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Identification of Human Intestinal Trefoil Factor

GOBLET CELL-SPECIFIC EXPRESSION OF A PEPTIDE TARGETED FOR APICAL SECRETION*

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Trefoil peptides are a recently recognized group of small peptides abundantly produced at mucosal surfaces that offer the opportunity to define mechanisms of mucosal cell-specific differentiation and to illuminate new mechanisms for the preservation of mucosal integrity. We report the cDNA cloning of a 75-amino acid human trefoil factor expressed in small and large intestinal mucosas that is highly homologous to the intestinal trefoil factor, with 70% identity at the amino acid level of the predicted mature protein. This human intestinal trefoil factor is also homologous, although to a lesser extent, to trefoil peptides expressed at other sites in the gastrointestinal tract in man, exhibiting absolute conservation of the P domain motif ($CX_9CX_9CX_4CCX_9WCF$) that defines this family of peptides. These findings indicate a high degree of evolutionary conservation of organ/region-specific members of this peptide family.

***In situ* hybridization of intestinal trefoil factor demonstrates a high degree of expression in mature small intestine villus and colonic epithelial goblet cells. Immunogold staining demonstrates high concentrations of intestinal trefoil factor in the rough endoplasmic reticulum and theca of goblet cells as well as throughout the mucosal surface, consistent with vectorial secretion of this factor by goblet cells onto the intestinal luminal surface. In addition, intestinal trefoil factor was also localized within columnar epithelial cells by immunogold labeling despite the absence of mRNA. These observations suggest that peptide secreted by goblet cells might be taken up from the luminal surface and transcytosed by enterocytes. Human intestinal trefoil factor expression was also detected in the HT-29N2 and HT-29H2 subclones in conjunction with the emergence of the goblet cell phenotype, but not in the CaCO2 cell line that exhibits enterocytic phenotype. In summary, these findings confirm the existence of a highly conserved family of peptides that are abundantly expressed in distinctive regions throughout the gastrointestinal tract in a highly cell-specific pattern reflecting a goblet cell differentiation pathway. They form one of the more abundant constituents of the interface between the mucosa and "outside" environment and may provide a new paradigm of regulation of the integrity of epithelial surfaces as well as a previously unrecognized dimension of goblet cell function.**

The mucosa of the gastrointestinal tract exhibits the most rapid constitutive cell turnover in the body. It must maintain exquisite balance between proliferation and cell loss while preserving the structural continuity and integrity of the monolayer that interfaces with a complex external milieu that includes microorganisms, antigens, dietary substances, and a mixture of host digestive enzymes. Thus, the gastrointestinal tract provides an excellent opportunity to delineate mechanisms regulating epithelial proliferation and cellular interface with the environment.

Recent studies in this laboratory and elsewhere have led to the recognition of a family of small peptides that might play a central role in these processes. In studies exploring proteins modulating growth in intestinal mucosa, this laboratory identified a small protein expressed in high concentrations in goblet cells within rat small and large intestinal epithelia (Suemori *et al.*, 1991). Histochemistry suggested that this protein, designated intestinal trefoil factor (ITF),[1] which we now rename rat intestinal trefoil factor (RITF), may be directed to the mucosal surface in conjunction with mucin glycoproteins. RITF is a member of a protein family of designated trefoil factors that can be distinguished by their regionalized expression within the gastrointestinal tract (Thim, 1987, Thim, 1989). In contrast to RITF, other members of this family have been characterized in man and include pS2 (found in the gastric epithelium) and HSP (similar to a porcine homologue PSP; found in the pancreas and gastric antrum) (Tomasetto *et al.*, 1990; Rio *et al.*, 1988).

Although the physiological function of the trefoil peptides will require further characterization, a number of observations suggest several biological activities. PSP stimulated proliferation of human colon cancer-derived cell lines *in vitro* (Hoosein *et al.*, 1989). In addition, PSP was found to both regulate motility and inhibit gastric acid secretion in rodents (Frandsen *et al.*, 1986). The cross-species biological activity of the porcine protein in rodent and human experimental systems indirectly suggests that these peptides and their actions may be highly conserved. This is consistent with recent studies in amphibians that have demonstrated the presence of close homologues of the mammalian protein in both frog skin and stomach (Hoffmann, 1988; Hauser and Hoffmann, 1991).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L08044.

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[1] The abbreviations used are: ITF, intestinal trefoil factor; RITF and HITF, rat and human intestinal trefoil factors, respectively; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HSP, human spasmolysin; PSP, porcine spasmolysin.

As noted, RITF was found to be expressed in goblet cells of rodent small and large intestinal mucosas. The secretory products of goblet cells are present as a continuous layer overlying the mucosal surface of the intestinal gastrointestinal tract, composing the apparent structural interface between the epithelial monolayer and the lumen. Mucin glycoproteins have generally been the only major recognized secretory product of the goblet cells (Neutra and Forstner, 1987). These large highly glycosylated glycoconjugates are an abundant constituent of mucus (which also contains smaller amounts of IgA secreted by columnar epithelium as well as water and electrolytes) and apparently contribute to its viscoelastic properties. Presumably, this gel subserves essential functions including protection from luminal agents such as microorganisms either as a physical barrier or through specific interactions perhaps mediated by the carbohydrate side chains. In addition, the gel could serve as a reservoir or depot that maintains concentrations of other secretory products of the goblet cell that might contribute to mucosal integrity including factors regulating mucosal turnover and structural integrity.

Interestingly, PSP, the one protein in this family that has been assessed directly, was found to be highly resistant to protease digestion (Jørgensen *et al.*, 1982a, 1982b; Thim *et al.*, 1982). This resistance is thought to derive from a distinctive secondary structure including three intrachain loops formed by disulfide bonding; recent NMR analysis has supported the predicted distinctive secondary structure (Carr, 1992). This characteristic is particularly noteworthy in suggesting that trefoil peptides may be structurally suited to retain functional activity in the luminal milieu that contains numerous proteases. Indeed, PSP was found to exhibit a range of biological activities whether given by an enteral or systemic route.

In aggregates, these earlier studies suggest that the trefoil family of peptides may be required throughout mucosal surfaces and reflect a central feature of goblet cell-specific differentiation. Although RITF was first cloned from rat tissue, the distribution suggested that this reflected a distinct member of the trefoil family rather than the rat counterpart of the human proteins simply expressed in a different tissue distribution. In this report, we confirm this supposition with the cDNA cloning of the human homologue, human intestinal trefoil factor (HITF). This study demonstrates a high degree of evolutionary conservation characteristic of the trefoil family. More important, characterization of ITF confirms that this factor is specifically expressed as a major product of intestinal and colonic goblet cells that is secreted onto the mucosal surface in conjunction with mucin glycoproteins.

MATERIALS AND METHODS

cDNA Cloning of HITF—A commercially available normal human colon cDNA library prepared in λgt11 by random priming (Clontech, HL1034b) was screened with a full-length cDNA clone for RITF (Suemori *et al.*, 1991). Nitrocellulose lifts from 15 150-mm plates containing 3×10^4 plaque-forming units/plate were hybridized with the T3411 RITF probe at 45 °C overnight in 30% formamide in 5 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate), 10% dextran sulfate, and 5 × Denhardt's solution in the presence of 100 μg of salmon sperm DNA/ml after initial prehybridization for 2 h. The probe was labeled by random priming using standard techniques and added at 1×10^5 cpm/ml (Sambrook *et al.*, 1989). Filters were washed twice in 2 × SSC, 0.1% SDS at room temperature for 15 min and finally washed in 5 × SSC, 0.1% SDS at 50 °C for 15 min. Seven independent but cross-hybridizing recombinant clones were obtained and plaque-purified. To obtain the isolated inserts from these clones, the inserts of the independent clones were amplified by PCR using primers from the λgt11 sequences flanking the insertion site. PCR was carried out for 35 cycles of 95 °C for 1 min, 48 °C for 1.5 min, and 78 °C for 2 min, followed by 72 °C for 8 min. The PCR reaction mixture included ~100 ng of the purified clone DNA in 50 μl containing 5 μl of 10 × PCR buffer, 1 μl each of 10 mM dNTPs, 1 mM $MgCl_2$, 1 μM each primer, and 2.5 units of thermostable DNA polymerase overlaid with mineral oil. The PCR mixture was subsequently electrophoresed on a 1% agarose gel. The amplified insert of 0.4 kilobase was ligated into the TA cloning vector using the conditions recommended by the manufacturer (Invitrogen). The amplification of the insert of interest was confirmed by hybridization of nitrocellulose lifts with the T3411 probe following transformation. The sequences of the individual clones were determined using the dideoxyribonucleotide chain termination reaction with ^{35}S-dATP and Sequenase (United States Biochemical Corp.). Reaction products were resolved on denaturing polyacrylamide gels by standard techniques (Sambrook *et al.*, 1989; Sanger *et al.*, 1977). Sequence comparisons were made using the ALIGN program and the program of Devereux *et al.* (1984) in conjunction with GenBank (release 68.14) and EMBL (release 26.0) data bases.

In Situ Hybridization—The cellular location of messenger RNA coding for RITF was evaluated using *in situ* hybridization in tissues from male Sprague-Dawley rats. After anesthesia with ether, a blunt 15-gauge cannula was inserted into the ascending aorta, and 200 ml of heparinized PBS (20 IU/ml) was then perfused, followed by 200 ml of fresh 4% paraformaldehyde in PBS (pH 7.4). The small and large intestines were dissected free of other viscera and fixed by immersion in neutral buffered formaldehyde (pH 7.4). The tissue was embedded in paraffin, and 4-μm sections were cut and floated onto 3-aminopropyltriethoxysilane-treated slides. The tissue was subsequently dewaxed, rehydrated with PBS, treated with proteinase K (20 μg/ml), post-fixed in 4% paraformaldehyde, acetylated with 0.1 M triethanolamine and acetic anhydride (0.25%, v/v), and dehydrated prior to hybridization. Hybridization was carried out overnight at 55 °C in a 25-μl solution containing 0.02% Denhardt's solution, 10% dextran sulfate, 50% formamide, 0.3 mg/ml bovine rRNA, 10 mM dithiothreitol, 300 mM NaCl, 10 mM Na_2HPO_4, 10 mM Tris-HCl, and 5 mM EDTA plus 1×10^6 cpm of ^{35}S-labeled RITF riboprobe (antisense or sense orientation). Following hybridization, the tissue was treated with ribonuclease A to remove excess probe, subjected to washes with increasing stringency to 0.5 × SSC at 65 °C, and dehydrated in alcohol containing 0.3 M ammonium acetate. Subsequently, the tissue sections were coated with autoradiographic emulsion (NBT-2, Kodak) and exposed for 4 days at 4 °C. The sections were then developed, fixed, and counterstained with haemotoxylin and eosin. Tissue blocks were prepared from each of five different rats (two to four blocks/rat), and multiple sections from each block (four to seven, total = 70) were examined. Patterns of hybridization were uniformly consistent.

Immunohistochemistry Staining—Small intestinal tissue (ileum) was fixed by perfusion and then immersion in paraformaldehyde/lysine/periodate (McLean and Nakane, 1974). Small blocks of fixed intestine were snap-frozen, and frozen sections (5 μm) were cut on a cryostat. Sections were rinsed in PBS and then incubated sequentially in anti-ITF rabbit serum (prepared as previously described (Suemori *et al.*, 1991)) diluted in PBS containing 1% bovine serum albumin and, following washing, in goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. Sections were mounted in PBS/glycerol containing *N*-propyl gallate to reduce fading and viewed on a Nikon Microphot microscope equipped for epifluorescence. In other experiments, a specific stain with anti-ITF was analyzed using a commercially available peroxidase-based method according to the instructions of the manufacturer (Vectastain, Vector Laboratories, Inc.). Control sections incubated in preimmune serum showed no specific staining. In total, 48 sections from 12 different rats were examined; intersample variability was not observed.

Immunogold Labeling—Immunogold staining was carried out on ultrathin frozen sections of rat intestine that were prepared according to the published methods of Tokuyasu (1980). Tissue blocks ($n = 8$) of rat intestine were fixed in paraformaldehyde/lysine/periodate containing 5% sucrose and then infiltrated in 2.3 M sucrose overnight. The blocks were snap-frozen in liquid nitrogen, and 60-nm frozen sections were cut on a Reichert FC4D ultracryomicrotome and mounted on copper grids. Sections on the grids were incubated sequentially with drops of the following solutions: PBS containing 20 mM glycine, the rabbit ITF antisera diluted in PBS, PBS with 0.1% bovine serum albumin, and then protein A-gold diluted in PBS with 1% bovine serum albumin. The sections were washed and post-fixed in 1% glutaraldehyde and then stained for 5 min in 2% uranyl acetate. The grids were destained and embedded in 2% methylcellulose, dried, and viewed on a Philips CM10 electron microscope.

Expression of HITF in Human Cell Lines—The HT-29N2, HT-29H2, and HT-29C18 lines, established by Dr. Daniel Louvard, were originally obtained as gift from Dr. M. Neutra (Neutra and Louvard, 1989; Huet *et al.*, 1987). HT-29N2 and HT-29H2 lines exhibit goblet cell phenotype as assessed by morphological differentiation and expression of mucin glycoproteins in the confluent state; the N2 cells used routinely were seeded on either 150-mm glass Petri dishes (for Northern blot analysis) or glass coverslips (for immunocytochemistry) and maintained in glucose-free medium to induce differentiation. HT-29N2 cells were grown on 100-mm plastic dishes. Differentiation was confirmed as previously described using anti-human colon mucin glycoprotein monoclonal antibodies established in this laboratory (Podolsky *et al.*, 1986; Phillips *et al.*, 1988). The HT-29C18 subclone was similarly maintained either in glucose-containing media on glass Petri dishes or in glucose-free media on glass Petri dishes in the confluent state to induce the enterocytic phenotype. CaCO2 cells, obtained from the American Type Culture Collection, were assessed in either the subconfluent or post-confluent state in which they also undergo enterocytic differentiation (Pinto *et al.*, 1982, 1983; Lesuffleur *et al.*, 1991; Zweibaum *et al.*, 1991). Enterocytic differentiation was confirmed by the demonstration of sucrase-isomaltase activity. Cells were routinely used at a minimum of 10 days post-confluence.

HITF mRNA expression was evaluated by Northern blot analysis essentially by methods previously described (Suemori *et al.*, 1991a, 1991b). mRNA was prepared by oligo(dT)-cellulose chromatography after isolation of total RNA by the guanidine isothiocyanate/CsCl cushion technique (Chirgwin *et al.*, 1979). mRNA was denatured with formamide, fractionated by electrophoresis on 1% formaldehyde-agarose gels, and transferred to a nylon membrane as described earlier (Koyama and Podolsky, 1989). The HITF insert was generated by combined *Eco*RI and *Hin*dIII digestion of the TA cloning vector and radiolabeled by random priming (Feinberg and Vogelstein, 1983).

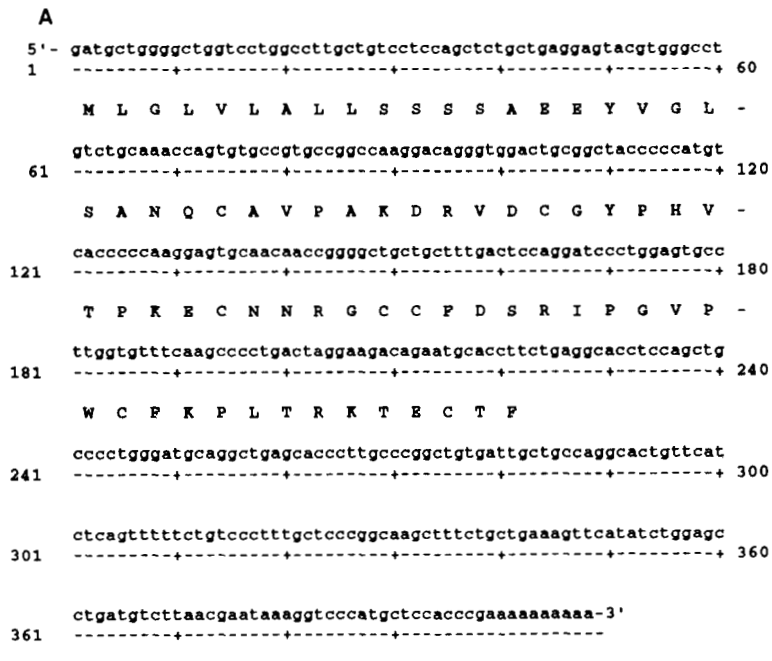

FIG. 1. **Structure of HITF.** Seven independent cross-hybridizing clones were obtained by screening a cDNA library prepared from a normal human colon using a full-length cDNA clone for the rat intestinal trefoil factor as described under "Materials and Methods." Sequencing of the inserts was accomplished using the dideoxynucleotide chain termination technique (Sanger *et al.*, 1977). *A*, nucleotide sequence of clones with amino acid sequence of encoded open reading frame; *B*, comparison of predicted amino acid sequence of HITF, RITF, and other human trefoil factors (pS2 and HSP) using ALIGN program; *C*, summary of overall homologies between HITF and other trefoil peptides. *RSP*, rat spasmolysin.

Blots were prehybridized and then hybridized as described above for the screening of the cDNA library. Approximately 3 μg of poly(A)+ RNA was loaded per lane and verified through rehybridization with a probe for the constitutively expressed transcript glyceraldehyde-3-phosphate dehydrogenase (Tso *et al.*, 1985). Six blots prepared from individual sets of cultured cells yielded the same relative abundance of ITF mRNA normalized to the constitutive probe with a coefficient of variation of ITF/glyceraldehyde-3-phosphate dehydrogenase of ≤6.4%.

HITF protein expression was evaluated by immunocytochemistry. Coverslips were first fixed with methanol and then incubated with either immune or preimmune rabbit anti-ITF antisera (diluted 1:100 in phosphate-buffered saline) for 30 min at room temperature. After washing off excess antibody, binding was visualized following incubation with peroxidase-conjugated anti-rabbit second antibody using commercially available reagents (Vectastain). Specificity of binding was confirmed by competitive incubation of the first antibody in the presence of an excess (25 μg/25 μl) of the synthetic peptide used as immunogen in the preparation of the antiserum.

RESULTS

Previous studies had resulted in the identification of RITF, a peptide expressed in small and large intestinal mucosas in rat, which exhibited substantial homology to previously identified members of the trefoil peptide family expressed in other regions of the human, porcine, and amphibian gastrointestinal tracts (Suemori *et al.*, 1991; Thim, 1989; Hauser and Hoffmann, 1991). To assess whether this represented a categorically distinct member of the trefoil family or, alternatively, the rodent equivalent of one of the previously identified trefoil proteins found in the stomach (pS2) and pancreas (HSP) of man, cDNA cloning techniques were used to determine whether a discrete homologue of rat ITF distinct from other human trefoil peptides was present in the human intestine. Screening of a cDNA library prepared from normal human colon at moderate stringency with a probe encoding RITF yielded several independent clones that contained an insert strongly hybridizing to the rat sequence. Nucleotide sequencing of the clones showed them to be identical and to encompass a 0.4-kilobase insert with an open reading frame encoding an 75-amino acid peptide following an ATG start site and a 0.15-kilobase 3′-untranslated region containing a polyadenylation consensus sequence and a poly(A) tail (Fig. 1*A*). This clone demonstrates the consensus sequence of cysteine residues that define the P domain characteristic of the trefoil peptide family. By analogy with other trefoil peptides, it appears to encode a 15-residue signal peptide and a mature 61-residue peptide. The predicted encoded mature protein exhibits a high degree of homology to RITF throughout its length (Fig. 1*B*). While the human protein appears to have a somewhat shorter putative signal sequence, the presumed mature peptide exhibits 70% identity to the rat protein. A particularly high degree of homology is present near the carboxyl terminus, with 18 of 20 residues identical and conservative substitutions at the two differing positions. Most important, as illustrated in Fig. 1 (*B* and *C*), whereas this clone also exhibits significant homology to the other P domain-containing human trefoil proteins, pS2 and HSP, these similarities are less marked than that between the rat and human intestine-derived factors. Thus, it is clear that the protein identified from the human colon library is distinct from the other human trefoil proteins. Collectively, these observations suggest that the human colon-derived clones represent the human structural counterpart of RITF and may therefore be designated HITF.

Northern blot analysis confirms a pattern of tissue expression of HITF similar to that of RITF. As indicated in Fig. 2, HITF mRNA is abundant in small intestinal and colonic mucosas, but cannot be detected at other sites in the gastrointestinal tract or its accessory organs, including stomach, pancreas, liver, and esophagus. Equivalent hybridization was observed in four Northern blots using mRNA prepared from separate sets of tissue samples. In addition, the pattern of HITF mRNA expression is paralleled by the presence of the HITF peptide as assessed by immunohistochemistry. A conventional antiserum prepared against the carboxyl-terminal 21 amino acids of RITF (identical to HITF at all but 3 residues) strongly stains human colonic and small intestinal epithelia (Fig. 3). Staining was not observed in other tissues including those of the gastrointestinal tract or other sites. The distribution of the HITF mRNA and peptide in the normal human gastrointestinal tract complements that reported for other human trefoil peptides expressed normally in the body of the stomach (pS2) as well as the antrum and pancreas (HSP). Taken together, these observations suggest that HITF and RITF are indeed evolutionarily conserved homologous trefoil factors, distinguished by their intestine-specific expression.

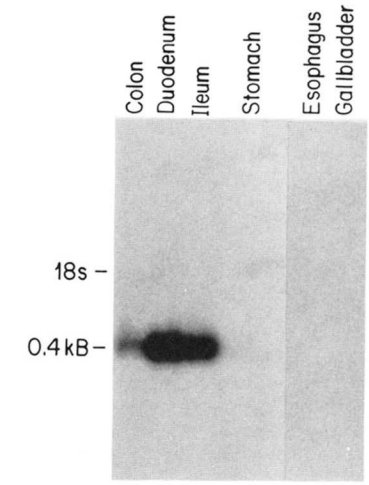

FIG. 2. **Northern blot analysis of human ITF expression.** Poly(A)$^+$ RNA purified after the initial preparation of total RNA from various human tissues was electrophoresed on 1% agarose gels and transferred to nitrocellulose paper, and the blots were hybridized sequentially with probes for HITF and the constitutively expressed transcript glyceraldehyde-3-phosphate dehydrogenase as described under "Materials and Methods." *kB*, kilobase.

While Northern blot analysis demonstrates the specific expression of HITF and RITF mRNAs in small and large intestinal mucosas, *in situ* hybridization permits a more refined assessment of the cellular basis of this tissue-specific expression. As demonstrated in Fig. 4*A*, ITF mRNA is essentially confined to the goblet cell population distributed throughout the epithelium. Thus, abundant ITF mRNA is present in the region of the rough endoplasmic reticulum of goblet cells, but is absent from other cell populations present in the epithelium when compared to control tissue incubated with a sense strand probe. Interestingly, ITF mRNA can be detected in cells present in the crypt region of the small intestinal mucosa before morphological features characteristic of distinctive epithelial cell populations including goblet cells can be appreciated (Fig. 4*B*). These findings suggest that ITF may be an early marker of commitment to goblet cell differentiation detectable before these cells accumulate the large amount of mucin glycoproteins that leads to the acquisition of their distinctive morphological appearance resulting from the mucin-filled theca. Alternatively, ITF may be broadly expressed in undifferentiated cells and then specifically suppressed in all differentiated populations beside the goblet cell. However, this seems less plausible with the selective expres-

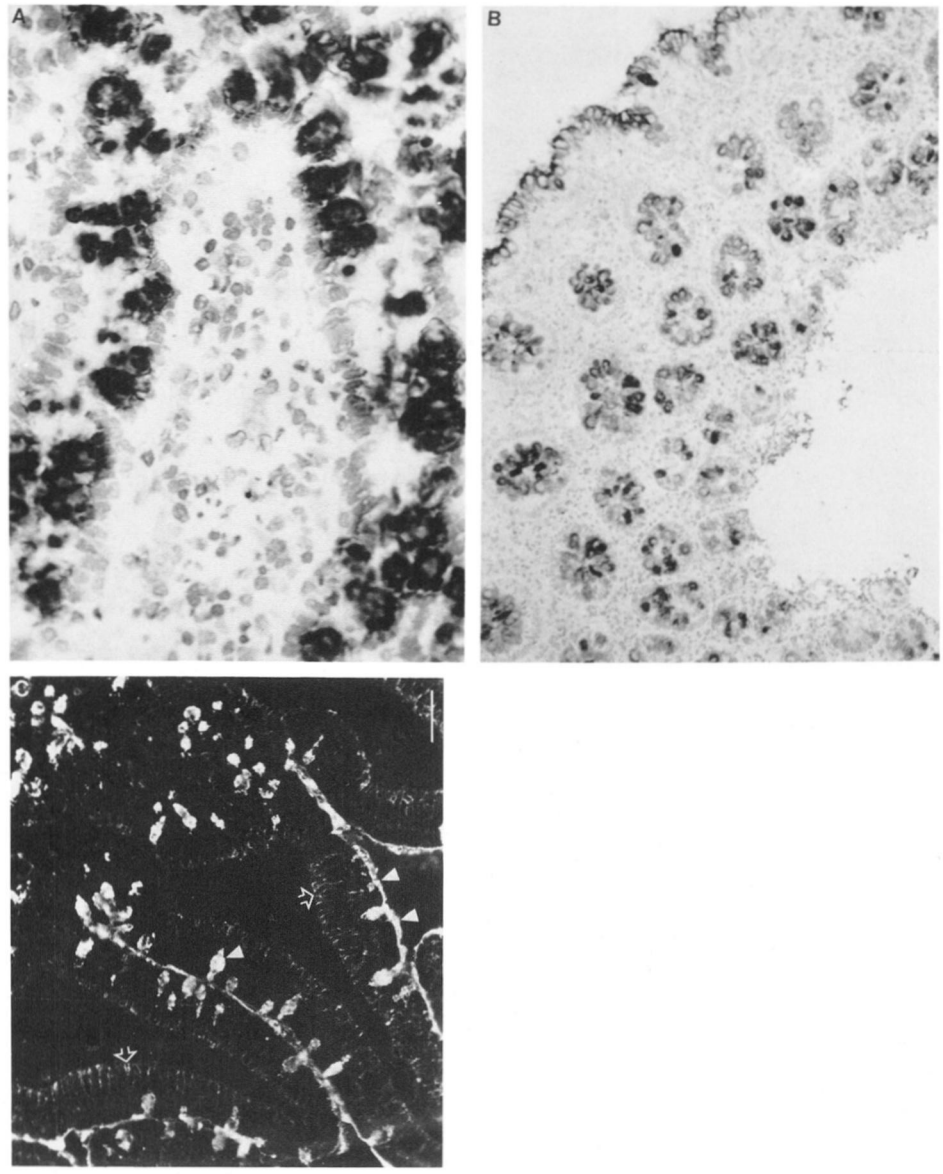

FIG. 3. *A* and *B*, immunolocalization of HITF and RITF. Frozen sections (4 μm) of mucosal biopsies were prepared from samples in OCT compound and incubated with rabbit anti-ITF antiserum (25 μl, 1:100 dilution) prepared by immunization with a synthetic peptide encoding the carboxyl-terminal 21-amino acids of RITF sharing 90% identity with HITF in the presence or absence of the peptide immunogen. Antibody-specific binding was visualized in representative sections using a peroxidase-based staining technique (Vectastain). *A*, human small intestinal (ileal) mucosa (magnification × 450) *B*, human sigmoid colonic mucosa (magnification × 100); *C*, immunofluorescence staining of ITF in rat intestine. Frozen sections of rat intestine were reacted with ITF antibody, followed by anti-rabbit IgG-fluorescein isothiocyanate. Intense staining of ITF is seen in goblet cells and in the mucin layer along the luminal borders (*arrowheads*). There is less intense staining lining the lateral membranes of absorptive cells (*open arrows*). *Bar* = 20 μm.

sion and secretion of ITF in mature goblet cells, consistent with the inference that ITF is a marker linked to commitment to a specific terminal differentiation pathway.

The translation of ITF mRNA into protein in goblet cells was confirmed by immunohistochemistry. As demonstrated in Fig. 3, dense staining is observed in representative tissue samples of both human small intestine and colon (by immunoperoxidase) as well as rat small intestine (by immunofluorescence) in the theca, known to contain mucin glycoproteins, previously the only proven secretory product of this cell population. In addition, specific immunoreactive material is present in the overlying secreted mucus layer. Immunogold electron microscopy is consistent with the results of light microscopy in demonstrating immunoreactive protein in the rough endoplasmic reticulum and theca of goblet cells (Fig. 5). In addition, ITF was present in the overlying secreted mucus of small intestinal mucosa (Figs. 5 and 6). Interestingly, despite the absence of detectable ITF mRNA by *in situ* hybridization within columnar absorptive enterocytes, some immunogold and immunofluorescence staining is observed within these cells, although in much smaller amounts than observed within the goblet cells and overlying mucus. In contrast to goblet cells, as illustrated in Fig. 7, ITF found in enterocytes was present within the Golgi apparatus, but not within the rough endoplasmic reticulum. In addition, ITF is localized along the lateral membranes of these cells. These observations together suggest that ITF may be specifically expressed by goblet cells and secreted onto the luminal surface. It is possible that some secreted ITF may be taken up by columnar absorptive cells. The appearance of ITF on the lateral surface suggests either that some ITF is secreted at the lateral surfaces of the goblet cells despite the localization of ITF to the theca within these cells or, alternatively, that ITF may be taken up by the enterocyte and transported via an unusual apical-to-basolateral transcytotic pathway.

To define further the relationship of ITF expression to the acquisition of the goblet cell phenotype, expression of HITF mRNA and protein was assessed in human colon cancer cell lines that can be induced to assume distinctive phenotypes. As demonstrated in Fig. 8, HITF protein could not be detected in subclones of the HT-29 cell line or the CaCO2 cell line when maintained under conditions in which they remained undifferentiated. However, both the N2 and H2 subclones of HT-29 were found to express a high level of HITF protein in parallel with other goblet cell phenotypic features, including the expression of mucin glycoproteins and typical morpholog-

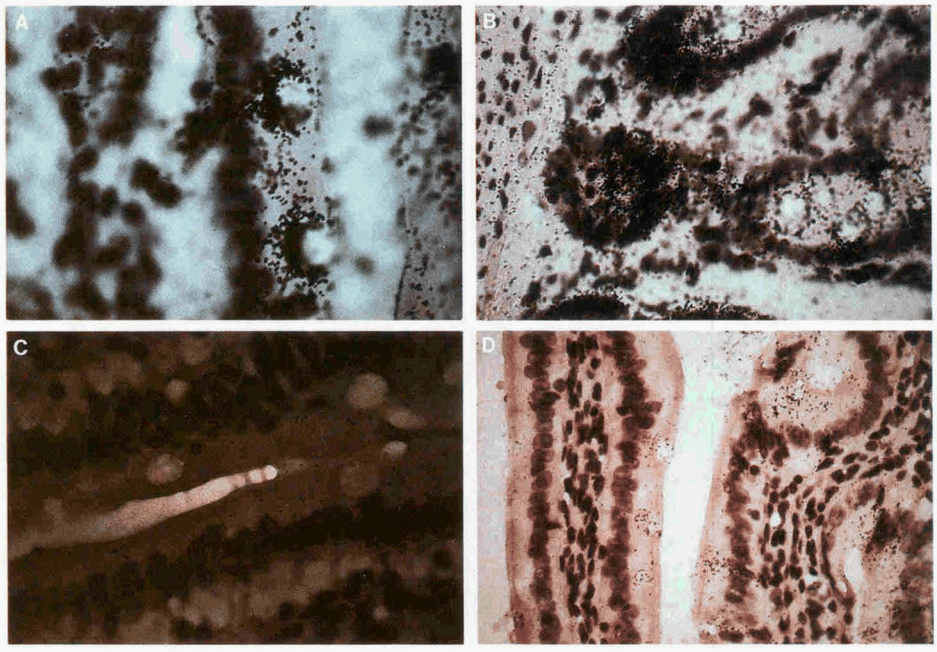

FIG. 4. **Localization of HITF mRNA expression.** *In situ* hybridization of small intestine was preformed with ^{35}S-labeled ITF riboprobe prepared and hybridized with antisense probe (*A*, *B*, and *D*) as described under "Materials and Methods." *A* and *D*, intestinal villus (magnification × 450 and 100, respectively). Note the selective hybridization over goblet cells. Shown are radioactive grains over non-goblet cells equivalent to control (*C*). *B*, high power view of intestinal crypt illustrating dense hybridization over crypt as well as morphologically distinguished goblet cells above. *C*, (control) hybridization with sense probe.

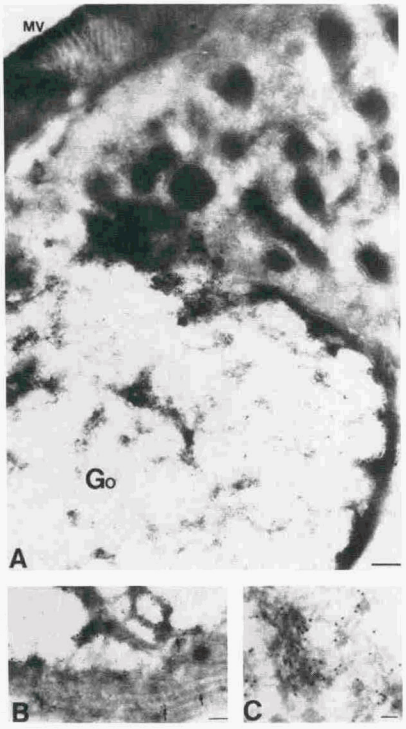

FIG. 5. **Immunogold localization of ITF in goblet cells.** *A*, ultrathin (60 nm) sections of rat intestine were stained with ITF antibody, followed by protein A-gold. The heaviest labeling was seen in goblet cells (*Go*), which showed heavy deposition of gold particles over the mucin-containing theca. There are also accumulations of ITF-gold over the entire microvillar (*MV*) surface of cells lining the intestinal lumen. *B*, ITF is synthesized in the goblet cells, and gold labeling was found in the rough endoplasmic reticulum in these cells (*arrows*). *C*, ITF-gold in the mucin granule was associated with web-like material that could represent wisps of accumulated mucin or very fine sections through membranes. *Bar* = 0.5 μm (*A* and *B*) and 0.1 μm (*C*).

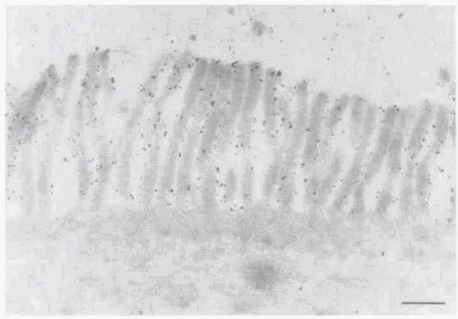

FIG. 6. **Immunogold localization of ITF in secreted mucin.** Ultrathin frozen sections of rat intestine showed heavy deposition of ITF-protein A-gold complexes throughout the area of the section corresponding to the intestinal lumen. Gold particles were also associated with the surface of microvilli on the absorptive cells along the length of the crypts. This labeling is consistent with the presence of ITF in secreted mucins lining the intestine. *Bar* = 0.5 μm.

ical features (data not shown) (previously reported by Phillips *et al.* (1988)). Interestingly, as demonstrated in a representative Northern blot in Fig. 9, HITF mRNA could be detected in relatively small amounts in the HT-29N2 line prior to phenotypic differentiation, but expression was significantly increased after emergence of the goblet phenotype in association with detectable peptide. Although some HITF mRNA could be detected after differentiation of the HT-29C18 line, these levels were much less than those observed in the HT-29N2 line. In contrast, the CaCO2 cell line did not express HITF either before or after induction of the enterocytic phenotype when evaluated by Northern blot analysis and immunocytochemistry, indicating that HITF expression is selectively associated with goblet cell differentiation among the various cell lines.

DISCUSSION

In the past several years, a number of proteins, designated trefoil peptides (Thim, 1989), that share important structural features have been identified within a number of animal species. These proteins share a characteristic structural motif designated a P domain resulting from a distinctive array of cysteine residues that can facilitate the formation of three intrachain loops. Earlier studies in this laboratory identified a member of the trefoil family (RITF) in small and large intestinal mucosas (Suemori *et al.*, 1991). This distribution contrasted with that of two other members of this family, pS2 and HSP, identified in human tissue. Thus, the two trefoil

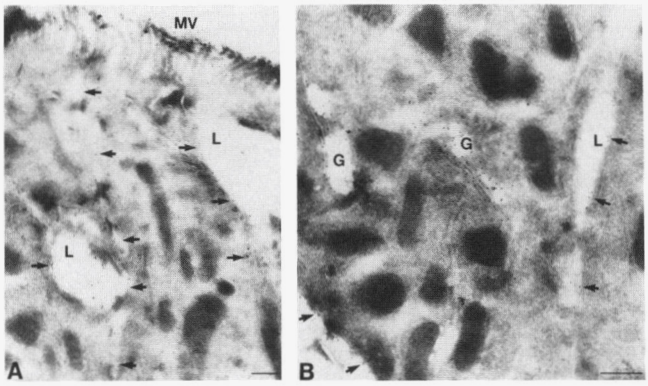

FIG. 7. **Immunogold localization of ITF in intestinal absorptive cells.** Ultrathin frozen sections of rat intestine were labeled with ITF antibody, followed by protein A-gold. *A*, labeling of ITF was seen associated with absorptive cells, although the heaviest labeling was found in the goblet cells. Gold labeling was associated with secreted mucin along the microvillus border (*MV*). There was also labeling along the entire lateral membranes between adjacent absorptive cells (*L*) (*arrows*). *B*, gold particles were also specifically deposited over the Golgi complex (*G*) in the absorptive cells, suggesting that some of ITF is processed or transcytosed in these cells. *Bar* = 0.5 μm.

peptides previously identified in man include pS2, which is normally expressed within the gastric mucosa, particularly the body and fundus, and HSP (the human homologue of PSP), expressed in the pancreas and gastric antrum (Rio *et al.*, 1988; Tomasetto *et al.*, 1990; Nunez *et al.*, 1989). Extrapolating across species, these observations suggested that the entire gastrointestinal tract may express specific members of the trefoil peptide family throughout its length. However, it was also possible that the so-called RITF was in fact the rat homologue equivalent to one or both of the human peptides and that their contrasting tissue distribution was a reflection of functional divergence between rat and man.

This report confirms that there is indeed a direct homologue of RITF in man, here designated HITF, distinct from both pS2 and HSP. This conclusion is supported by the high degree of homology between RITF and HITF (70%), which is significantly greater than the still considerable homologies between HITF and the two other human trefoil peptides, pS2 (36%) and HSP (28%). Similarly, the homology between RITF and HITF is greater than the significant homology between RITF and rat spasmolysin (45%), the recently cloned rat homologue of HSP.[2] All of the trefoil peptides exhibit conservation of the P domain motif ($CX_9CX_9CX_4CCX_9WCF$).

HITF also exhibits a pattern of tissue-specific expression that parallels that of RITF in rodent. In contrast to pS2 and HSP, HITF is present exclusively throughout the small and large intestinal mucosas. These observations indicate that each region of the gastrointestinal tract appears to express a specific member of the trefoil family that has been individually conserved through evolution in mammals and in animals at least as evolutionarily remote as amphibians (Hoffmann, 1988; Hauser and Hoffmann, 1991).

Most important, this study shows a close association between ITF expression and commitment to goblet cell differ-

[2] G. P. Jeffrey, P. S. Oates, T. C. Wang, M. Babyatsky, and S. J. Brand, submitted for publication.

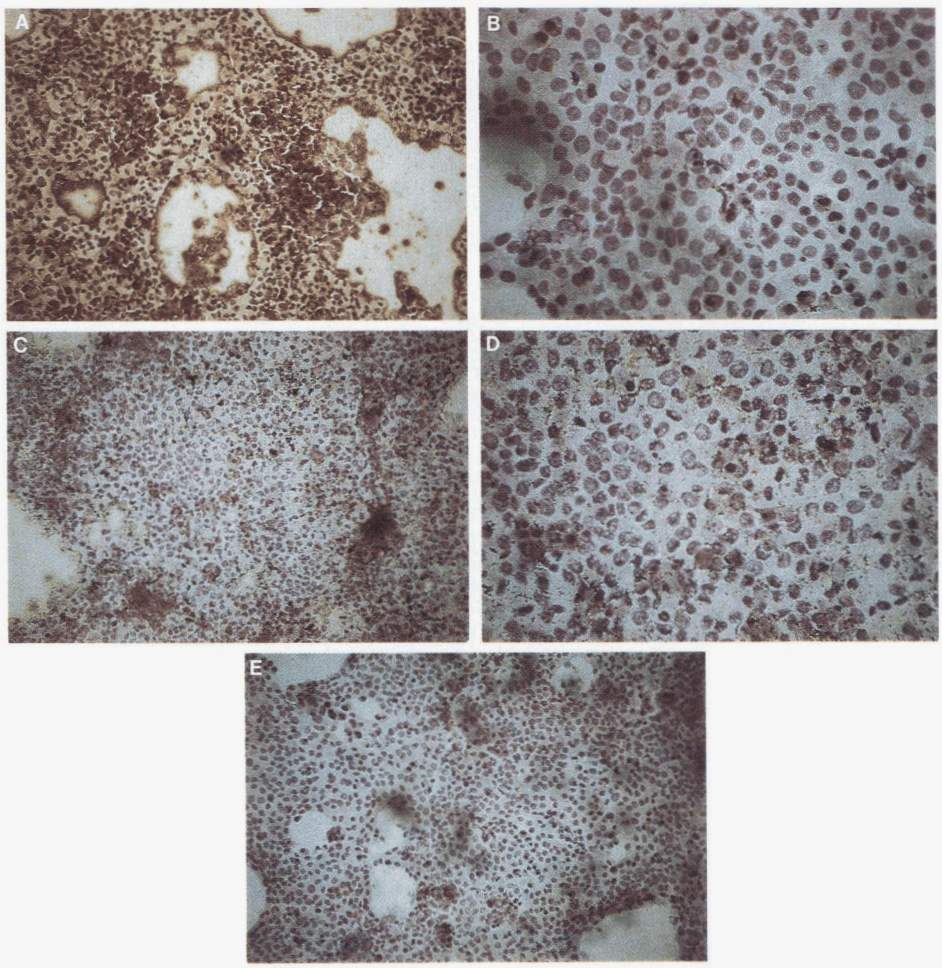

FIG. 8. **Expression of HITF peptide in human colon cancer-derived cell lines and relationship to differentiation.** Monolayers of two subclones (designated C18 and N2) of the HT-29 cell line were maintained in an undifferentiated state or under conditions that promoted acquisition of goblet cells (N2) or enterocytic phenotype (C18) as previously described; differentiation was confirmed by demonstration of mucin glycoprotein production (N2) or sucrase activity (C18). Monolayers on glass coverslips were fixed in 10% methanol and then stained for ITF protein as described under "Methods and Methods" and in the legend to Fig. 3. *A*, N2, undifferentiated; *B*, N2, differentiated; *C*, C18, undifferentiated; *D*, C18, differentiated; *E* N2, differentiated (preimmune control).

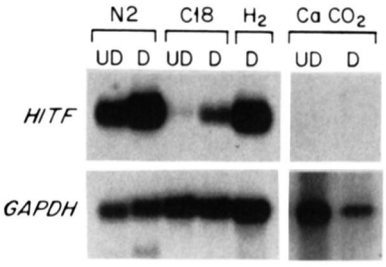

FIG. 9. **Expression of HITF mRNA in human colon cancer-derived cell lines.** Northern blot analysis was performed on poly(A)$^+$ RNA (~4 μg/lane) prepared from HT-29 subclones H2 (which exhibits goblet cell phenotype) and C18 (which exhibits columnar absorptive cell phenotype) as well as the CaCO2 cell line (which acquires a columnar enterocytic phenotype) in undifferentiated (*UD*) and differentiated (*D*) states. Goblet cell differentiation of H2 cells and enterocytic differentiation of C18 and CaCO2 cells were validated by demonstration of mucin glycoprotein and sucrase activities, respectively. Nitrocellulose blots prepared after agarose gel electrophoresis were sequentially hybridized to probes for HITF and the constitutively expressed transcript glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) under the conditions described under "Materials and Methods."

entiation. Thus, both ITF mRNAs were found exclusively in goblet cells among the mature intestinal and colonic mucosa of rat and man. The presence of ITF mRNA in a subpopulation of cells within the crypt epithelium prior to the development of distinctive morphological features suggests that ITF is indeed an early marker of commitment to goblet cell differentiation. In this context, the ITF gene offers a powerful tool to define the molecular basis of goblet cell-specific gene expression and differentiation. The relationship of ITF expression and goblet cell differentiation is further validated by similar goblet cell phenotype-specific association of ITF expression in a series of colon cancer-derived cell lines that undergo goblet cell-like differentiation *in vitro*. These conclusions are also supported by the apparent absence of ITF mRNA in enterocytes as assessed by *in vitro* hybridization and the absence of either ITF mRNA or immunoreactive protein in cell lines exhibiting enterocyte-like differentiation (Figs. 8 and 9).

The functional properties of ITF and, to large extent, those of the entire family remain to be defined. The localization of these proteins implies a role in maintaining the integrity of the mucosal surface. This supposition is consistent with the observed resistance to protease digestion demonstrated for PSP, the one member of the family directly characterized (Frandsen *et al.*, 1986). The properties of ITF within this gel remain to be determined. PSP has been found to regulate both motility and secretion. It has also been suggested that trefoil factors may function as growth factors (Hoosein *et al.*, 1989; Wright *et al.*, 1990; Rio *et al.*, 1991). However, the high level of expression and the abundance of the peptide contrast with the generally low levels of expression of most conventional peptide growth factors. An alternative possibility that is consistent with the abundance of ITF in the intestinal and colonic mucus coats is that ITF contributes to the stability of the viscoelastic gel that is essential to the preservation of the integrity of the mucosal surface. This concept of ITF function is also consistent with the apparent induction of enhanced expression of trefoil peptides in proximity to mucosal ulceration at various sites in the gastrointestinal tract (Wright *et al.*, 1990; Rio *et al.*, 1991). If trefoil factors facilitate continuity of the mucus coat that represents the interface of the mucosal surface and the luminal milieu, induction of expression could help reestablish coverage over the mucosal defect.

While this study clearly demonstrates goblet cell expression of ITF and apical secretion onto the luminal surface, immunogold staining reveals the presence of small amounts of peptides within enterocytes and at the lateral surfaces of these cells. These localization studies imply potential uptake of ITF from the apical surface and translocation to the lateral membrane. Although apical-to-lateral protein transcytosis of immunoglobulin is an important functional feature of enterocytes in the neonatal period prior to weaning, uptake and transport of intact proteins in this direction have not been generally appreciated as a trafficking pathway. However, these findings are consistent with earlier observations documenting the apparent uptake of intact ribonuclease in rodents following ligation of the pancreatic duct (Alpers and Isselbacher, 1967). Trefoil peptides produced by the mucosa that are intrinsically resistant to protease digestion might be natural substrates for the pathway implied by the earlier study. Alternatively, the presence of the ITF peptide at the lateral surface below the intact tight junctions could reflect a low level of secretion of the peptide through the basolateral as well as the apical pole of goblet cells in a manner similar to the bipolar secretion of alkaline phosphatase exhibited by enterocytes (Sussman *et al.*, 1989). While it is also possible that enterocytes themselves express ITF at low levels and at levels much below those observed in goblet cells, this appears unlikely; ITF mRNA was not observed within enterocytes by *in situ* hybridization, and ITF could not be detected in cell lines exhibiting an enterocyte-like differentiated phenotype. Nonetheless, these data cannot exclude the possibility that the ITF present within enterocytes reflects endogenously produced peptide remaining after transient mRNA expression (and subsequent degradation) at an earlier stage of absorptive cell maturation. Detailed characterization of the synthesis, secretion, and uptake of HITF in the HT-29 subclones exhibiting specific differentiated phenotypes should permit clarification of pathways of ITF trafficking.

Collectively, these studies provide an insight into a previously unappreciated dimension of goblet cell function and the complexity of the mucosal surface interface with the lumen. The high level of evolutionary conservation of distinctive trefoil peptides implies the importance of this class of peptides for normal mucosal function throughout the gastrointestinal surface. Moreover, these genes would seem to offer powerful tools for the delineation of the molecular basis of region- and cell-specific gene expression within the gastrointestinal tract. The elements responsible for the characteristic tissue- and cell-specific pattern of expression of these gastric and pancreas-associated trefoil factors have yet to be determined. Detailed analysis of ITF may provide further insight into the molecular mechanisms of tissue- and cell-specific expression that are responsible for the goblet cell phenotype.

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