cDNA cloning of rat pS2 peptide and expression of trefoil peptides in acetic acid-induced colitis

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By using a combination of the methods of reverse transcription-PCR and rapid amplification of cDNA ends, a cDNA for rat pS2 peptide (rpS2) was successfully cloned and sequenced from rat stomach. By RNA blot analysis, the gene was shown to be expressed abundantly in the stomach and only faintly in the duodenum, but not in other tissues including the distal small and large intestines. rpS2 expression was also examined in the rectum during the course of acetic acid-induced colitis; rpS2 mRNA was detected during the acute phase of colitis but not in normal controls or during the recovery phase. On the other hand,

expression of rat intestinal trefoil factor (rITF) was down-regulated during the acute phase of colitis and then up-regulated during the recovery phase, whereas rat spasmolytic peptide was not detectable throughout the course of the induced colitis. These results indicate that the patterns and timing of the expression of these trefoil peptides are different from each other. rpS2 may play an important role in the proliferation of intestinal epithelial cells during the acute phase of mucosal ulceration, whereas rITF may be involved in differentiation of the cells, particularly to form goblet cells, during the recovery phase.

INTRODUCTION

The trefoil peptides constitute a family of regulatory peptides that have between one and six P-domains or trefoil motifs [1–4]. The pS2 peptide, which is the canonical peptide of the trefoil peptide family and contains a single trefoil motif, was originally identified as a breast cancer-associated peptide by differential screening of a cDNA library from the human breast carcinoma cell line MCF-7 [5,6]. Indeed, the pS2 peptide is widely expressed in various malignant tissues, including breast [7], pancreas [8], stomach [9] and colon [10] cancers. In normal tissues, however, pS2 and another trefoil peptide termed spasmolytic polypeptide (SP), which contains two trefoil motifs, have been identified only in the stomach, and not in the mammary gland or the distal small and large intestines [11–13]. On the other hand, the expression of intestinal trefoil factor (ITF), which also belongs to the trefoil peptide family and contains a single trefoil motif, has been reported to be confined to goblet cells of the small and large intestines [14–18].

During mucosal ulceration in the gastrointestinal tract, these three trefoil peptides (pS2, SP and ITF) were strongly expressed in an ulceration-associated cell lineage (UACL) that developed adjacent to the ulcer [16,19–21]. However, alteration of the expression of these peptides during the course of mucosal ulceration and recovery has never been explored in detail. In the present study we have cloned and sequenced rat pS2 (rpS2) cDNA, and examined its expression in comparison with those of rat ITF (rITF) and rat SP (rSP) in rat rectum during the course of acetic acid-induced colitis, which might represent UACL [22,23].

MATERIALS AND METHODS

Animals and treatments

Male Wistar rats were purchased from Seiwa Experimental Animals Ltd. (Fukuoka, Japan) and kept in clean conventional conditions in the Experimental Animal Center, Miyazaki Medical College. They were 8–10 weeks old at the start of experiments. Experimental colitis was induced by intrarectal administration of 5% acetic acid under ether anaesthesia [22,23]. Briefly, the rectum was lavaged with 2 ml of saline for enema and then 2 ml of 5% acetic acid was administered into the rectum 4 cm proximal to the anus. At 1, 2, 4 or 6 days after administration, rats were killed by an overdose of ether and the rectal tissues showing macroscopic erosion were used for histological examination and RNA extraction. Three rats were used at each designated time point, and the histological and RNA samples were prepared separately from each rat. Control rats (day 0) were only lavaged with 2 ml of saline. In addition to the rectum, various tissues from control rats were used for RNA extraction to examine the tissue distribution of rpS2.

RNA extraction and histological examination of resected tissues

Each rectum resected from an acetic acid-treated or control rat was longitudinally divided into two pieces, one for total RNA extraction and the other for histological examination. Total RNAs from the rectum and various other tissues, including the stomach, were extracted with TRIZOL® (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) according to the manufacturer's protocol. Tissues for histology were fixed with neutral buffered

Abbreviations used: rpS2, rat pS2; mpS2, mouse pS2; hpS2, human pS2; ITF, intestinal trefoil factor; rITF, rat ITF; SP, spasmolytic polypeptide; rSP, rat SP; UACL, ulceration-associated cell lineage; RACE, rapid amplification of cDNA ends; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; EGF, epidermal growth factor.

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under accession no. D83231.

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1:  gatcactcgtggtcttccctggaagctgccatggagcacaaggtagacctgtgtcctcgcc
(-21)                                     M E H K V T C V L A
                                     P1
61:  atggtcctcatgctggctctcagcagccttgccagaaaccaggaagaacatgtgccgtg
(-11) M V L M L A L S S L A Q N Q E E T C A V
      P2                                     P4
121: atccccgggagagagaataaattgtggttccccgggtgtcaccgcccagcagtgcaaggaa
(10) I P R E R I N C G F P G V T A Q Q C K E
      P3
181: aagggttgctgttttgatgacagtgtccgggggttcccatgggtgcttccgacctctggtc
(30) K G C C F D D S V R G F P W C F R P L V
241: attgagaaccagcaagaagaagaatgtcccttctaagggtccatccgagagaactggttac
(50) I E N Q Q E E E C P F *
301: atcaagactcggcaccctcccgccaaacctggacacctggagccacctggcccacctg
361: cttcatacacacctgttcggtggctggatctggcctggtgacacagttcaacctctcaga
421: cttttaaccctcaaattcggcctgattattaaagatatgaatgct

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Figure 1 Nucleotide and deduced amino acid sequence of rpS2

The proposed signal peptide is underlined, the stop codon is marked with an asterisk, and the polyadenylation signal is indicated by a shaded box. The amino acids are numbered (in parentheses) with position +1 set at the start of the predicted mature peptide. The direction and locations of primers P1–P4 are indicated.

formalin overnight and then embedded in paraffin wax. Serial 4 µm-thick sections were deparaffinized, rehydrated and stained with Alcian Blue and periodic acid–Schiff reagent.

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

A 10 µg sample of total RNA extracted from the stomach of a control rat was reverse-transcribed by a DT7 primer and SuperScript reverse transcriptase (Life Technologies, Inc.). The DT7 primer contains oligo(dT)₁₇ at the 3' end followed by a PT7 anchor primer sequence. For 3' RACE [24,25], the first-strand cDNA was amplified by PCR with primers P1 and PT7, and the resulting first-round RACE products were amplified again with primers P2 and PT7. Primers P1 and P2 were degenerate, and were designed based on well conserved regions of mouse (mpS2) and human pS2 (hpS2) cDNA sequences [6,12]. Each thermal cycle profile was 1 min at 94 °C, 1 min 30 s at 50 °C and 2 min 30 s at 72 °C, for 35 cycles. After electrophoresis, the 3' RACE product of the expected size was cut from a 2% agarose gel, purified using 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 [26]. After sequencing of this 3' RACE product, primers P3 and P4 were designed from rat-specific inner regions of the oligonucleotide, and then 5 µg of total RNA extracted from rat stomach was reverse-transcribed with the P3 primer for subsequent 5' RACE [18,25,27]. For 5' RACE we used a 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 primer P4 and the anchor primer P5. The 5' RACE products were purified, cloned and sequenced as described above.

Primers and anchor sequences used were as follows: primer P1 (sense), 5'-CCATGGAGA(C)ACAAGGTGAC(T)CTG-3'; primer P2 (sense), 5'-CCTCATGCTGGCCC(T)TCGGCA-3'; primer P3 (antisense), 5'-GGGAAGCCACAATTTATTCT-3'; primer P4 (antisense), 5'-ACATGTTTCTTCCTGGTTCT-3'; primer P5, 5'-CTGGTTCGCCCCACCTCTGAAGGTTCCA-GAATCGATAG-3'; primer PT7, 5'-AATACGACTCACT-

ATAG-3'; primer DT7, 5'-TAATACGACTCACTATAGGG-ATTTTTTTTTTTTTTTT-3'; 5' anchor, 3' NH3-GGAGAC-TTCCAAGGTCTTAGCTATCACTTAAGCAC-p-5'. The direction and location of primers P1–P4 are shown in Figure 1.

RNA blot analysis

Samples of 30 µg of total RNA from various tissues were denatured with formamide, fractionated by electrophoresis on a 1% formaldehyde/agarose gel and transferred to a Hybond-N nylon membrane (Amersham) as described [26]. Four identical membranes containing rectum total RNA from the colitis rats at determined time points were prepared for hybridization with rpS2, rITF, rSP and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probes. Hybridization was performed under high-stringency conditions using ³²P-labelled probes prepared by a random-primed DNA labelling method [26]. The rpS2 probe used was the 3' RACE product corresponding to nucleotide positions 66–466. The rITF and rSP probes, corresponding to positions 1–265 (rITF) and 13–427 (rSP), were made by PCR. The control human G3PDH probe, which strongly cross-hybridizes to rat G3PDH mRNA, was purchased from Clontech. In the time course experiments, the intensities of signals were directly quantified using a Bioimaging Analyzer, Fujix BAS 2000 system (Fuji Photo Film, Tokyo, Japan), and the ratio of each trefoil peptide (rpS2, rITF and rSP) to G3PDH was calculated [18,28]. The results (see Figure 5) are means ± S.D. from three rats at each designated time point.

Statistical analysis

Data from RNA blot analysis were analysed using Student's *t* test. A *P* value of < 0.05 was considered significant.

RESULTS

Sequencing of rpS2 cDNA

To obtain full-length rpS2 cDNA, we first performed 3' RACE with the degenerate primers P1 and P2, which were designed based on the well conserved region of mpS2 and hpS2 cDNAs [6,12]. A 3' RACE product of the expected size (approx. 400 bp) was obtained and sequenced. Based on this partial cDNA sequence, 5' RACE was performed using primers P3 and P4,

Figure 2 Comparison of the deduced amino acid sequence of rpS2 with those of hpS2 and mpS2 (A), and of the other rat trefoil peptides rITF and rSP (B)

Identical amino acid residues are indicated by asterisks, and the well conserved trefoil motif $CX_6RX_2CGX_8CX_4CCX_9WCF$ is indicated by boxes. The percentage identities of rpS2 with hpS2 and mpS2 (predicted mature peptide) (A) and with rITF and rSP domain 1 (D1) and 2 (D2) within the trefoil motif (B) are also indicated.

which were specific for rpS2. By these steps, the full-length cDNA sequence was obtained. To obtain maximum accuracy, after 3' and 5' RACE at least six individual clones obtained at each step were separately sequenced and confirmed to be completely identical with each other in both directions. The total rpS2 nucleotide sequence of 466 bp and its deduced amino acid sequence are shown in Figure 1. A presumed initial ATG codon was found 31 nucleotides downstream of the 5' end. A stop codon (nucleotide 274) and a further 193 bp of non-coding region including a presumed polyadenylation signal, ATTAAA (nucleotides 448–453), were found at the 3' end. Thus a peptide of 81 amino acids was encoded by a single open reading frame. The first 21 amino acids probably represent the signal peptide, based on comparisons with hpS2 and mpS2 [6,12]. Consequently the initial Met was numbered –21, and the Gln considered to be the N-terminal residue of the mature peptide was numbered +1.

Comparison with other trefoil peptides

When the deduced amino acid sequence of rpS2 was compared with those of mpS2 and hpS2 (Figure 2A) [6,12] and of the other rat trefoil peptides rSP and rITF (Figure 2B) [13,14], the cysteine-rich P-domain or trefoil motif, $CX_6RX_2CGX_8CX_4CCX_9WCF$, was well conserved (boxes in Figure 2). The amino acid sequence identity of rpS2 mature peptides with mpS2 and hpS2 was 81.7% and 66.7% respectively. When the trefoil motif of rpS2 was compared with that of other rat trefoil peptides, the identity was 44.9% with rITF [14], and 51.0% and 32.7% with domain 1 and domain 2 respectively of rSP [13]. Therefore the cDNA reported here was thought to represent the rat homologue of mpS2 and hpS2, but was clearly distinct from the other rat trefoil peptides rITF and rSP.

rpS2 expression in various tissues

The tissue distribution of rpS2 transcripts in the normal rat was examined by RNA blot analysis. As shown in Figure 3, the rpS2 mRNA signal with an approximate size of 500 bases was

Figure 3 RNA blot analysis of rpS2 expression in various tissues

Samples of 30 μ g each of total RNA derived from the tissues shown were loaded on to the membrane. Hybridization was performed under high-stringency conditions, and the membrane was probed with 3' RACE products corresponding to nucleotide positions 66–466. The membrane was exposed at -80°C for 2 days. Expression of the control G3PDH probe is also shown.

detected strongly in the stomach (2 days exposure) and faintly in the duodenum (10 days exposure; results not shown), but not in other tissues including the distal small and large intestines, even after a long exposure (10 days). These results were confirmed by two separate experiments using RNA preparations from different control rats. Such an expression pattern of rpS2 was basically consistent with those of mpS2 and hpS2 [6,12]. Since the probe used for RNA blot analysis hybridized to the digested rat genomic DNAs as single bands in Southern blot analysis under the high-stringency conditions used (results not shown), and

Figure 4 Histology of rat rectum on days 0 (A), 1 (B), 2 (C), 4 (D) and 6 (E) after acetic acid administration

Note the marked decrease (B) followed by a gradual recovery (C–E) in the number of mucin-secreting cells compared with the control (A). Staining was with Alcian Blue and periodic acid/Schiff reagent. Magnification $\times 65$.

Figure 5 RNA blot analysis of the expression of rpS2, rITF and rSP, and their peptide/G3PDH expression ratios, in the rectum during the course of acetic acid-induced colitis

Left panels: samples of 30 μ g each of total RNA derived from the tissue at the time points indicated (days) were loaded and hybridization was performed under high-stringency conditions. The transblotted membrane was probed with rpS2, rITF, rSP and control G3PDH, and exposed at -80°C for 7, 2, 14 and 2 days respectively. The results shown are from one of three independent experiments using different rats giving similar results. Right panels: the radioactivity ratios of the trefoil peptides to control G3PDH expression were calculated using a Bioimaging Analyzer BAS 2000. The results are means $\pm$ S.D. from three rats at each time point. * $P < 0.01$ compared with day 0.

since the expression patterns of rITF [14] and rSP [13] were different from that of rpS2 in this study, the signal detected was thought to represent only rpS2.

Expression of trefoil peptides during the course of acetic acid-induced colitis

Experimental colitis showing mucosal ulceration with marked inflammatory infiltrate and haemorrhage was induced in the

rectum of rats shortly after administration of acetic acid. After acetic acid treatment, the numbers of goblet cells and columnar cells drastically decreased as early as 1 day after administration, and then recovered gradually to the normal level by day 6 (Figure 4). The mRNA signals and the ratio of expression of rpS2, rITF and rSP to that of G3PDH at each time point are shown in Figure 5. rpS2 mRNA was detected during the acute phase of colitis (days 1 and 2), but not in normal control rats (day 0) or during the recovery phase of colitis (days 4 and 6). The

ratio of rpS2 to G3PDH expression was increased significantly at days 1 and 2 after treatment ($P < 0.01$ compared with day 0). rITF expression was down-regulated during the acute phase of colitis (days 1 and 2) and then up-regulated during the recovery phase (days 4 and 6). The ratio of rITF to G3PDH expression was decreased to about 25% of that of controls ($P < 0.01$ compared with day 0) at days 1 and 2 after treatment, and then increased up to 2.5-fold ($P < 0.01$ compared with day 0) at day 6 after treatment. rSP mRNA was not detected throughout the time course examined.

DISCUSSION

In this study, we have cloned and sequenced rpS2 cDNA. The deduced amino acid sequence is very similar to those of hpS2 and mpS2 [6,12], but very different from those of other rat trefoil peptides, rITF and rSP, except for the well conserved cysteine residues of the trefoil motif (see Figure 2) [13,14]. rpS2 is thought to be produced as a propeptide encoding 21 residues of signal peptide and 60 residues of a mature peptide. The predicted cleavage site between the signal peptide and the mature peptide of rpS2, Ala⁻¹ and Gln¹, is in accordance with the rules of von Heijne [29].

In the present study, rpS2 was expressed strongly in the stomach and faintly in the proximal small intestine, but not in the distal small and large intestines of normal rats. Such expression patterns are the same as those reported in humans [11] and mice [12], and are basically similar to those of hSP and mSP [11–13]. In addition to the normal expression sites, we demonstrated that rpS2 mRNA was induced during the acute phase of colitis, but was not detected in normal controls or during the recovery phase of colitis. In contrast, rITF was down-regulated during the acute phase of colitis and then gradually up-regulated during the recovery phase. These trefoil peptides might operate sequentially in repairing the damaged mucosa: rpS2 for the proliferation of intestinal epithelial cells and rITF for differentiation of the cells, particularly to give goblet cells. In contrast with rpS2 and rITF mRNAs, rSP mRNA was not detected throughout the time course examined. Since the three trefoil peptides, including SP, were co-expressed in UACL of human gastric ulcer and Crohn's disease [2,16,19,20] and of rat gastric ulcer [21], the lack of rSP expression in the ulcerated colon of rats may be a unique feature of colonic UACL in this animal. Further studies are required to elucidate the tissue distributions of trefoil peptides in different animal species in health and disease.

Concerning the regulation of expression of pS2, the regulatory region of the pS2 gene contains a complex enhancer region that is responsive to oestrogen, epidermal growth factor (EGF), phorbol 12-myristate 13-acetate and the proto-oncogene products c-Ha-ras and c-jun [30]. Although pS2 expression in breast cancer is induced by oestrogen, its expression in the stomach is thought to be induced by EGF or a similar growth factor [19,20,31] because the gastric mucosa does not have oestrogen receptors [32]. Since the regulatory regions of the rITF and hITF genes do not contain an EGF-responsive element [33,34], the mechanisms of regulation of expression of pS2 and ITF might be different from each other.

Little is known about the biological roles of the trefoil peptides, except that SP causes a reduction in intestinal motility by its spasmolytic effects [35], inhibits gastric acid secretion [35] and has mitogenic activity with some cultured cancer cells [36]. pS2 and ITF, both of which contain only a single trefoil motif, can easily form covalent dimers [37,38] and are also thought to be putative growth factors by analysis of the crystal structure of the

trefoil motif [39]. Recently, trefoil peptides were found to promote epithelial migration through a transforming-growth-factor- β -independent pathway [40] and to protect the gastrointestinal mucosa from a variety of insults via interactions with mucin glycoproteins [41]. Therefore trefoil peptides as a group seem to play an important role in repairing the damaged mucosa, co-operatively interacting with mucin glycoproteins [41] and some growth factors such as EGF [32], fibroblast growth factor [42], hepatocyte growth factor [43,44] and keratinocyte growth factor [44].

In summary, we have isolated rpS2 cDNA and compared its expression patterns with those of other trefoil peptides during the course of experimental colitis in rats. rpS2 may play an important role in the proliferation of intestinal epithelial cells during the early phase of mucosal ulceration, whereas rITF may be involved in the differentiation of the cells during the recovery phase.

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