Molecular cloning of mouse intestinal trefoil factor and its expression during goblet cell changes

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A cDNA encoding mouse intestinal trefoil factor (mITF) was successfully cloned and sequenced from the small intestine of C57BL/6 mouse by using the combination of reverse transcription-PCR and rapid amplification of cDNA ends methods. The gene was, similar to rat and human ITFs, mainly expressed in the small and large intestine. The mITF expression was up-regulated during the recovery phase after depletion of goblet cells in acetic acid-induced colitis. On the other hand, the expression in the

jejunum was not altered, while goblet cell hyperplasia was induced by *Nippostrongylus brasiliensis* infection. These results suggest that the mITF expression did not simply correlate with the number of goblet cells. The mITF may play an important role in the maintenance and repair of mucosal function of the rectum. Additionally, the mITF in the jejunum may play a role in alteration of the physicochemical nature of goblet cell mucins, thereby affecting the establishment of intestinal helminths.

INTRODUCTION

Intestinal trefoil factor (ITF), a new member of the trefoil peptide family (reviewed in [1–4]), was first cloned from rat intestinal villus epithelial cells and has one cysteine-rich P domain or trefoil motif [5]. Subsequently, human ITF (hITF/hP1.B) was cloned and sequenced [6,7]. By immunohistochemical study using a polyclonal antiserum to the C-terminal 21 amino acids of rat ITF (rITF), expression of ITF was confined to goblet cells throughout the small and large intestine [5,6]. Furthermore, ITF mRNA was detected in the mucus-producing intestinal goblet cells by *in situ* hybridization [6–8]. ITF was shown to promote epithelial migration through a transforming growth factor β -independent pathway [9]. Therefore, ITF seems to play an important role in goblet cell differentiation and proliferation. However, its expression during goblet cell changes has never been explored in detail. In the present study, we cloned and sequenced mouse ITF (mITF), and examined its expression during the course of infection with *Nippostrongylus brasiliensis*, a strong inducer of goblet cell hyperplasia in the jejunum of mice [10–12], and during the course of an acetic acid-induced experimental colitis, which may represent an ulceration-associated cell lineage (UACL) [13–15].

MATERIALS AND METHODS

Animals and their treatments

Inbred C57BL/6 mice were raised and kept in clean conventional conditions in the Experimental Animal Center, Miyazaki Medical College, and were used at 8–10 weeks of age at the start of experiments. *N. brasiliensis* infection was performed as described [11,12]. Briefly, adult worms obtained from the jejunum of infected Wistar rats were washed several times with physiological saline, adjusted to 100 worms/ml, and were introduced surgically into the proximal duodenum of recipient mice with a 1 ml syringe

fitted with a 19G needle. One, 3, 5 and 7 days after infection, mice were killed by cervical dislocation under ether anesthesia. Tissues from various organs were used for histological examination and RNA extraction. Uninfected mice served as controls. Experimental colitis was induced by intrarectal administration of 5% acetic acid under ether anesthesia [13–15]. Briefly, rectum was lavaged with 0.2 ml of saline for enema and then 0.1 ml of 5% acetic acid was administered into the rectum 3 cm proximal from the anus. Control mice were given 0.1 ml of saline in the same manner. Mice were killed 1, 3 and 5 days after administration and the rectal tissues showing macroscopic erosion were used for histological examination and RNA extraction. Three mice were used at each designated time point, and the histological and RNA samples were prepared separately from each mouse.

RNA extraction

On the designated days, various tissues obtained immediately after death were used for RNA extraction. Total RNAs were extracted by ISOGEN (Nippongene, Tokyo, Japan), according to the manufacturer's protocol. Poly(A)⁺ RNA selection was performed by oligo(dT) latex (Oligotex-dT30; Nippon Roche, Tokyo, Japan).

Cloning and sequencing by rapid amplification of cDNA ends (RACE)

A sample (1 μ g) of poly(A)⁺ RNA extracted from the small intestine of a normal mouse was reverse-transcribed by a DT7 primer and SuperScript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, U.S.A.). The DT7 primer contains oligo(dT)₁₇ at the 3' end followed by PT7 anchor primer sequence. For 3' RACE [16,17], the first-strand cDNA was amplified by PCR with the primers P1 and PT7, and the resulting first-round RACE products were amplified again with the primers P2 and

Abbreviations used: ITF, intestinal trefoil factor; mITF, mouse ITF; hITF, human ITF; rITF, rat ITF; UACL, ulceration-associated cell lineage; mSP, mouse spasmodic polypeptide; RACE, rapid amplification of cDNA ends; PAS, periodic acid-Schiff; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HPF, high-power field.

The nucleotide sequence data will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number: D38410.

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PT7 (Figure 1a). The primers P1 and P2 were designed in the well-conserved regions of rat and human ITF cDNA sequences [5-7]. Each thermal cycle profile was 45 s at 94 °C, 1 min 30 s at 50 °C, 2 min 30 s at 72 °C for 35 cycles. After electrophoresis, the 3' RACE product of the expected size was cut from 2% agarose gel, purified by GeneClean II (Bio 101 Inc., La Jolla, CA, U.S.A.), and cloned into a TA cloning vector, pCR II (Invitrogen, San Diego, CA, U.S.A.). The nucleotide sequence was determined by the dideoxy chain-termination method as described previously [18]. After sequencing of this 3' RACE product, the primers P3 and P4 were designed in mouse-specific inner regions, and then 5 µg of total RNA extracted from mouse small intestine was reverse-transcribed with P3 primer for subsequent 5' RACE [17,19]. For 5' RACE, we used 5'-AmpliFINDER RACE Kit (Clontech, Inc., Palo Alto, CA, U.S.A.). Briefly, the single-stranded oligonucleotide anchor (5' anchor) was directly ligated to the 3' end of the first-strand cDNA with T4 RNA ligase, and the resulting anchor-ligated cDNA was amplified by PCR with the primer P4 and the anchor primer P5 (Figure 1a). The 5' RACE products were also purified, cloned and sequenced as described above. Primers and anchor sequences were as follows: primer P1 (sense), 5'-GAAGTTTTCGTGCTGCCATG-3'; primer P2 (sense), 5'-ACAAATGTCAGAGTGGACTG-3'; primer P3 (antisense), 5'-GCTTCAAAATGTGCATTCTG-3';

To obtain the full-length mITF cDNA, we first performed 3' RACE with the primers P1 and P2, which were designed within the well-conserved region of rat and human ITF cDNAs (Figure 1a) [5–7]. Fortunately, the expected size (approximately 300 bp) of the 3' RACE product was obtained and it was sequenced. Based on this partial cDNA sequence, 5' RACE was performed using the primers P3 and P4 (Figure 1a), which were specific for mouse. By these steps, the full-length cDNA sequence was obtained. At least six individual clones, each from 3' and 5' RACE products, were sequenced, and all sequencing was done once or more in both directions to obtain maximum accuracy for the sequence given. Total (450 bp) mITF nucleotide sequence and its deduced amino acid sequence are shown in Figure 1(b). An ATG codon was found 36 nucleotide residues downstream of the 5'

Figure 2 Comparison of the deduced amino acid sequence of mITF with those of (a) rITF and hITF/hP1.B and (b) other mouse trefoil peptides

Identical amino acid residues are indicated by an asterisk, and the well-conserved P domain or trefoil motif, CX₆RX₂CGX₈CX₄CCX₉WCF, is indicated by shadowed boxes. The percentage identities of mITF with rITF, hITF and hP1.B in the propeptides (mature peptides in parentheses) (a) and those with mpS2 and mSP domain 1 (D1) and 2 (D2) within the P domain (b) are also indicated.

end. A stop codon (numbered 279), and the following 172 bp non-coding region including two polyadenylation signals, AATAAA (numbered 384–389 and 409–414), were found at the 3' end. Thus, the peptide with 81 amino acids was encoded by a single open reading frame. The first 22 amino acids probably represent the signal peptide, based on the comparison with rITF and hITF/hP1.B [5–7]. Consequently the initial Met was numbered –22, and the following Ala, considered to be the N-terminus of the mature peptide, was numbered 1.

Comparison with other trefoil factors

When the deduced amino acid sequence of mITF was compared with those of rITF and hITF/hP1.B (Figure 2a) [5–7], and other mouse trefoil factors (Figure 2b) [21,22], the cysteine-rich P domain or trefoil motif, CX₆RX₂CGX₈CX₄CCX₉WCF, was well-conserved (shadowed boxes in Figure 2). The percentage identity of mITF precursor and mature peptides with rITF was 87.6 and 93.2%, whereas with the hITF/hP1.B, these values were 69.1 and 73.3/76.2%, respectively. When the trefoil motif of the mITF was compared with those of other mouse trefoil peptides, the percentage identity was 51.2% against mouse pS2 (mpS2) [21] and 45.0/33.3% against the domain 1/domain 2 of the mouse spasmolytic polypeptide (mSP) [22]. Therefore, the cDNA reported here was thought to represent the mouse homologue of rITF and hITF/hP1.B but was clearly distinct from other mouse trefoil peptides, mpS2 and mSP.

mITF expression in various tissues

Tissue distribution of mITF transcripts in normal mouse was examined by RNA blot analysis. The mITF mRNA signal with an approximate size of 500 bases was detected strongly in jejunum, ileum and colon, weakly in stomach, but not at all in lung, pancreas or kidney (Figure 3). These results were confirmed by two separate experiments using RNA preparations from

Figure 3 RNA blot analysis of mITF expression in various tissues

Samples (30 µg each) of total RNAs derived from the tissue described above the lane were loaded. Hybridization was performed under high-stringency conditions and was probed with 3' RACE products corresponding to the nucleotide positions 144–450. The control G3PDH is also shown.

Figure 4 RNA blot analyses of mITF expression during the course of goblet cell changes

The mITF expression is shown in jejunum before and after *N. brasiliensis* infection (a) and in rectum before and after 5% acetic acid administration (b). Aliquots (30 µg each) of total RNAs derived from the tissue at the determined time point were loaded and the hybridization was performed under the same conditions as described in Figure 3. The transblotted membrane was probed with both mITF and control G3PDH. The number of goblet cells per 10 villi in the jejunum (a) and those per high-power field (HPF) in the rectum (b) are indicated as open circles in the bottom Figures. The ratio of mITF and control G3PDH expression calculated by Bioimaging analyser BAS 2000 are indicated as closed circles in the Figure. These results are shown as the mean ± S.D. from three mice. **P* < 0.01 against day 0.

Figure 5 Histology of the jejunum 5 days after *N. brasiliensis* infection (a) and the rectum 1 day after acetic acid administration (c)

Note marked increase (a) or decrease (c) in the number of goblet cells in contrast to respective controls, (b) and (d). (PAS staining, $\times 120$).

different mice. Such an expression pattern of mITF along the digestive tract was basically consistent with that of rITF and hITF/hP1.B [5–7]. Since the probe used for RNA blot analysis hybridized to the digested mouse genomic DNAs as single bands in Southern blot analysis under the high-stringency conditions (results not shown), and since the expression patterns of mpS2 [21] and mSP [22] were different from those of mITF in this study, the signals detected were thought to represent only mITF.

mITF expression during *N. brasiliensis* infection

Marked goblet cell hyperplasia was induced in the jejunum of mice by *N. brasiliensis* infection (see Figures 5a and 5b). The number of goblet cells before infection was 91.75/10 villi. After infection, their number gradually increased, reaching a peak on day 5 (259.5/10 villi, $P < 0.01$ compared with day 0), and then decreased with time (Figure 4a). These results were consistent with our previous observation [11]. The mRNA signal and the ratio of mITF and G3PDH expression estimated by Bioimaging analyser at each determined time point are also shown in Figure 4(a). Interestingly, the mITF expression did not change significantly during the course of *N. brasiliensis* infection and the ratio of mITF and G3PDH expression remained constant in spite of goblet cell hyperplasia.

mITF expression during experimental colitis

Marked goblet cell depletion was induced in the rectum shortly after administration of acetic acid (Figures 5c and 5d). The number of goblet cells in the rectum of normal control mice was 149.0/HPF. After acetic acid-treatment, their number drastically decreased as early as 1 day after administration (42.2/HPF, $P < 0.01$ compared with day 0) and then recovered gradually to the normal level by day 5 (Figure 4b). The mRNA signal and

the ratio of mITF and G3PDH expression at each time point were also shown in Figure 4(b). The mITF expression was up-regulated during the recovery phase of the colitis and the ratio of mITF and G3PDH expression increased about 2.5-fold at day 5 after the treatment ($P < 0.01$ compared with day 0).

DISCUSSION

In this study, we have cloned and sequenced mITF and have examined its expression during goblet cell changes in the small and large intestine of mice. The deduced amino acid sequence of mITF is very similar to that of rITF. Indeed, the mITF peptide is only one amino acid different from rITF among the P domain or trefoil motif, though it is almost entirely different from other mouse trefoil peptides, mpS2 and mSP, except for the well-conserved cysteine residues of the P domain (see Figure 2). The mITF is, like rITF and hITF/hP1.B [5–7], thought to be produced as a propeptide encoding a 22-residue signal peptide and a mature 59-residue peptide. The predicted cleavage site of signal peptide and mature peptide of mITF, Ala⁻¹ and Ala¹, followed the rules of von Heijne [23].

The expression pattern of mITF along the digestive tract was basically similar to that of rITF and hITF/hP1.B. The mITF mRNA was strongly detected in jejunum, ileum and colon, and only faintly in stomach. Although mITF mRNA was not detected in kidney, lung or pancreas of mice, the rITF mRNA was detected in rat kidney [5] and the hITF/hP1.B mRNA was detected in human lung and pancreas [3,7]. This might indicate the different usage of ITF in various organs of each animal, or merely be due to the difference in the sensitivity of the assay methods used for each study.

The hITF is strongly expressed in cultured enterocytes showing goblet cell differentiation [6] and UACL of inflammatory bowel disease [7]. Therefore, ITF is considered to be an early marker of

goblet cell differentiation [5–7]. In the present study, mITF expression in the rectum was augmented at the recovery phase of acetic acid-induced colitis. On the other hand, its expression in the jejunum remained constant in spite of goblet cell hyperplasia during the course of *N. brasiliensis* infection (Figure 4). These results suggest that the mITF expression did not simply correlate with the number of goblet cells. The mITF may play an important role in the maintenance of normal mucosal function and the repair of the damaged mucosa but not be involved in the hyperplasia of goblet cells. Alternatively, the usage of mITF might be different between small and large intestines.

The intestinal mucin genes, MUC 2 and 3, are almost exclusively expressed in the goblet cells of small and large intestine [24], where ITF peptides have been detected. On the other hand, another mucin gene, MUC 1, is expressed mainly in breast, stomach and salivary glands where pS2 and/or SP transcripts have been detected [2,4]. Such co-expression of mucin and trefoil peptide genes indicates mutual interaction of these gene products [8,25]. Since the seventh cysteine residue of all trefoil peptides including ITF, present near the C-terminal end, is considered to interact with mucin glycoprotein [3,7], ITF may be able to act as a link peptide between mucin glycoproteins such as MUC 2 and 3 products. The ITF specifically enhanced viscosity in mixtures with mucin glycoprotein (unpublished work cited in [9]). In the present study, mITF expression in the jejunum of *N. brasiliensis*-infected mice remained constant in spite of goblet cell hyperplasia. Therefore, the viscosity of jejunal goblet cell mucin might decrease when goblet cell hyperplasia was induced by *N. brasiliensis* infection. Since our previous studies demonstrated the importance of goblet cell mucins as the selective effector molecule in the immune-mediated expulsion against *N. brasiliensis* [11,12,26–29], ITF might be an important determinant of the physicochemical nature of goblet cell mucins for expulsion of intestinal parasites.

Apart from a possible role of ITF in the mucosal defence in the jejunum, ITF is constitutively expressed in the goblet cells of colonic mucosa [5–8]. Furthermore, previous reports have shown that hITF/hP1.B was strongly expressed in UACL [4,7]. In the present study, indeed, mITF mRNA was up-regulated during the recovery phase of the acetic acid-induced colitis. This finding supports the concept that ITF plays an important role for maintenance and repair of mucosal function [7] as a growth-promoting factor for goblet cells in the damaged rectum. Other trefoil peptides, pS2 and SP, are known to have a growth-promoting activity for epithelial and/or cancer cells [1–4]. In addition, in the repairing phase of colitis, the mITF might enhance the viscosity of rectal goblet cell mucin because mITF was up-regulated in spite of goblet cells depletion. Increased viscosity of mucin might be important for the protection of the damaged mucosa.

In conclusion, we isolated a cDNA encoding mITF and showed its expression pattern during goblet cell changes. The mITF expression in the rectal mucosa was up-regulated in the recovery phase of experimental colitis, whereas its expression in the jejunum remained constant during the course of *N. brasiliensis*

infection in spite of goblet cell hyperplasia, indicating that mITF expression is not simply correlated with the number of goblet cells. These results suggest that mITF plays an important role not merely in the maintenance and repair of mucosal function but also in the alteration of the physicochemical nature of goblet cell mucins in causing expulsion of intestinal parasites. Further analysis using experimental models showing goblet cell changes would facilitate the understanding of biological roles of ITF.

The excellent technical assistance of Ms. Eri Ohno is gratefully acknowledged. This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists No. 06770160, from the Ministry of Education, Science and Culture, Japan, and by grants from the Ichiro Kanehara Foundation, the Japan Research Foundation for Clinical Pharmacology, and the Uehara Memorial Foundation.

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