by FACScan

Confluent IEC-18 cells were incubated with bITF (0.25 μM; broken line), bTag (0.25 μM; dotted line) or binding buffer (solid line) for 30 min on ice, then reacted with PE-conjugated anti-biotin polyclonal antibody. The fluorescence of the cells was assessed by FACScan as described in the Materials and methods section. Each histogram was based on 50000 events acquired in the assay.

microscopy of bITF-labelled IEC-18 cells (to characterize ITF-binding molecules on the cell surface) showed that unstimulated IEC-18 cells had barely discernible ITF-binding sites on their surface (Figures 2a and 2b). The amount of ITF binding to the cell surface was increased after pretreating IEC-18 cells with mucin (5 mg/ml) for 90 min (Figures 2c and 2d). The negative control probe (bTag) did not bind to either unstimulated or mucin-treated IEC-18 cells (Figures 2e and 2f). In addition we found that an excess of unlabelled ITF (25 μM) competed for the binding of bITF (0.25 μM) to DMEM- and mucin-treated cells (Figures 2g and 2h). Ligand blot analysis confirmed the induction of ITF-BP by treatment with mucin (5 mg/ml for 90 min; Figure 3). These observations indicated that specific ITF-binding molecules are present on the IEC-18 cell surface and are regulated by mucin.

Table 3   Effect of mucin on binding of ITF to IEC-18 cells

Cells were detached with an enzyme-free cell dissociation solution, then processed for the ITF-binding assay (see the Materials and methods section). Results are means ± S.E.M., $n = 4$. Specific binding units were calculated by subtracting non-specific binding from total binding as described in the text. *$P < 0.05$, **$P < 0.01$ compared with the DMEM group.

Treatment	ITF bound (specific binding units)
DMEM	54.75 ± 10.92
Mucin (1 mg/ml, 90 min)	155.00 ± 12.84*
Mucin (5 mg/ml, 90 min)	298.00 ± 19.36**
Mucin + CHX[1]	134.00 ± 0.58
Mucin + AD[2]	119.00 ± 8.21

[1] Cells were treated with CHX (0.1 mM, in DMEM) for 18 h, then stimulated with mucin (5 mg/ml) for 90 min in the presence of CHX (0.1 mM).
[2] Cells were treated with AD (5 μM, in DMEM) for 18 h, then stimulated with mucin (5 mg/ml) for 90 min in the presence of AD (5 μM).

Mucin increases ITF-binding molecules on IEC-18 cell surfaces

Initially we examined whether ITF-binding molecules on IEC-18 cell surfaces could be characterized by flow cytometry with bITF, a biotinylated probe [22]. As shown in Figure 4, the histogram of IEC-18 cells incubated with a bITF probe then reacted with PE-conjugated goat anti-biotin polyclonal antibody (broken line) shifted to the right, as compared to with that of cells incubated with the antibody only (solid line). In contrast, the histogram of IEC-18 cells treated at 4 °C for 30 min with bTag (dotted line) or with unlabelled ITF (25 μM) followed by 0.25 μM of bITF (results not shown), did not display a shift to the right after incubation with anti-biotin antibody (Figure 4). This observation suggests (1) that ITF-binding molecules on the IEC-18 cell surface could be determined by flow cytometry with a bITF probe, and (2) that non-specific binding could be determined by using a bTag probe instead of adding an excess of unlabelled ITF. We therefore quantified ITF-binding molecules on the IEC-18 cell surface by flow cytometry and the binding was expressed as specific binding units (PE molecules per cell) calculated as described in the Materials and methods section. Table 3 shows that the specific binding of bITF to unstimulated IEC-18 cells was low. Mucin treatment rapidly enhanced bITF binding to the IEC-18 cell surface in a dose-dependent manner, compared with medium alone.

Mucin up-regulates ITF-binding molecules on the IEC-18 cell surface via transcriptional and translational mechanisms

To investigate further the mechanism by which mucin regulates ITF-binding molecules on the intestinal epithelial surface, we treated the cells with mucin (5 mg/ml) in the presence or absence of either cycloheximide (CHX, 100 μM), a protein synthesis inhibitor, or actinomycin D (AD, 5 μM), an RNA synthesis inhibitor. As shown in Figure 5, the histogram of bITF binding to IEC-18 cells was shifted to the right after treatment with mucin (compare Figures 5A and 5B). The mucin effect was attenuated by pretreatment with CHX (compare Figures 5B and 5C) or AD (compare Figures 5B and 5D). To confirm this observation, a series of experiments were done to assay ITF-binding molecules on IEC-18 cell surface by flow cytometry. As shown in Table 3, the effect of mucin on ITF binding to the IEC-18 cell surface was attenuated by pretreatment with CHX or AD.

DISCUSSION

NO, which is produced by NOS during the conversion of L-arginine to L-citrulline, has an important role in the regulation of physiological functions of mammalian cells, and is also a mediator of inflammation [30]. This dual role of NO is supported by the observations that NO mediates intestinal injury, whereas under other experimental conditions it serves as a cytoprotective agent in the gastrointestinal system [19,31]. ITF is a cytoprotective peptide secreted by intestinal goblet cells [16]. ITF has an important role during intestinal epithelial migration [12,17], a
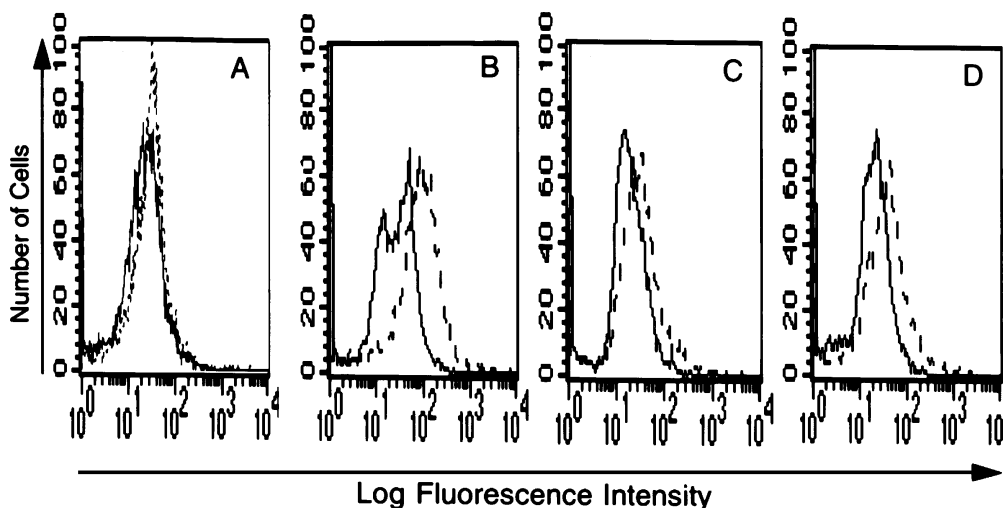
Figure 5   Flow cytometric analysis of ITF-binding molecule expression on IEC-18 cells treated with mucin, AD plus mucin, and CHX plus mucin

Cells were: cultured in medium only (**A**); cultured in medium and treated with mucin (5 mg/ml) for 90 min (**B**); cultured in medium containing CHX (100 μM) overnight then treated with mucin (5 mg/ml) for 90 min (**C**); or cultured in medium containing AD (5 μM) overnight then treated with mucin (5 mg/ml) for 90 min (**D**). The expression level of ITF-binding molecules was analysed with a FACScan as described in the Materials and methods section. Each histogram was based on 10 000 events acquired in the assay. Broken line, cells incubated with bITF (0.25 μM) then reacted with an FITC-conjugated anti-biotin mAb; solid line, cells incubated bTag (0.25 μM) then reacted with the mAb.

major step of the restitution process [32], but its mechanism of action remains unclear. Previous investigations showed that intestinal epithelial cells constitutively release NO *in vitro* [33,34]. In the present study we found that IEC-18 cells (a cell line from rat small intestinal crypts) constitutively generate nitrite, the major metabolite of NO [35]. Furthermore, we demonstrated that ITF induces the production of NO by IEC-18 cells. Interestingly, this is low, though significant, NO production when compared with cytokine-induced NO synthesis, which typically ranges from 10 to 100 μM after an 18 h stimulation [36]. The effect of ITF on NO release is attenuated by 1400W, a selective inhibitor of NOS2. We also found that IEC-18 cells express NOS2, which is up-regulated by ITF. These observations suggest that ITF increases NO production via the up-regulation of NOS2 protein. NO has been found to mediate epithelial restitution [37,38]. Dysregulation of restitution interferes with the closure of superficial epithelial defects after injury. A recent investigation showed that wound healing was delayed in NOS2-deficient mice, emphasizing the requirement of NO (from NOS2) in the process of wound healing and repair [39]. Thus NO released by the intestinal epithelium in response to ITF stimulation might mediate the biological effect of ITF *in vivo*.

The molecular mechanism by which ITF interacts with intestinal epithelium is unclear. Previous observations suggest the presence of ITF-BP on the intestinal epithelial cells [24]. Our recent study further identified this ITF-BP in the crypt cells of rat small intestine [22], and characterized it as a 50 kDa glycosylated membrane protein [22]. The present study demonstrated (1) that the IEC-18 cell expresses the 50 kDa ITF-BP and (2) that the effect of mucin in regulating ITF binding to intestinal epithelial cells could be blocked by inhibitors of transcription and translation, thereby suggesting that the ITF-binding molecule on IEC cells is a protein, most probably ITF-BP. By using confocal microscopy, we localized this ITF-binding molecule to the surface of intestinal epithelium and ascertained that its binding by bITF was augmented after treatment with mucin. Moreover we found that mucin-primed IEC cells are more sensitive to ITF stimulation, speculating that the up-regulation of ITF-binding molecules might be physiologically relevant. Several groups have proposed the existence of a receptor-like molecule that is able to mediate ITF action [1,2,18,22–26]. Further isolation and characterization of ITF-BP will shed light on the molecular mechanism of ITF action and intestinal epithelial cell biology.

Epithelial cell-associated mucin has an important protective role in the gastrointestinal tract [40]. In co-operation with mucin glycoproteins, ITF protects cultured intestinal epithelial cells against a variety of injurious agents [12,14]. However, the mechanism by which mucin, ITF and the epithelium interact with one another is understood only partly. It is possible that ITF binds to mucin, which adheres to cells. Alternatively, mucin might regulate the expression of ITF-binding molecules in intestinal epithelial cells. Here we found that mucin primes intestinal epithelial cells to enhance ITF-induced NO release. Examination by confocal microscopy revealed (1) a greater number of ITF probes bound to the epithelial cell surface after treatment with mucin compared with untreated cells, and (2) excess unlabelled ITF competed with bITF for binding to IEC-18 cells. By using ligand blot analysis we found that ITF-BP in IEC-18 cells is up-regulated by mucin. Inhibitors of transcription and translation blocked this effect of mucin. Furthermore we found that other epithelial cells such as CHO-K1 do not bind to bITF; neither does mucin treatment promote bITF binding to these cells (X.-D. Tan, Q.-P. Liu and M. Olszewski, unpublished work). Taken together, our observations suggest that the up-regulation of ITF-BP by mucin is unique to intestinal epithelial

cells. Moreover, mucin has a 'priming effect' on the epithelial cells to enhance ITF binding, which might account, at least in part, for the protective function of epithelium-associated mucin in the gastrointestinal tract. Intestinal mucin has been shown to be depleted in patients with IBD [41]. The composition of mucin in patients with Crohn's disease is significantly different from that of normal controls, suggesting that an altered profile of mucin glycoprotein might be present in IBD patients [42,43]. Thus our present findings might provide one explanation for the depletion of intestinal mucin and pathogenesis of IBD. However, a determination of whether the expression of ITF-BP in the intestinal epithelium of IBD patients is also dysregulated awaits future studies.

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