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ORIGINAL ARTICLE

Ileal Mucosal and Fecal Pancreatitis Associated Protein Levels Reflect Severity of Salmonella Infection in Rats

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Abstract *Background* Microbial infections induce ileal pancreatitis-associated protein/regenerating gene III (*PAP/RegIII*) mRNA expression. Despite increasing interest, little is known about the PAP/RegIII protein. Therefore, ileal mucosal PAP/RegIII protein expression, localization, and fecal excretion were studied in rats upon *Salmonella* infection. *Results* *Salmonella* infection increased ileal mucosal PAP/RegIII protein levels in enterocytes located at the crypt-villus junction. Increased colonization and translocation of *Salmonella* was associated with higher ileal mucosal PAP/RegIII levels and secretion of this protein in feces. *Conclusions* PAP/RegIII protein is increased in enterocytes of the ileal mucosa during *Salmonella* infection and is associated with infection severity. PAP/RegIII is excreted in feces and might be used as a new and non-invasive infection marker.

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Introduction

Pancreatitis associated protein (PAP) is a type III member of the regenerating (Reg) gene family and was originally identified as a lectin-related secretory protein present in rat pancreatic juice during experimental pancreatitis [21]. Since then, considerable attention has been given to the Reg family and its structurally related molecules. Recently, the complex terminology of the Reg family and its isoforms was elegantly reviewed [16], and a combined term of PAP/RegIII was coined to foster a concerted effort in the investigation of PAP and the isoforms. In this study we focused on ileal PAP/RegIII in rats, represented by the genes *PAP1* and *PAP3*. Both genes encode for a protein of similar size, and these genes are paralogues, which means that they derive from the same ancestral gene. The rat PAP3 (alias RegIIIγ) and mouse RegIIIγ are orthologues, which means that they are on the amino acid level the most identical genes between two species (bidirectional best hit). The other isoform, the rat PAP1 (alias PAP), is the orthologue of the mouse PAP1 (alias RegIIIβ).

Expression of *PAP/RegIII* mRNA has been shown in the pancreas of human [40], mouse [39], and rat [19]. Moreover, *PAP/RegIII* was described as constitutively expressed in the rat [20] and human [9] small intestine. Intestinal expression was not altered during acute pancreatitis [20], indicating that intestinal and pancreatic expressions are differentially regulated. Interestingly, increased levels of intestinal *PAP/RegIII* mRNA have been detected during active inflammatory bowel disease (IBD), Crohn's disease,

and ulcerative colitis in humans [11, 25], and in animal models of IBD [13, 25, 36]. We recently reported a time-dependent increase in intestinal *PAP/RegIII* mRNA, represented by the genes *PAP1* and *PAP3,* in *Salmonella*-infected rats [30, 31]. These results are in line with studies showing increased *PAP/RegIII* mRNA upon bacterial colonization of the porcine small intestine with *Salmonella* [24] and enterotoxigenic *Escherichia coli* (ETEC) [23]. Hence, it is suggested that PAP/RegIII expression is triggered by increased microbial-epithelial contact and reflects a state of enhanced host defense [8]. Hitherto, few studies focused on PAP/RegIII protein levels, and it was not investigated whether PAP/RegIII expression actually reflects infection severity or an improved host defense status.

Furthermore, data on localization of ileal PAP/RegIII are controversial. *PAP/RegIII* mRNA is reported to be present in epithelial cells of the lower villus part [20], whereas other studies limit expression to Paneth cells in the ileal crypt bottoms [8].

To investigate intestinal localization and protein levels of PAP/RegIII, we studied PAP/RegIII expression in the rat small intestine upon infection with *Salmonella enteritidis*, which is a common foodborne pathogen. Our study focused on the distal ileum, since this area is important in *Salmonella* infection pathology and sensitive for infection-induced up-regulation of PAP/RegIII genes [30]. In addition, we determined whether ileal PAP/RegIII expression relates to infection severity. Modulation of this infection severity can be achieved by inoculating animals with different doses of the pathogen, but it has been shown that *Salmonella* translocation to organs [17] and the magnitude of immune response can be irrespective of the inoculated dose [35]. On the other hand, we have shown in previous studies that dietary calcium has profound resistance-enhancing effects and protects against *Salmonella* and ETEC infection in rats [2–5] and humans [5]. Therefore, we performed a dietary calcium intervention in rats to modulate *Salmonella* infection severity and to relate well-established infection markers with intestinal PAP/RegIII protein expression. More importantly, we determined the presence of PAP/RegIII in feces and studied the proposed antimicrobial activity of PAP/RegIII in vitro using fecal water incubates.

Methods

Diets, Infection, and Dissection of the Rats

The experimental protocol was approved by the animal welfare committee of Wageningen University (Wageningen, The Netherlands). Specific pathogen-free male outbred Wistar rats (WU, Harlan, Horst, The Netherlands), 8 weeks old and with a mean body weight of 245 g, were housed individually in metabolic cages as described [6]. Rats were fed purified diets containing per kg: 200 g acid casein, 326 g cornstarch, 174 g glucose, 160 g palm oil, 40 g corn oil, 50 g cellulose, and vitamin and mineral mix (without calcium) according to AIN-93 [29]. To mimic the composition of a Western human diet, the prepared diets were relatively low in calcium and high in fat compared to standard rodent diets [29]. Diets were supplemented with $CaHPO_4 \cdot 2H_2O$ (Merck, Darmstadt, Germany), at the expense of glucose, to a final concentration of 30 mmol/kg (control diet) or 120 mmol/kg (calcium diet). Food intake was recorded daily, and demineralized drinking water was supplied ad libitum. Body weight was measured every 2 days before infection and daily after infection. Two groups were fed the control diet, and another two groups were fed the calcium-supplemented diet ($n = 9$ per group). In addition, five rats were fed the control ($n = 3$) or calcium ($n = 2$) diet, and served as non-infected controls.

Animals were acclimatized to housing and dietary conditions for 14 days, after which they were orally infected with 0.5 ml of saline containing 3.10^9 colony-forming units of *Salmonella enteritidis* (clinical isolate, phage type 4; strain NIZO1241, NIZO food research, Ede, The Netherlands) as described elsewhere [6]. Animals in the non-infected group orally received 0.5 ml saline only.

On day 3 or 4 after oral infection, rats from one control and one calcium-supplemented group were randomly selected and killed by carbon dioxide inhalation. The non-infected control rats were also killed by this procedure. During the dissection, the distal 12 cm of the ileum was excised. A 2-cm piece from an identical location in each sample was cut out and preserved in 10% formalin (Sigma–Aldrich, St Louis, MO) and embedded in paraffin for histological analysis. The remaining parts were cut open longitudinally and, after flushing with saline, the mucosa was scraped off and immediately frozen in liquid nitrogen for RNA isolation and protein analyses, as described below.

The other infected control and calcium groups were followed until day 7 after infection to collect fresh fecal samples with time for *Salmonella* quantification, as described elsewhere [4]. In addition, 24-h feces (pooled per animal per 2 days) and urines were collected before infection and 7 consecutive days after oral infection. All 24 h feces and urine samples were stored at −20°C until further analysis. Oxytetracycline (Sigma-Aldrich) was added to the urine collection vessels of the metabolic cages to prevent bacterial deterioration. Bacterial translocation was quantified by measuring urinary NO_x (sum of nitrate and nitrite) excretion by using a colorimetric enzymatic kit (Roche Diagnostics, Basel, Switzerland), as described [38].

Urinary NO_x is a more sensitive and quantitative marker of intestinal bacterial translocation than culturing of extra-intestinal organs [12, 26].

Myeloperoxidase Analysis in Ileal Mucosa

Frozen mucosal scrapings of the ileum were pulverized under liquid nitrogen. Approximately half of the pulverized tissue was suspended in a 0.2-M sucrose buffer of pH 7.4 containing 20 mM trishydroxymethylaminomethane (Tris), 1 mM dithiothreitol (DTT), and a protease inhibitor cocktail (Complete, Roche Diagnostics). After mixing and centrifugation at 14,000*g* for 20 min, the pellet was resuspended in acetate-HETAB buffer [0.5% hexadecyltrimethylammonium bromide (HETAB) at pH 6.0, 50 mM sodium acetate, 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.25 M sucrose] and sonicated on ice for 30 s at level 2–3 (Sonicator XL2020, Heat Systems, Farmingdale, NY). The protein concentration of the samples was determined using BC Assay (Omnilabo, Breda, The Netherlands) according to the manufacturer's protocol. A mouse myeloperoxidase (MPO) ELISA test kit (Hycult Biotechnology, Uden, The Netherlands), which is cross-reactive with rat MPO, was used according to the manufacturer's guidelines to determine the concentration of MPO in mucosal scrapings.

Quantitative Real-Time PCR Analysis of PAP/RegIII, Represented by *PAP1* and *PAP3* mRNA, in Ileal Mucosa

The other half of the pulverized ileal mucosal scrapings was dissolved in TRIzol reagent (Invitrogen, Carlsbad, CA) to isolate and purify total RNA as described before [30]. By using TaqMan Reverse Transcription reagents (Applied Biosystems Inc., Foster City, CA), copy DNA was created from 1 μg of RNA on a Perkin Elmer DNA Thermal Cycler 480, followed by SYBR green-based real-time PCR on a 7500 Fast Real-Time PCR system (Applied Biosystems). PCR conditions used were 95°C for 10 min, followed by 40 amplification cycles (95°C for 15 s, 60°C for 1 min). Data were normalized against β-actin and aldolase. Controls, methods, and primer design were performed as described [30]. The primer sequences are listed in Table 1.

Analysis of PAP/RegIII Protein in Ileal Mucosa

Ileum paraffin sections were immunostained with a goat polyclonal antibody against rat PAP/RegIII (1:50) (PAP/RegIII #AF 1996; R&D Systems, Minneapolis, MN). This antibody cannot discriminate between PAP1 and RegIII (PAP3), but since both *PAP1* and *PAP3* fall within the PAP/RegIII group, this discrimination is not essential for our investigation [16]. Western blot analysis of total mucosal homogenates showed that the antibody bound to a single size protein band only, indicating specific binding of the antibody. Localization of Paneth cells was confirmed by immunostaining for lysozyme with ready-to-use anti-lysozyme (N1515; Dako). DAB peroxidase substrate kit (Dako) was used for signal detection of the horseradish peroxidase (HRP)-labeled secondary antibody, according to the manufacturer's protocol.

PAP/RegIII protein expression in the ileal mucosa was semi-quantified by light microscopy as follows: total length of (bottom) crypt to villus (tip) and part of this length stained positive for PAP/RegIII were measured. The length stained positive for PAP/RegIII was expressed as percentage of total crypt-villus length. For each tissue section three completely visible crypt-villus axes were analyzed, scored, and averaged for that particular rat. All histological slides were recoded before microscopy to ensure blind scoring and to prevent observer's bias.

Analysis of PAP/RegIII Protein in Feces

Total 24-h feces were lyophilized in a manifold freeze dryer (FD5515; Ilshin Laboratory Co., Ltd, Seoul, South Korea) and pooled per treatment group on the basis of individual daily fecal dry weight excretion. Proteins were isolated from lyophilized feces pools as described elsewhere [18] with a few modifications. Briefly, 100 mg of feces was homogenized in 500 μl buffer containing 50 mmol/l Tris–HCl (pH7.5), 100 mmol/l NaCl, 1 mmol/l EDTA, and Complete Protease Inhibitor Cocktail (Roche). After centrifugation (2 min at 15,000*g*), the supernatant

Table 1 Primer sequences

Gene	Acc. no.	Forward primer (5′→3′)	Reverse primer (5′→3′)
PAP1	NM_053289	GACTCCATGACCCCACTCTTG	GCAGACGTAGGGCAACTT CAC
PAP3	NM_173097	GCTTCCTTTGTGTCCTCCTTGATT	TACTCCACTCCCATCCACCTCTG
β-actin	NM_031144	CTTTCTACAATGAGCTGCGTGTG	GTCAGGATCTTCATGAGGTAGTCTGTC
Aldolase	NM_012495	ATGCCCCACCCATACCCAGCACT	AGCAGCAGTTGGCGGTAGAAGCG

Sequences of primers used for quantitative real-time PCR analysis. Acc. no. describes the accession number of the used sequence ID

was taken, and its protein concentration was determined using DC protein assay kit (Bio-rad Laboratories, Veenendaal, The Netherlands) according to the manufacturer's protocol. Then 45 µg of protein was denatured at 100°C for 3 min in Tricine sample buffer (Bio-rad Laboratories), subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (4% stacking gel, 14% separation gel), and transferred to a PVDF membrane (Bio-rad Laboratories). After blocking, the membranes were incubated with the PAP/RegIII antibody (1:100). The signal of the secondary HRP-conjugated antibody was detected using the ECL Plus chemiluminescent detection kit (GE Healthcare, Den Bosch, The Netherlands).

To evaluate recovery of PAP/RegIII protein in feces, 3 × 50 mg of the pre-infection feces pool, from animals fed the control diet, was homogenized in PBS. One of these three samples was spiked with 1 µg recombinant rat PAP (recPAP alias PAP1; pre-release reagent from R&D Systems Inc.). The second sample was heat inactivated by incubation at 75°C for 10 min, then cooled down to room temperature and identically spiked with the recombinant protein. PBS was added to the third sample and used as negative control. Subsequently, the three samples were incubated at 37°C for 1 h, and protein was isolated and analyzed by immunoblotting as described above.

Analysis of Antimicrobial Activity

To assess antimicrobial activity of rat PAP/RegIII against gram-negative *S. enteritidis* and gram-positive *Listeria monocytogenes* (strain EGD-e, strain NIZO2364, NIZO Food Research, Ede, The Netherlands), analysis was performed exactly as described [8]. Briefly, target organisms were grown in brain-heart infusion (BHI) broth and resuspended in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6), containing 25 mM NaCl. The initial bacterial concentration of the incubates was 10^6 CFU/ml, and recPAP was added to a final concentration of 4 and 10 µM. After incubation for 2 and 24 h at 37°C, viable bacteria were quantified by plating ten-fold dilutions on BHI agar plates.

To mimic the environment in which PAP/RegIII is proposed to function as antimicrobial peptide, we performed a similar antimicrobial activity assay, but replaced the MES buffer by sterile fecal water extracts from non-infected animals fed the control diet. Fecal water was prepared as described [37]. Purified recPAP was added to fecal water incubates to a final concentration of 4 and 10 µM, and viable pathogens were quantified after 2 and 24 h of incubation as described above. Experiments were performed in duplicate.

A control experiment was performed to check correct folding and thus binding activity of recPAP as described elsewhere [8]. Briefly, 10 µg recPAP was added to 50 µg insoluble *Bacillus subtilis* peptidoglycan (Sigma–Aldrich) and pelleted. Pellet and supernatant fractions were analyzed on recPAP content by SDS-PAGE (as described above).

Statistical Analysis

Data from the non-infected rats fed the control or calcium diet were pooled as no diet-induced differences were observed. All data are expressed as means ± SE, except for PCR results, which are individually plotted in addition to indication of groups' mean. Data were tested for normality by the Kolmogorov-Smirnov test. If normally distributed, differences between means were tested for significance using one-way ANOVA and/or Student's *t* test (two-tailed). For non-normally distributed data, differences between means were tested for their significance using the non-parametric Kruskall-Wallis ANOVA and/or Mann–Whitney *U* test (two-tailed). Statistical significance was set at $P < 0.05$.

Results

Animals and Food Intake

All data from one animal in the calcium group (followed until day 7 or 8 after infection) were excluded from the study results because that rat suffered from pneumonia due to oropharyngeal reflux of the *S. enteritidis* suspension. At the start of the experiment, mean body weight of the animals was 245 g. Average body weight gain (mean before infection 5 g/day, after infection 3.3 g/day) and food intake (mean 17 g dry weight/day; not affected by *Salmonella* infection) were not affected by dietary treatment.

Fecal Excretion and Translocation of *Salmonella*

As expected, no *Salmonella* could be detected in feces collected before infection of the animals. The first days after *Salmonella* infection, rats fed the calcium diet had approximately ten-fold less *Salmonella* in their feces than rats fed the control diet ($P < 0.05$; Table 2), reflecting reduced intestinal colonization of this pathogen. Furthermore, translocation of *Salmonella* was inhibited in rats on the calcium diet as measured by the infection-induced increase in urinary NO_x excretion. Total infection-induced urinary NO_x excretion (area under the curve) of the calcium group was significantly lower than that of the control group ($P < 0.05$; Table 2). Results are in accordance with previous infection studies of our laboratory [3, 4, 26].

Table 2 Fecal *Salmonella* excretion, translocation-induced urinary excretion of nitric oxide metabolites (NO_x) and mucosal myeloperoxidase

	Day after infection	Non-infected	Infected	
			Control	Calcium
Fecal *Salmonella* (log CFU/g)	Day 1	n.d.	7.2 ± 0.3[a]	6.3 ± 0.3[b]
	Day 3	n.d.	6.2 ± 0.4[a]	4.9 ± 0.3[b]
	Day 7	n.d.	4.7 ± 0.4[a]	4.2 ± 0.5[a]
Infection-induced urinary NO_x (μmol/7 days)	Days 1–7	0	73 ± 14[a]	25 ± 5[b]
Ileal mucosal myeloperoxidase (ng/mg protein)	Day 3/4	11 ± 3[a]	127 ± 52[b]	60 ± 17[b]

Values are means ± SE, $n = 8$ per infected group and $n = 5$ for the non-infected control group. Differences between groups were tested for statistical significance using Student's *t* test (two-sided), except for myeloperoxidase data, which were tested by non-parametric Kruskall–Wallis ANOVA followed by Mann–Whitney *U* test (two-sided). Values in the same row not sharing the same letter are significantly different ($P < 0.05$); n.d. indicates not detected

Ileal Mucosal Inflammation

Compared to non-infected animals, ileal MPO levels increased 12-fold in the *Salmonella*-infected control group ($P < 0.05$; Table 2) and 5-fold in the calcium group on days 3–4 post-infection. Although post-infection MPO levels in the calcium-supplemented rats were less than half of those detected in the control group, the difference did not reach statistical significance. This was likely due to the relatively large inter-individual variation observed in the *Salmonella*-infected control group.

Effect of Infection and Dietary Calcium Intervention on PAP/RegIII mRNA and Protein Expression in Ileal Mucosa

During infection, ileal *PAP1* was expressed three-fold higher than *PAP3* (*PAP1/actin* was 0.8 and 0.7 and *PAP3/Actin* was 0.29 and 0.27 in control and calcium groups, respectively). *Salmonella* infection of rats fed the control diet increased *PAP1* mRNA 2.4-fold, whereas no up-regulation was observed in calcium-supplemented infected rats ($P < 0.05$, Fig. 1a). Furthermore, compared to non-infected rats, a 3.6- and 1.8-fold induction of *PAP3* mRNA was observed in infected rats fed the control and calcium diet, respectively (Fig. 1b).

Little PAP/RegIII protein was detected in the ileum mucosa of non-infected rats, whereas *Salmonella* infected animals showed an increased number of cells staining positive for PAP/RegIII protein (Fig. 2). PAP/RegIII protein was detected specifically in enterocytes at the crypt-villus junction. The protein was not detected within goblet cell thecae. After infection the number of PAP/RegIII-positive enterocytes increased towards both villus tips and crypts' bottom. So, a larger area of the surface epithelium stained positive for PAP/RegIII after infection. Semi-quantification showed that the percentage of total crypt-villus length stained positive for PAP/RegIII protein increased 34% after *Salmonella* infection in the control group ($P < 0.05$; Fig. 3). In contrast, no significant increase in PAP/RegIII protein was observed in calcium-supplemented infected animals (Fig. 3). PAP/RegIII

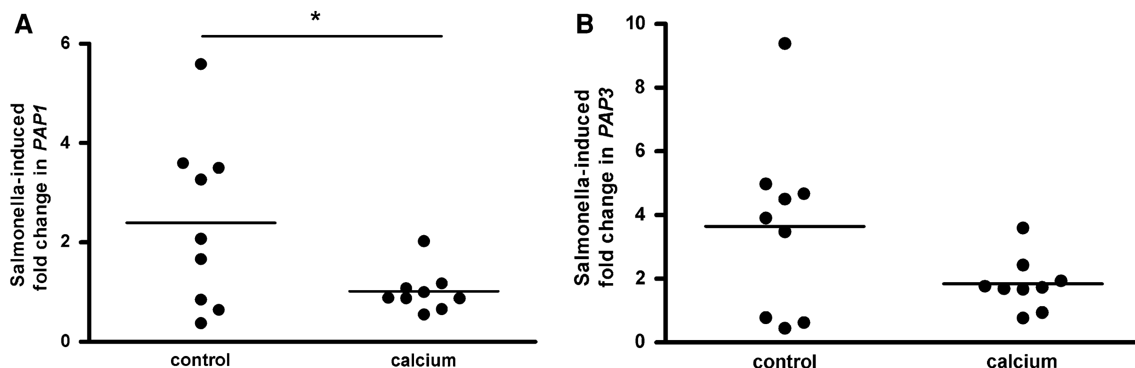

Fig. 1 Relative pancreatitis-associated protein 1 (*PAP1*, panel A) and pancreatitis-associated protein 3 (*PAP3*, panel B) mRNA expression in ileal mucosa due to *Salmonella* infection in rats fed the control or calcium-supplemented diet. Individual values were first normalized to β-actin expression. These normalized data were used to calculate relative expression levels by setting the mean expression level of non-infected rats fed the identical diet at one. The mean of each diet group ($n = 9$) is indicated by a line. Differences between the calcium and control group were tested for statistical significance using Student's *t* test (two-sided). The asterisk indicates $P < 0.05$. Identical results were found when signals were normalized to aldolase (data not shown)

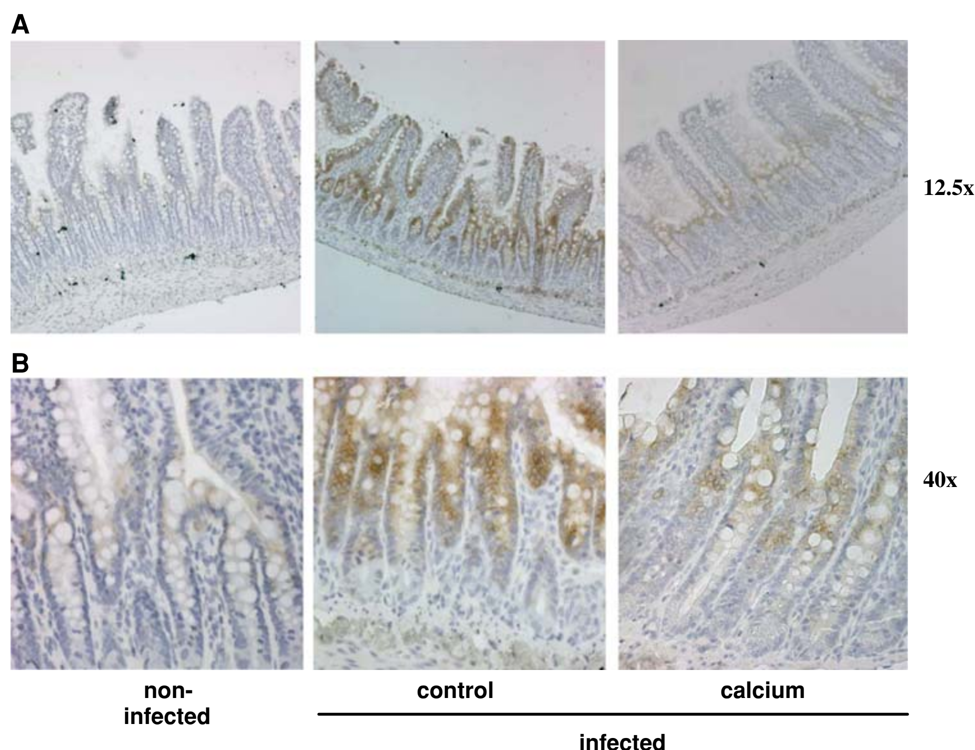

Fig. 2 Pancreatitis-associated protein/regenerating gene III (PAP/RegIII) protein expression in the ileal mucosa of non-infected and *Salmonella*-infected rats fed either the control or calcium-supplemented diet. PAP/RegIII protein was present in enterocytes at the crypt-villus junction (panel A, ×12.5; panel B, ×40). The number of PAP/RegIII-positive cells increased after infection, especially in the direction of villus tips (panel A). Dietary calcium reduced PAP/RegIII expression in infected animals. No staining was observed in sections incubated without primary antibody (data not shown). All sections were counterstained with haematoxylin to visualize nuclei

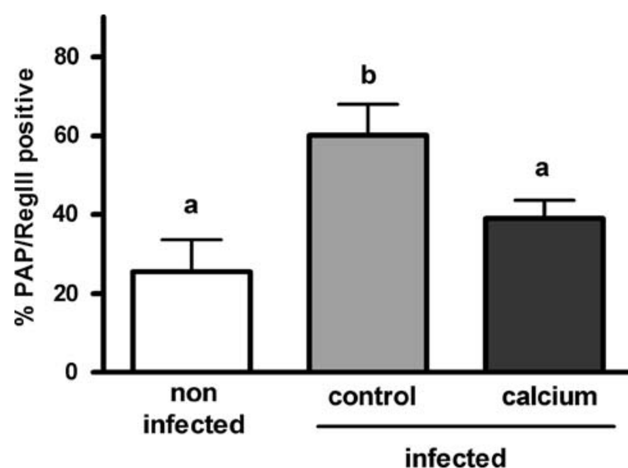

Fig. 3 Pancreatitis associated protein/regenerating gene III (PAP/RegIII) protein expression in the ileum mucosa of non-infected ($n = 5$) and *Salmonella*-infected rats fed either the control ($n = 9$) or calcium-supplemented ($n = 9$) diet. Immunohistochemical slides (representative images are shown in Fig. 2) were used to quantify the percentage of total crypt-villus length stained positive for PAP/RegIII. Total crypt-villus length was not affected by infection or calcium supplementation. Slides were recoded before microscopy to guarantee blind scoring. Results are expressed as means ± SE. Differences between groups were tested by non-parametric Kruskall–Wallis ANOVA followed by Mann–Whitney *U* test (two-sided). Different letters indicate significant differences ($P < 0.05$)

protein was not detected in the ileal Paneth cells located at the deep crypt bottom as confirmed by Paneth cell-specific staining of the lysozyme (data not shown).

Presence of PAP/RegIII in Feces

Immunoblotting revealed the presence of PAP/RegIII protein in feces (Fig. 4a, lanes 1–10). Similar to the PAP/RegIII levels in the ileum mucosa, PAP/RegIII protein in feces of rats fed the control diet was considerably increased from 3 to 4 days and remained steady until day 7 after infection. This infection-induced increase in fecal PAP/RegIII was less in calcium-supplemented infected animals. However, basal fecal PAP/RegIII levels in non-infected rats were slightly higher in the calcium group than in the control group.

Purified recPAP protein showed a single band of approximately 16 kDa, which is identical to the size of PAP/RegIII detected in ileum mucosa (Fig. 4b, lanes 4 and 5). When recPAP was added to feces it was hydrolyzed into two smaller forms. RecPAP incubated with pre-infection feces formed identical-sized bands to that seen in feces of infected rats (data not shown). Adding recPAP to heat-inactivated feces largely prevented subsequent hydrolysis and merely preserved the 16-kDa band (Fig. 4b, lanes 2 and 3).

Antimicrobial Activity

No antimicrobial activity could be detected when 4 or 10 μM recPAP was incubated with *L. monocytogenes* or *S. enteritidis* in a MES buffer system (Fig. 5). Also, no bactericidal or bacteriostatic activity of rat PAP was noticed

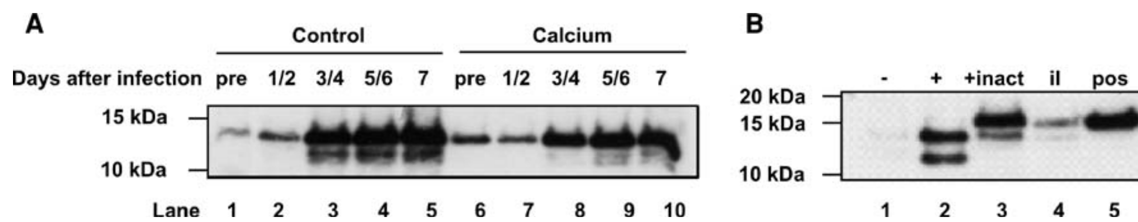

Fig. 4 Effect of *Salmonella* infection and dietary calcium on pancreatitis-associated protein/regenerating gene III (PAP/RegIII) secretion in feces with time. Lyophilized feces were pooled per group ($n = 9$ and $n = 8$ for control and calcium group, respectively) and per 2 days. SDS-PAGE gels were loaded with equal quantities of fecal protein and analyzed for PAP/RegIII (panel A, lanes 1–10) by using PAP/RegIII anti-serum. Recombinant rat pancreatitis-associated protein (recPAP) showed a single band (panel B, lane 5), similar to PAP/RegIII detected in the ileum mucosa of animals fed the control diet (panel B, lane 4). RecPAP incubated with pre-infection feces from rats fed the control diet was hydrolyzed into two smaller fragments (panel B, lane 2). When run on one gel, these fragments were of identical size as those found in fecal extracts of infected rats (data not shown). Heat inactivation of feces before addition of recPAP largely prevented hydrolysis of the protein (panel B, lane 3). Non-spiked feces (from non-infected rats) was added to lane 1 and served as negative control. No signal was detected when immunoblots were incubated without primary antibody (data not shown)

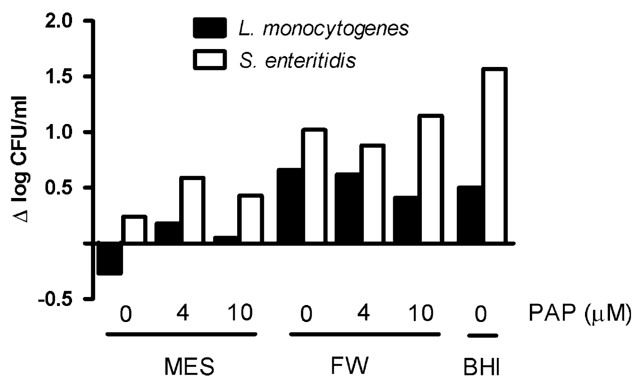

Fig. 5 Rat PAP lacks anti-bacterial activity against *L. monocytogenes* (black bars) and *S. enteritidis* (*white bars*) in vitro. Bacteria were incubated in MES buffer or fecal water extract (FW) with 0, 4 or 10 μM recombinant rat PAP (PAP). Pathogens were also grown in brain–heart infusion (BHI) broth as positive control. The initial bacterial concentration in the incubates was 10^6 colony-forming units per ml (CFU/ml). After incubation for 2 h at 37°C, viable bacteria were quantified by plating serial dilutions. The increase (positive values) or decrease (negative values) in viable pathogen counts in comparison with $t = 0$ is expressed as Δ log CFU/ml. Elongation of the incubation time to 24 h resulted in similar results (data not shown). The assays were performed in duplicate

against these pathogens when fecal water was used (Fig. 5). When the incubation time was prolonged from 2 to 24 h, results similar to that in Fig. 5 were obtained. To investigate whether absence of antibacterial activity was due to potential incorrect folding of the recombinant protein, binding activity was tested as described by Cash et al. [8]. The recPAP used in the present study bound to bacterial peptidoglycan (data not shown).

Discussion

This study shows that upregulation of PAP/RegIII protein expression by *Salmonella* infection parallels infection severity as quantified by intestinal *Salmonella* colonization and translocation. Interestingly, PAP/RegIII protein is also present in feces, and these fecal levels reflect the concentration of this protein in infected ileal mucosa.

In support of findings reported [7, 20, 25], our experiments clearly showed that PAP/RegIII protein was present in enterocytes at the crypt-villus junction of the ileal mucosa. The protein was not detected in the goblet cells, goblet cell thecae, or Paneth cells. Others have reported expression of *RegIIIγ* in mouse ileal Paneth cells isolated by laser capture microdissection before mRNA analysis [8]. In that study possible production of *RegIIIγ* by other mucosal cell types was not described. Moreover, the latter study was performed in (ex-)germ-free mice, whereas our and other [20] studies were performed in animals with a conventional flora. First-time exposure of germ-free animals to micro-organisms initiates a period of intense contact between microbes and the gut mucosa, until their naïve immune system has matured and, e.g., secretion of sIgA is normalized [32]. Therefore, results obtained in germ-free animals should be extrapolated with caution, as those might not be relevant for the normal host with a conventional flora. Furthermore, differences between animal species and the various RegIII isoforms should be considered. However, the ileal PAP/RegIII localization results of the present study are in line with those reported in mice [25], so there is no evidence that mice behave differently from rats in this respect. Other studies reporting Paneth cells as expression site mostly concerned so-called metaplastic Paneth cells in the colon of IBD patients [11, 13]. As Paneth cells are absent in the healthy colon, it can be questioned whether these cells are identical to ileal Paneth cells.

Increased mRNA levels of *PAP* or *Reg* in the intestinal mucosal after bacterial infection have been reported by our and other groups [23, 24, 30]. We now confirmed that this increase actually occurs at the protein level. The former

and our results indicate that *PAP/RegIII* expression is triggered by increased microbial-epithelial contact at mucosal surfaces. In order to establish infection, foodborne pathogens like *S. enteritidis* and ETEC bind to the small intestinal mucosa by using adhesins or colonization factors, which are known virulence factors [10, 27, 28]. Situations of increased host epithelium-microbial contact often coincide with gut mucosal inflammation, especially when pathogenic bacteria are involved [1]. Inflammation in general is also suggested as the trigger for PAP/RegIII upregulation, as increased mRNA levels have been documented in inflamed colonic mucosa from patients with active IBD [11, 25] as well as in experimental models of colitis [25, 36]. It should be realized that during active inflammation the mucosa might be damaged, its barrier function impaired, and mucosal cells are likely more exposed to micro-organisms from the gut lumen in comparison to a healthy non-inflamed mucosa [22, 33, 34]. Hence, it is very difficult to point out whether it is the inflammation or the microbe-host contact only that induces PAP/RegIII upregulation. In a previous study we have shown that *PAP/RegIII* mRNA is not upregulated till day 3 after oral *Salmonella* administration [30]. This is in line with the time-dependent fecal PAP/RegIII excretion pattern of the present study (Fig. 4a). As translocation of *Salmonella* already takes place on day 1–2 [3, 30], initial bacterial contact is less likely the main driver for PAP/RegIII upregulation. From day 3 after oral *Salmonella* administration, infection-induced mucosal inflammation is starting, and many genes of immune function are upregulated [30]. Together, these results suggest inflammation as the trigger for PAP/RegIII increase. It is becoming increasingly recognized that epithelial cells, besides being crucial for absorption of nutrients and maintenance of the gut barrier, function through an interactive process with components of the underlying immune system. Intestinal epithelial cells play an important role in both innate and adaptive immune responses. Intraepithelial lymphocytes, triggered by bacterial translocation, exist in direct contact with epithelial cells, and their interaction can lead to epithelial expression of a variety of regulatory molecules, e.g., involved in adhesion, bacterial recognition and antigen presentation, chemotaxis, and mucosal inflammation [27]. Follow-up infection studies, for instance in immuno-deficient animal models, can reveal whether this interplay is important for PAP expression and functionality. Furthermore, a study in which SPF animals are inoculated with an organism known to adhere to the gut mucosa without causing inflammation can show whether inflammation is indeed required for PAP/RegIII upregulation.

The function of intestinal PAP/RegIII is of most interest. Studies suggesting antimicrobial activity of PAP/RegIII have shown that mouse RegIIIγ was able to bind to gram-positive bacteria in vitro [8]. Analogous to defensins, PAP/RegIII is a small protein ($\pm$16 kDa); it consists of a secretion signal and a carbohydrate-binding motif. Carbohydrates are present in the gut mucosa (for example, mucins) and on the surface of gram-positive bacteria. Up to now, actual secretion of PAP/RegIII to the gut lumen has only been briefly mentioned without showing data [20]. The results of our infection study clearly show that PAP/RegIII is present in feces and is upregulated within the same timeframe as the mucosal levels of this protein. This suggests that fecal PAP/RegIII originates from ileal mucosa, and the gut lumen might be the functional site. Our recovery experiments identified hydrolysis of PAP/RegIII in intestinal contents (Fig. 4b). RecPAP incubated with pre-infection feces formed identical-sized bands to that seen in the feces of infected mice. The hydrolysis of PAP might activate its function, as shown for pancreatic-derived PAP/RegIII [15]. Therefore, the proposed antimicrobial properties of PAP/RegIII reported by others [8] were further explored. Besides repetition of the bactericidal activity assay in MES buffer as performed [8], we also included incubates of *L. monocytogenes* and *S. enteritidis* in fecal water in the presence or absence of PAP/RegIII. Fecal water, containing intestinal enzymatic activity and gut surfactants like bile acids and fatty acids, better represents the natural (in vivo) environment than a clean MES buffer system. The concentration of recPAP used in our experiments was 4 μM, as this was the estimated concentration in feces after *Salmonella* infection as detected by immunoblotting. Moreover, this concentration fits well in the range applied and found active in reported experiments [8]. In addition, a higher PAP concentration (10 μM) and a longer incubation time were included. Remarkably, we found no evidence for any bactericidal or bacteriostatic activity against *L. monocytogenes* or *S. enteritidis*. These results are in line with unpublished results from another research group, who also failed to confirm the antimicrobial activities of rat PAP as well as human PAP (J. L. Iovanna, personal communications). In view of the suggested exclusive antimicrobial function against gram-positive bacteria [8], it also remains unexplained why gram-negative bacterial pathogens, like *S. enteritidis* in our present and previous [30] studies and ETEC [23], are the most potent inducers of intestinal PAP/RegIII, whereas this mucosal protein has no anti-bacterial effect against its inducers. It might indicate that PAP/RegIII functions only at the mucosal interface, where most of the protein would be unhydrolyzed still containing its signal sequence. On the other hand, it cannot be excluded that hydrolysis of PAP/RegIII in the intestinal lumen reflects activation, as is suggested for pancreatic derived PAP/RegIII [15], rather than inactivation of a function yet to be discovered. Furthermore, PAP/RegIII will face a myriad of carbohydrates

associated with the mucosa upon epithelial secretion. In that respect, binding of PAP/RegIII to mucins and effects on gut barrier function are areas to be investigated in the near future. It should be noticed that PAP1, or RegIIIß, applied in our in vitro experiments is a homologue, but not the orthologue of mouse RegIIIγ, which was used in published in-vitro experiments on bactericidal activity. It might well be that PAP1 (RegIIIβ) and RegIIIγ have different functions. Binding assays showed that the recombinant-rat-PAP protein we used was able to bind peptidoglycan, indicating proper folding of the protein.

Diet is known to modulate gene and protein expression in the gut. As we used a calcium intervention to modulate severity of *Salmonella* infection in rats, we cannot exclude a direct effect of dietary calcium on intestinal PAP expression. In fact, the Western blots seemed to indicate that PAP protein in feces of non-infected rats fed the calcium diet was higher than that of non-infected rats fed the control diet (Fig. 4a, lanes 1 and 6). Despite a possible difference in basal excretion, the situation was totally reversed after infection, showing the highest ileal and fecal PAP/RegIII protein levels in the low-calcium control group.

In conclusion, this study demonstrates that PAP/RegIII protein levels present in the ileal mucosa and feces reflect infection severity. As fecal PAP/RegIII levels can be monitored non-invasively and with time, its use as a new infection and/or inflammation marker in animal and in human studies is worthwhile to further explore. Considering the growing interest in intestinal PAP/RegIII, elucidation of its functionality is scientifically important and might be achieved by the generation of PAP/RegIII knock-out animals. Recently, a PAP/HIP knock-out mice study on experimentally induced pancreatitis was published [14]. Investigation of the reaction of these mice to intestinal infection and inflammation would be very interesting.

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