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Author(s)	Nakamura, Kiminori; Sakuragi, Naoya; Ayabe, Tokiyoshi
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Instructions for use

A monoclonal antibody-based sandwich enzyme-linked immunosorbent assay for detection of secreted α-defensin

Short title: Quantification of cryptdin-4 in the bowel lumen

Kiminori Nakamura, Naoya Sakuragi and Tokiyoshi Ayabe *

Department of Cell Biological Science, Faculty of Advanced Life Science, Graduate School

of Life Science, Hokkaido University

Corresponding Author: Tokiyoshi Ayabe M.D., Ph.D.
Innate Immunity Laboratory, Department of Cell Biological Science, Graduate School of Life Science, Faculty of Advanced Life Science, Hokkaido University
21-Kita, 11-Nishi, Kita-ku, Sapporo, Hokkaido 001-0021, Japan
Telephone: +81-11-706-9049
Fax: +81-11-706-9053
E-mail: ayabe@sci.hokudai.ac.jp

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Abstract

Paneth cells at the base of small intestinal crypts secrete α-defensins, which contribute to innate immunity and shape composition of enteric microbiota. Efforts to establish relationship between secreted α-defensins and disease have been hampered by a lack of sensitive assays to quantify luminal α-defensins. Here we report on a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for the mouse Paneth cell α-defensin cryptdin-4 (Crp4) in varied sources, including luminal contents rinsed from stomach to distal colon, as well as fecal pellets. One pair of monoclonal antibodies (mAbs), selected from 10 rat hybridomas secreting Crp4-specific mAbs, was optimized for Crp4 detection and specificity in the sandwich ELISA. In CD1 mice, luminal Crp4 levels gradually increased from 6.8 ± 5.2 ng/ml in proximal to 54.3 ± 10.3 ng/ml in distal small intestine, and the peptide was detected in colonic lumen and feces. Secreted Crp4 was reduced significantly in feces of IL10-null mice, a model of inflammatory bowel disease (IBD) when compared with wild-type controls. This Crp4 sandwich ELISA enable accurate determinations of luminal α-defensins as biomarkers of Paneth cell function and enteric integrity in diverse disease states such as IBD, infectious disease, graft-versus-host-disease, and obesity in association with dysbiosis of the intestinal microbiota.

Keywords: sandwich ELISA; monoclonal antibodies; paneth cell; α-defensin; cryptdin; dysbiosis; inflammatory bowel disease; biomarker.

Introduction

Innate immunity is at the front line of host defense in plants, invertebrates, and mammals [1], and antimicrobial peptides (AMPs) are major effectors of innate immunity [2-4]. In the small intestine, α-defensins, termed cryptdins (Crps) in mice and human defensin (HD)5 and HD6 in humans, are the predominant AMP family found in the cytoplasmic granules of Paneth cells which are located at the base of the crypts of Lieberkühn. Crps are secreted into the lumen of intestinal crypts in response to cholinergic and microbial stimuli [5]. Once secreted, α-defensins elicit potent bactericidal activity and contribute to innate immunity in the small intestine [6, 7], as suggested by previous *in vivo* studies. For example, DEFA5+/+ transgenic mice, which express HD5 at the same level as endogenous Crps, are immune to oral infection by *Salmonella enterica serovar* Typhimurium infection. On the other hand, mice deficient in matrix metalloproteinase-7 (MMP7), the intracellular Paneth cell pro-α-defensin convertase, show increased susceptibility to oral *S*. Typhimurium infection [6-9].

In the intestinal lumen, huge numbers, over 10^{11} cells per gram [10], of commensal bacteria colonize and elicit beneficial effects in the host. Alteration of those microbial communities, termed dysbiosis, in patients or experimental animals is associated with many dis-

ease states including IBD, obesity, cancer, diabetes, GVHD and allergy [11-16]. Also,

α-defensins regulate intestinal homeostasis by modifying the composition of the commensal

microbiota, influencing intestinal mucosal immunity [17]. Moreover, Masuda et al.

[18] reported that Crp4 showed only attenuated bactericidal activity against commensal bac-

terial species but potent activities against the non-commensal bacterial species tested *in vitro*.

Collectively, these studies support the suggestion that dysbiosis induced by changes in lu-

minal α-defensin concentrations contributes to disease pathogenesis. Studies also show that

onset of obesity and Crohn's disease are related with the reduction of α-defensins mRNA and

protein expression in the small intestinal tissue [19, 20].

Human Paneth cells express two α-defensin peptides, HD-5 and HD-6 [21, 22], but

the mouse α-defensin gene family is larger, coding for approximately 20 different Crp

isoforms in mouse [23]. Crps 1-6, have been purified and characterized from mouse small in-

testine [12,13], showing several features of Crp4 that distinguish it from other Crps.   For

example, the Crp4 has the highest antimicrobial activity of the known mouse enteric

α-defensins in *in vitro* assays [23]. Also, the Crp4 gene has a unique pattern of expression

along the longitudinal axis of the small intestine. In contrast to Crp1 and Crp5, whose

mRNAs and proteins occur at approximately equivalent levels along the length of the mouse

small bowel, Crp4 mRNA and protein levels increase along the proximal-to-distal axis to reach maximal concentration in the distal ileum [24]. These findings suggest that Crp4 may play a pivotal role in intestinal innate immunity and maintenance of enteric homeostasis.

At present, determining levels of α-defensins relied on quantitative reverse transcription polymerase chain reaction (qRT-PCR) or immunohistochemical analyses of tissue samples with peptide-specific antisera. However, these approaches lack the capability of accurately determining how modest changes in α-defensin concentration influence the microbiota composition. To establish a method that enables such measurements of luminal α-defensins, and to understand roles for α-defensins in intestinal homeostasis, we developed a highly sensitive and specific sandwich enzyme-linked immunosorbent assay (ELISA) for Crp4 based on two optimal monoclonal antibodies (mAbs) raised against native Crp4. To measure the secreted Crp4 using this sandwich ELISA, we also optimized sample preparation methods *in vitro* and *in vivo*. In addition, to assess α-defensin involvement in the onset of IBD, we quantified luminal Crp4 with the ELISA in IL10-/- mice, a representative model of IBD [25].

Materials and Methods

Animals

BALB/c and CD1 mice were purchased from Charles River Laboratories Japan (Yokohama, Japan), AKR/J mice from Kyudo (Saga, Japan), and IL-10-/- mice on a BALB/c background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were males and kept in conventional conditions under 12 h light cycles. Animal experiments were approved by the Committee on Animal Experimentation, Hokkaido University.

Preparation of native, oxidized-Crps and reduced-Crps

Oxidation of synthetic Crp1, 2, 3, 4 and 6 (Medical and Biological Laboratories, Nagoya, Japan) to form three intramolecular disulfide bonds was performed as described previously [18]. Crps were then purified to homogeneity using reverse-phase high-performance liquid chromatography (RP-HPLC). Oxidized Crps, termed n-Crps, were purified by a C-18 column (SepaxHP-C18, 4.6 X 150 mm, 5 µm; Sepax Technologies, Inc, Newark, Del, USA) in 0.1% trifluoroacetic acid with an 18-36% acetonitrile gradient developed over 30 min at 1 ml/min. The purified n-Crps were lyophilized and stored at -30 °C until use. Reduced-Crp4 (r-Crp4), in which the three disulfide bonds were reduced, was prepared by dissolving n-Crp4 in 500 mM dithiothreitol and left to stand overnight at 4 °C. The reaction mixture was then

applied to a C-18 RP-HPLC column and under the same conditions used in the purification of n-Crp4. The purified r-Crp4 was lyophilized and stored at -30 °C until use.

Production of mAbs against Crp4.

Rat monoclonal antibodies that specifically recognized Crp4 were generated based on"GLLCYCRKGHCKRGERVRGTCGIRFLYCCPRR" the rat lymph node method [26]. A Sprague Dawley (SD) rat was immunized with n-Crp4 (GLLCYCRKGH-CKRGERVRGTCGIRFLYCCPRR). After ten days, rat lymphocytes were fused with mouse myeloma P3U1 cells in the presence of PEG. Hybridoma clones producing Crp4-specific antibody were screened by indirect ELISA and subcloned four times by the limiting dilution method. Isotype of mAbs was determined by applying the rat Ig isotyping kit (Serotec, Oxford, UK).

Indirect ELISA

The wells of the flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) were coated with 1 µg/ml of n-Crp4 in Ca^{2+}/Mg^{2+}-free Phosphate Buffered Saline (PBS) and incubated overnight at 4 °C. After three washes with PBS containing 0.1% Tween 20 (PBS-T), mAbs against Crp4 were added to the wells, and incubated for 1 h at 37 °C. After extensive washing, horseradish peroxidase (HRP)-conjugated goat anti-Rat IgG (GE Healthcare Bio-

sciences, Piscataway, NJ, USA) was added to the wells and incubated for 45 min, followed by another wash. Finally, color development was performed by adding 100 μL of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate buffer (KPL, Gaithersburg, MD, USA). The plates were incubated for 30 min at 25 °C, and the absorbance at 405 nm was detected with a micro-plate reader (Multiscan FC, Thermo Scientific, Waltham, MA, USA).

Pairwise antibody selection

Each of the ten mAbs was labeled with biotin and then utilized as either the capture or the detection antibody to determine the optimum combination. Briefly, 100 μl of coating Crp4 mAb (30 μg/ml) was added to each well of a microtiter plate and incubated overnight at 4 °C. The plate was then washed three times with PBS-T. After three washes, 100 μl of n-Crp4 (10 ng/ml or 0.01 ng/ml) with PBS-T, was added to each well and incubated for 1 h at 25 °C. After three washes, the biotin-labeled detection antibody (0.5 μg/ml) was added to the wells and the plate was incubated for 1 h at 25 °C. Coupling of biotin to Crp4 mAbs was per-formed according to the manufacturer's protocol (Dojindo Laboratories, Kumamoto, Japan). After extensive washing, the color development was carried out by the addition of 100 μl of TMB substrate buffer. The plates were incubated for 30 min at 25 °C, and the absorbance at 450 nm (absorption wavelength) and 620 nm (reference wavelength) was detected using a

microplate reader.

Sandwich ELISA procedure

The sandwich ELISA was carried out in microtiter plates. Microtiter plate wells were coated overnight at 4 °C with 100 μl of the capture antibody at a concentration of 1 μg/ml in 50 mM sodium carbonate-bicarbonate buffer (pH 9.6). The plate was then washed 3 times with PBS-T and blocked for 1 h at 25 °C with 200 μl of 25% Block Ace (DS Pharma Biomedical Co, Suita, Japan). To prepare an ELISA standard curve, a serial dilution of synthesized n-Crp4 was prepared in the range 0.02–5 ng/ml. One hundred microliters n-Crp4 or samples were added to the wells and incubated at 25 °C for 2 h. After washing in PBS-T, 100 μl of 0.5 μg/ml biotinylated detection antibody was added at 25 °C for 1 h. Subsequently, the wells were incubated with 100 μl of Streptavidin-HRP conjugate (GE Healthcare Biosciences, Piscataway, NJ, USA) in a 1:5000 dilution at 25 °C for 1 h. After final washes, 100 μl of TMB chromogen substrate buffer was added and incubated at 25 °C for 30 min. The reaction was stopped by the addition of 100 μl of 0.6 N H_2SO_4 and absorbance values were determined at 450 nm using a microplate reader. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated through 10 measurements of the blank. We applied the following formulas: LOD = 3.3 SD/S, LQ = 10 SD/S, where SD is the standard deviation of the response

and *S* is the slope of the calibration curve, according to the guidelines of International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

Immunohistochemistry

The small intestinal tissue specimens from the CD1 mice were fixed in 10% neutralized buffered formalin, embedded in paraffin, placed on poly-L-lysine-pretreated slides. For immunohistochemistry, after deparaffinization and rehydration, the antigens were retrieved in an autoclave at 120 °C for 10 min with Tris-EDTA buffer (pH 9.0). After the antigen retrieval, endogenous non-specific peroxidases were quenched with 0.3% hydrogen peroxide. Nonspecific binding was blocked with Block Ace. Sections were incubated overnight with each of the primary antibodies at 4 °C. After rinsing in PBS-T, the slides were incubated with HRP- labeled anti-rat IgG (HISTOFINE, Simple stain MAX-PO (Rat), Nichirei, Tokyo, Japan) for 30 min at room temperature. Thereafter, staining was developed with 3, 3`-diaminobenzidine tetrahydrochloride (DAB) solution.

Immunoblotting

Native-Crp4 was separated by electrophoresis on polyacrylamide gel (TGX Gel Any-kD, BioRad, Hercules, CA, USA). After electrophoresis, the proteins were transferred to

polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) by electroblotting.

Blots were blocked with StabilGuard (SurModics, Eden Prairie, MN, USA) for 1 h at room

temperature and incubated at 4 °C overnight with anti-Crp4 antibodies diluted 1:1,000 in

PBS-T. After several washes with PBS-T, the membranes were incubated for 1 h with goat

anti-rat IgG-HRP (Imgenex, San Diego, CA, USA) diluted 1:5,000 in PBS-T. After washing,

proteins were detected using chemiluminescent substrate (Chemi-Lumi One, Nacalai tesque,

Kyoto, Japan).

Preparation and analysis of isolated mouse crypts

Crypts were prepared by EDTA treatment of everted small intestine segments as

described [4]. Briefly, segments of adult mouse small bowel were agitated in buffered 30 mM

EDTA (pH 7.4), and eluted crypts were deposited by centrifugation and resuspended in

ice-cold Ca^{2+} /Mg^{2+} -free buffer.

Bactericidal assays

Bactericidal assays were performed on secretions collected from crypts exposed to

10 μM CCh using a defensin-sensitive strain of *S. enterica* serovar Typhimurium Δ*phoP* [27].

In triplicate, 1000 colony-forming units (CFU) of exponentially growing cells were deposited

by centrifugation, resuspended in 10 μl of 10 mM PIPES, pH 7.4, supplemented with 0.01

volume (1%, v/v) of Trypticase soy broth (PIPES/TSB) and combined with 10 µl of secretions collected from crypts. After 60 min at 37 °C, 10 µl of each incubation mixture were diluted 1:100 with PIPES/TSB and 50 µl of the diluted samples were plated on Trypticase soy-agar plates using an Autoplate 4000 (Spiral Biotech Inc, Bethesda, MD, USA). Surviving bacteria were quantitated as CFU/ml on plates after incubation at 37 °C for 18 h.

Preparation of luminal contents in separated gastrointestinal tract

The specimens were obtained from the following portions of the CD1 mouse gastrointestinal tract: stomach, duodenum (distal to the pylorus, approximately 3 cm length), jejunum (distal to the duodenojejunal flexure, approximately 20 cm length), ileum (proximal to the ileocecal junction, approximately 20 cm length), cecum (distal to the ileocecal junction, approximately 2,5 cm length), proximal colon (distal to the cecocolonic junction, approximately 5 cm length) and distal colon (proximal to the anus, approximately 5 cm length). For these samples, the luminal contents were flushed from the segments with deionized distilled water and the washed solution was collected. Samples were vortex mixed for 1h at 4 °C and centrifuged at 630 X g for 10 min to yield a clear solution and lyophilized.

Preparation of fecal samples

Fecal samples were collected from CD1, AKR/J, BALB/c and IL-10 KO mice,

air-dried and powdered using a bead beater-type homogenizer (Beads Crusher µT-12, TAITEC, Saitama, Japan). Ten milligram samples of powdered stool were mixed with 100 µl of PBS for 10 min and then were vortex mixed for 1 h at 4 °C. After clarification by centrifugation at 20,000 g for 20 min. Crp4 in supernatants was measured by the sandwich ELISA.

Statistical Analysis

All measured values are presented as the mean ± standard deviation, the statistical examination was performed with Student's t-test, and a P-value of < 0.05 was considered statistically significant.

Results

Ten hybridoma cell lines secreting monoclonal antibody against Crp4 were obtained by immunization of SD rats with n-Crp4. These hybridomas were propagated, the monoclonal antibodies were harvested and characterized in various immunoassays as shown in Table 1. A representative immunohistochemistry image of normal mouse ileum tissue is shown in Fig. 2A. The immunohistochemical staining shows that, after retrieval, the antigen is distributed uniformly in Paneth cell granules in which Crp4 is localized.

To determine optimal combination of antibodies for the establishment of a sandwich-ELISA, we performed pairwise interaction analyses. Ten different affinity-purified antibodies (cf. Tables 1) were used separately as capture reagents and each was tested in combination with ten different biotin-labeled monoclonal antibodies that were used for detection of captured n-Crp4 antigen. The results of 10 x 10 pairwise interaction analyses are presented in Fig.1. When the antigen concentration was 10 ng/ml, A_{450} values greater than 3.0 were measured for seven paired mAbs (Fig.1A). Further, the seven mAb combinations were evaluated at 1 ng/ml n-Crp4.   Under these conditions, the highest A450 value was obtained when mAb 74-6 was the capture antibody and biotin-labeled mAb 74-14 was used as the detection antibody (Fig.1B). When Crp4 concentrations and absorbance were expressed logarithmically,

detection was linear for Crp4 values from 0.04 to 2.5 ng/ml (Fig. 2A). The linear regression

obtained from the standard curve after polynomial transformation showed a good correlation

coefficient ($R^2 = 0.99$). According to ICH guidelines, the LOD and LOQ were determined

using standard curve around the blank (Fig. 2B). The LOD was 80 pg/ml and LOQ was 233

pg/ml for this sandwich ELISA. The dynamic range for this sandwich ELISA was between

0.23 and 2.5 ng/ml (Fig. 2C).

To test the Crp isoform specificity of the sandwich ELISA, assays were performed

on n-Crps 1, 2, 3, 4 and 6.   Positive immunoreactivity was detected only for n-Crp4, indi-

cating that the combination of mAb 74-6 and mAb 74-14 was highly peptide-specific, lacking

cross-reactivity with other Crp isoforms (Fig. 2D).   Because α-defensins are characterized

by three invariant disulfide bonds, we evaluated the conformational specificity for native

Crp4 in this ELISA by testing the immunoreactivity of reduced Crp4 (r-Crp4), which lacks

disulfide bonds. r-Crp4 had no immunoreactivity, even at 10 ng/ml of peptide, under these

assay conditions (Fig. 2E). Thus, this ELISA is highly specific for n-Crp4 and constitutes a

highly sensitive detection system for n-Crp4.

To test the applicability of the Crp4 ELISA to the analysis of biological samples,

we assayed for Crp4 secreted by Paneth cells *in vivo* and *in vitro*. First, we isolated small in-

testinal crypts *in vitro* by EDTA treatment from adult CD1 mice as described previously [5] and stimulated them with the muscarinic receptor agonist carbamyl choline (CCh), which selectively modulates Ca^{++} dynamics in Paneth cells. The isolated intact crypt method is useful for characterizing Paneth cell secretory responses and to define the role of Crps in elicited secretions. The level of secreted Crp4 released in response to CCh stimulation increased in a manner that was dependent on crypt number, as determined by sandwich ELISA measurements (Fig. 3A). The levels of secreted Crp4 released in response to CCh stimulation were 1.22 ± 0.11, 5.49 ± 0.46, 20.45 ± 0.68 ng/ml per 1000 crypts, 10.30 ± 0.49, 30.85 ± 0.69, 54.46 ± 2.28 ng/ml per 5000 crypts, 22.28 ± 2.50, 40.36 ± 2.66, 76.75 ± 9.87 ng/ml per 10000 crypts and 109.98 ± 1.98, 125.72 ± 4.91, 161.09 ± 8.51 ng/ml per 20000 crypts, respectively from three separate experiments. Released Crp4 was not detected for unstimulated crypts (data not shown). The bactericidal activities of the elicited supernatants against small intestinal noncommensal bacteria, *S*. Typhimurium *ΔphoP* were consistent with the Crp4 ELISA determinations, showing potent bactericidal activities as a function of crypt number (Fig. 3B). Although additional microbicides are released by Paneth cells, these data indicated that Crp4 could be quantitated in Paneth cell secretions stimulated by CCh and that bactericidal activity corresponded to Crp4 levels.

To determine whether the Crp4 sandwich ELISA can measure Crp4 in the intestinal lumen *in vivo*, Crp4 was measured in samples prepared by rinsing the luminal contents of segments of the lower gastrointestinal tract. Luminal contents were prepared separately from stomach, duodenum, jejunum, ileum, cecum, proximal colon and distal colon (Materials and Methods), and Crp4 levels in each sample were measured by the sandwich ELISA (Fig. 4). The concentrations of Crp4 were 6.81 ± 5.26 ng/ml, 29.07 ± 8.37 ng/ml and 54.36 ± 10.33 ng/ml in duodenum, jejunum and ileum, respectively, showing that luminal (Fig. 4A) Crp4 peptide concentrations increase almost 8-fold in the distal small bowel relative to the duodenum. Replicates of the same samples were highly reproducible, but luminal Crp4 levels was variable in different mouse samples. On the other hand, Crp4 was not detected in stomach luminal rinses, where Paneth cells are lacking. In segments distal of the terminal ileum, Crp4 concentrations of cecum and proximal and distal colon rinses were 1.18 ± 0.32 ng/ml, 0.38 ± 0.21 ng/ml and 1.04 ± 0.05 ng/ml, respectively (Fig. 4B). These large bowel values reflect the presence of Crp4 molecules that persist after having been secreted by small intestinal Paneth cells.   These results show that the ELISA allows measurement of secreted Crp4 in the intestinal lumen, where levels are markedly lower than corresponding small intestinal tissue levels.

To monitor changes in the level of secreted Crp4 at different time points without sacrificing mice and to test the feasibility of using Crp4 as a biomarker of small intestinal health and integrity, we measured Crp4 in feces. Fecal pellets were dried and pulverized allow the efficiency of Crp4 extraction to be measured precisely. Crp4 levels in normal CD1 and BALB/c mouse fecal extracts were 7.12 ± 1.87 ng/ml and 6.82 ± 1.74 ng/ml, respectively. In contrast, Crp4 was not detected in AKR/J fecal pellets, because the AKR/J strain lacks Crp4 (Masuda & Ayabe, unpublished). Thus, the Crp4 sandwich ELISA detects Crp4 in fecal samples and would be appropriate for quantitative determination of secreted Crp4 as a potential biomarker without euthanizing mice.

To test the concept that measurement of Crp4 as a potential biomarker with this sandwich ELISA may provide valuable insight into enteric integrity in IBD or other intestinal diseases, we measured Crp4 concentrations in the feces of interleukin (IL)-10-null mice, a representative model of IBD. The spontaneous onset of gastrointestinal inflammation is reported to become evident in around 7-week-old IL10-null mice [28]. The feces were collected at 7 weeks of age. Extracts of feces from IL10 null mice (IL10KO, n = 5), and wild type (BALB/c, n = 5) contained 4.37 ± 2.50 ng/ml and 10.23 ± 2.54 ng/ml of Crp4, respectively (Fig. 6). Secreted Crp4 was significantly reduced in IL10 null mice compare to that in wild

type. This result suggests that reduction of α-defensin released into the intestinal lumen might

be related to pathology of IBD.

Discussion

The Paneth cell, an intestinal epithelial lineage that resides at the base of the small intestinal crypts plays important roles in innate immunity [29]. Paneth cells contain the major effectors in innate immunity in their apically oriented secretory granules, and α-defensins are abundant Paneth cell granule components are known to contribute to maintain intestinal homeostasis [17, 18]. Moreover, Paneth cell numbers and α-defensin mRNA levels in the small intestine have been reported to be lower in obese subjects compared to lean subjects, suggesting possible relationship between α-defensins and obesity [20].

Sensitive, reliable methods for determining α-defensin peptide levels have not existed until now, making it difficult to assess their actual roles and underlying mechanisms in health and disease. One major reason for this deficiency has been the lack of specific and high-affinity monoclonal antibodies that recognize differing α-defensin epitopes. The novel sandwich ELISA we have developed for measurement of Crp4 secreted by Paneth cells in highly varied samples has been validated, using these newly established monoclonal antibodies. The ELISA system was highly sensitive in detecting Crp4 concentrations at a minimum concentration of 0.04 ng/ml and to have no cross-reactivity with other Crp4 isoforms. Furthermore, the sandwich ELISA only detected native Crp4 and not the linearized reduced form.

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When crypts dissociated from mouse small intestine were stimulated with CCh, crypt Paneth cells released Crp4 into the supernatant in a crypt number-dependent manner. Although Paneth cells release bactericidal secretions that contain Crps when stimulated with bacterial products such as muramyl dipeptide, a component of bacterial peptidoglycan [5, 30], or by cholinergic agonists [31, 32], the actual concentration of secreted Crp4 has not previously been measured. Since total numbers of stimulated crypts were known in each experiment, we can calculate levels of released Crp4 per one crypt. Crp4 level released from single crypt was estimated to be 0.46 – 0.90 pg under our experiment condition. The precise machinery in Paneth cell secretion or other cellular responses is yet to be clearly determined [33, 34]. To unravel the various mechanism of Paneth cells, it is vitally important to know what kind of substance regulates the secretion of α-defensins. By using the crypts dissociated from mouse small intestine and the newly established sandwich ELISA, the simple and high-throughput screening of defensin inducers may be feasible.

In this study, we first showed concentration of Crp4 throughout the enteric lumen. The gradient of secreted Crp4 concentration was observed when comparing duodenum, jejunum and ileum. The expressions of Crp4 analyzed by quantitative RT-PCR and immunohistochemistry in the tissue were known to be most abundant in the ileum [24, 35]. Our re-

sults confirmed that secreted Crp4 concentrations increase along the proximal-distal axis, with

highest levels in the distal ileum [36]. In this study, Crp4 were able to be determined in the

luminal contents of cecum and colon where Paneth cells are absent (Fig. 4). This observation

is in agreement with the finding that processed Crps were detected in the whole tissue plus

luminal contents of colon and cecum respectively [37, 38]. These findings confirmed that se-

creted α-defensins influence microbiota not only in small intestine but also in colon. Because

the sandwich ELISA reacts with native, fully folded Crp4, it is clear that certain secreted by

Paneth cells in the small intestine travel the full length of the bowel, and accumulate in the

feces as functional Crp4. The measurement of α-defensins secreted into the lumen using this

high sensitivity sandwich ELISA is essential to understand possible correlation between gut

microbiota and α-defensins.

Studies in humans and mice provide evidence that changes in Paneth cell α-defensin

expression may contribute to Crohn's disease pathogenesis and obesity, and that changes in

the microbiota may mediate these pathogenic mechanisms [11-16]. Therefore, to correlate

disease and α-defensin levels, it is important to monitor sequential changes in the levels of

α-defensins secreted into the intestinal lumen in mouse models of disease. Our sandwich

ELISA is able to measure Crp4 concentration in the feces, making sequential measurements

of secreted Crp4 possible without euthanizing mice and enabling reponses to therapeutic intervention to be followed prospectively. Secreted Crp4 levels in the feces were significantly reduced in IL10 null mice compare to those in wild type (Fig. 6), consistent with findings that Crp4 expression was significantly decreased using quantitative RT-PCR and AU-PAGE at 7-week in the IL10 null mice [28]. Our data suggested that the low levels of secreted Crp4 lead to possible dysbiosis, and may have a link with pathogenesis of IBD. The measurement of α-defensins in stool specimens by the sandwich ELISA may be a useful biomarker for small intestinal homeostasis and integrity.

In the present study, the sandwich ELISA for Crp4 was designed to provide a simple, precise, and highly sensitive method to detect Crp4 secreted from Paneth cells. The ELISA could potentially be used to study the α-defensin secretion mechanisms of the Paneth cells *in vitro*. Therefore, the results obtained with this ELISA may open new perspectives for using defensins as biomarkers in the study, diagnosis, and follow-up of diseases associated with Paneth cells α-defensin such as inflammatory bowel disease and obesity.

Acknowledgments

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Figure Legends

Fig. 1. Evaluation of the optimal antibody combination. (A) Sandwich ELISA for pairwise

interaction analysis. Each of the ten mAbs was used as the capture antibody or as the detection antibody. The absorbance values were measured for each antibody combination using native-Crp4 at 10 ng/ml. (B) The absorbance values were measured for seven antibody combinations which showed high absorbance value using native-Crp4 at 0.01 ng/ml.

Fig. 2. Establishment of the sandwich ELISA and its specificity and sensitivity. (A) Immunohistochemical localization of Crp4 in Paneth cells of mouse small intestine. Sections of adult CD1 mouse ileum were analyzed for the presence of the Crp4, by using purified monoclonal antibody 74-14 and 74-6 (see Table 1). In each panel, arrows indicate immunoperoxidase localization in Paneth cells of the crypts. (B) Determination of the limit of detection (LOD) and limit of quantitation (LOQ). The figure shows a very good linear response in the lower concentration range from 0 to 150 pg/ml. LOD was calculated with the equation: 3.3 * SD / S and LOQ was calculated with the equation: 10 * SD / S where: SD = the standard deviation of the blank, S = the slope of the calibration curve around detection limit. (C) Representative calibration curve of native-Crp4 starting at a concentration of 2.5 ng/ml with serial dilutions. Ln means natural log. (D) The isoform specificity of the sandwich ELISA was confirmed by using native-Crp4 (●), native-Crp1 (○), native-Crp2 (■), native-Crp3 (□), and native-Crp6 (▲).

(E) The conformational specificity of the sandwich ELISA was confirmed by using native-Crp4 (●) and reduced-Crp4 (○).

Fig. 3. Measurement of Crp4 in bactericidal secretions released by Paneth cells in isolated crypts stimulated with CCh. (A) Isolated crypts from an adult CD1 mouse were stimulated with 10μM CCh. Sample of collected secretions were assayed for the sandwich ELISA for Crp4. Three different experiments were done. Different symbols represent different experiments. n.d. means not detected. (B) Bactericidal activities of the same secretion samples against *S. enterica* serovar Typhimurium Δ*phoP*was performed. Isolated crypts were stimulated with 10μM CCh (●) or PBS alone (○). Data are expressed as means SD; n = 3. Experiments were performed triplicate.

Fig. 4. Measurement of Crp4 concentration in luminal contents in separated gastrointestinal (GI) tract from stomach to colon. The GI tract was segmented into (A) stomach, duodenum, jejunum, (B) ileum, cecum and proximal colon and distal colon. The contents of the lumen were removed by gentle pressure by PBS (-). Three different experiments were done. Different symbols represent different experiments. Data are expressed as means SD. n.d. means not

detected.

Fig. 5. Measurement of Crp4 concentration in fecal extracts of various mice. The fecal samples were collected from normal CD1 mice, BALB/c mice and AKR/J mice (n = 5). Crp4 concentration was determined by the developed sandwich ELISA. The horizontal bars indicated the mean concentrations.*, $p < 0.01$.

Fig. 6. Mesurement of secreted Crp4 concentration in the interleukin10 null mouse, a model of IBD. The fecal samples were collected from IL10 null mice (IL10KO, n = 5), and wild type (BALB/c, n = 5). Both WT and IL10KO mice were on a pure BALB/c genetic background. Crp4 concentration was determined by the developed sandwich ELISA. The horizontal bars indicated the mean concentrations.*, $P < 0.01$.