A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis

Kristina K. Hansen*, Philip M. Sherman[†], Laurie Cellars*, Patricia Andrade-Gordon[‡], Zhengying Pan[§], Amos Baruch[§], John L. Wallace*, Morley D. Hollenberg*[¶], and Nathalie Vergnolle*^{||}

*Proteinases and Inflammation Network, Mucosal Inflammation Research Group, Department of Pharmacology and Therapeutics, and [¶]Department of Medicine, Faculty of Medicine, University of Calgary, Calgary, AB, Canada T2N 4N1; [†]Hospital for Sick Children, University of Toronto, Toronto, ON, Canada M5S 1X8; [‡]Drug Discovery, Johnson & Johnson Pharmaceutical Research and Development, Spring House, PA 19477; and [§]Celera Genomics, South San Francisco, CA 94080

Edited by Pedro M. Cuatrecasas, University of California at San Diego School of Medicine, La Jolla, CA, and approved April 25, 2005 (received for review December 20, 2004)

Citrobacter rodentium is a bacterial pathogen that causes a murine infectious colitis equivalent to enterohemorrhagic *Escherichia coli* infection in humans. Colonic luminal fluid from *C. rodentium*-infected mice, but not from sham-infected mice, contains active serine proteinases that can activate proteinase-activated receptor-2 (PAR₂). We have identified granzyme A and murine trypsins to be present in *C. rodentium*-infected luminal fluid, as determined by mass spectrometry and Western blot analysis. Inflammatory indices (colonic mucosa macroscopic damage score, increased intestinal wall thickness, granulocyte infiltration, and bacterial translocation from the colonic lumen to peritoneal organs) were all increased in *C. rodentium*-infected mice, compared with sham-infected mice. Soybean trypsin inhibitor-treated wild-type mice and untreated PAR₂-deficient (PAR₂^{-/-}) mice (compared with their wild-type littermates) both had substantially reduced levels of *C. rodentium*-induced inflammation. These data point to an important role for both pathogen-induced host serine proteinases and PAR₂ in the setting of infectious colitis.

colitis | inflammation | trypsin | granzyme

Proteinase-activated receptors (PARs) are seven-transmembrane G-protein-coupled receptors that are activated by proteolytic cleavage of their N-terminal domain (1, 2). To date, there are four members (PARs 1–4) in this receptor family. PAR₂, which can be activated by “trypsin-like” serine proteinases, plays both a proinflammatory and a protective role in inflammation (3). PAR₂ is highly expressed in the intestinal tract, where its activation induces acute inflammation of the mouse colon (4, 5). Conversely, triggering of PAR₂ in the setting of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis exerts a pronounced antiinflammatory action (6).

Bacterial infection of the intestinal tract also causes mucosal inflammation (7). For instance, enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) colonize the lumen of the intestinal epithelium and cause diarrhea and hemorrhagic colitis, respectively. Human-specific EPEC and EHEC strains share conserved virulence mechanisms, which involve attaching-effacing pedestal formation beneath adherent bacteria. *Citrobacter rodentium* is an attaching-effacing murine enteropathogen that serves as an animal model to study pathogen-induced host cell responses (e.g., cytoskeletal rearrangements) that confer colonization and infection of the host (8, 9).

Apart from an understanding of the mechanisms by which pathogens infect the hosts, a growing body of evidence suggests that disease symptoms are largely due to pathogen-induced host inflammatory and immune responses (10–12). An understanding of the mechanisms by which the host immune system is activated by these enteric pathogens is essential for the development of new therapeutic modalities for treating infectious

colitis. Because *E. coli* strains are known to release extracellular serine proteinases (12) and proteinase-mediated activation of PAR₂ can play a role in modulating intestinal inflammation, we hypothesized that *C. rodentium* infection in mice would lead to the release of PAR₂-activating proteinases and that this pathogen-induced activation of PAR₂ would contribute to host inflammatory responses.

Experimental Procedures

Reagents. All peptides were synthesized by the peptide synthesis facility at the University of Calgary. *tert*-Butyloxycarbonyl (Boc)-Gln-Ala-Arg-7-amino-4-methylcoumarin (AMC) was from Bachem (Hauptstrasse, Switzerland). Porcine pancreatic trypsin (T7418), soybean trypsin inhibitor (STI) (T9128), recombinantly expressed murine granzyme A (G6041), and STI-agarose (T0637), were from Sigma. All antibodies were from Santa Cruz Biotechnology unless indicated otherwise.

Bacterial Strains, Mouse Treatments, and Collection of Luminal Fluid.

C. rodentium was kindly provided by David Schauer (Massachusetts Institute of Technology, Cambridge) and stored at –80°C in 10% glycerol. After growth on MacConkey and Luria Bertani (LB) agar plates (PML Microbiologicals, Mississauga, ON, Canada) for 24 h at 37°C, *C. rodentium* was subcultured into LB broth overnight at 37°C. Six-week-old C57BL/6 wild-type mice (Charles River Breeding Laboratories, St. Constant, QC, Canada) and PAR₂-deficient (PAR₂^{-/-}) mice (C57BL/6 background and their wild-type littermates, bred at the University of Calgary) were used. Institutionally approved animal care and intervention protocols were used. Mice were challenged orogastrically with *C. rodentium*, 10⁷ colony-forming units in 0.1 ml, or an equal volume of broth culture. Colonization was confirmed by culture of rectal swabs and/or luminal contents on MacConkey agar plates for 24 h at 37°C. For STI treatments, mice were orally gavaged (100 μ l) with STI or its vehicle (distilled water) at a dose of 40 mg per mouse per day. The first dose was given 1 h before *C. rodentium* infection. At time of death, the colon was sterilely removed and, after removing fecal pellets, the lumen was flushed with 0.5 ml of sterile PBS, pH 7.4. Luminal contents were held at –80°C, until used in the *in vitro* assays.

Assessment of Inflammation and Bacterial Translocation. At different times after infection, mice were killed and colonic tissues were excised to assess macroscopic damage score using criteria de-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PAR₂, proteinase-activated receptor-2; STI, soybean trypsin inhibitor; AMC, 7-amino-4-methylcoumarin; Boc, *tert*-butoxycarbonyl.

^{||}To whom correspondence should be addressed. E-mail: nvergnol@ucalgary.ca.

© 2005 by The National Academy of Sciences of the USA

scribed in ref. 5. Bowel wall thickness was measured with a caliper, and myeloperoxidase (MPO) activity, an index of granulocyte infiltration, was assayed as described (5). Bacterial translocation was assessed as described (5), by plating blood collected under sterile conditions from mice 10 days after sham or *C. rodentium* infection. Samples were plated onto blood and MacConkey's agar and incubated for 24 and 48 h before counting the number of colony-forming units.

Preparation of Colonic Homogenates. Colonic homogenates were prepared with 30–40 mg of colonic tissue in 1.5 ml of 0.5% hexadecyltrimethylammonium bromide (Fluka) in potassium phosphate buffer and stored at -80°C until used in the *in vitro* assays.

Cell Culture. Previously described Kirsten virus-transformed rat kidney cells (KNRK; American Type Culture Collection, Manassas, VA) permanently vector-transfected with rat PAR₂ (KNRK-PAR₂) were grown to 90% confluence with antibiotic-containing 10% FBS growth medium (Invitrogen), supplemented with 0.6 mg/ml geneticin to maintain vector-selective pressure (13–15).

Proteinase Activity Assay. Trypsin-like activity was determined by using a Fluoroskan Ascent microplate fluorometer (Thermo Electron, Franklin, MA) (excitation = 355 nm; emission = 460 nm). The hydrolysis rate over 20 min at room temperature of a luminal fluid sample (5 μl) added to 200 μl of substrate solution (75 μM Boc-Gln-Ala-Arg-AMC in 50 mM Tris/20 mM CaCl₂ buffer, pH 7.4) was standardized to the rate generated by known concentrations of trypsin.

Calcium Signaling Assay. Fluorescence signals caused by the addition of test agonists (*C. rodentium*-infected luminal fluid, *C. rodentium*-infected colonic homogenates, or PAR₂-activating peptides) to a suspension of KNRK-PAR₂ cells ($\approx 1 \times 10^6$ cells per ml) were compared with the fluorescence peak heights yielded by replicate cell suspensions treated with 2 μM A23187 (Sigma) (15–18). PAR₂ cross-desensitization was done as outlined in ref. 19. For this experiment, desensitization at the ligand activation site of PAR₂ was achieved by treatment of the KNRK-PAR₂ cells with two sequential additions of the PAR₂ agonist peptide SLIGRL-NH₂ (50 μM), at 5-min intervals, before the addition of the putative PAR₂ agonist. The calcium response generated by the putative PAR₂ agonist after desensitization of PAR₂ was then compared to the calcium response generated by the putative PAR₂ agonist alone.

In-Gel Fluorescence Detection of Trypsin-Like Activity. In-gel trypsin-like activity after SDS/12% PAGE resolution was detected in 200 μM Boc-Gln-Ala-Arg-AMC-containing gels as described (20–22). After sample separation by SDS/PAGE at 4°C , the gel was washed with shaking in cold distilled water (seven times, 5 min), and incubated in trypsin assay buffer (0.2 M glycine, pH 8.9) at 37°C for 30 min. Fluorescent bands generated by substrate proteolysis were immediately visualized and recorded by a Gel Doc 2000 transilluminator (Bio-Rad).

Affinity Purification of *C. rodentium*-Infected Luminal Fluid with STI-Agarose. A column of STI-agarose (2 ml) was equilibrated with 10 ml of buffer (10 mM Tris/500 mM NaCl, pH 8). *C. rodentium*-infected luminal fluid (325 μl) was applied to the column and eluted with 75 mM glycine/500 mM NaCl, pH 3, at a flow rate of 0.5 ml/min. Ten 1-ml fractions were collected in tubes containing 50 μl of 2 M Tris, pH 8. Trypsin-like activity in the fractions was measured as described above. Most of the activity was contained in eluent fractions 4–6.

SDS/PAGE. Western blotting detection of activity based probe-modified proteins. The activity-based probe, biotin-Pro-Lys-diphenylphosphonate (Bio-PK, Celera Genomics) was used to identify serine proteinases in the luminal samples. Two 60- μl aliquots of trypsin (0.1 μM) *C. rodentium*- and sham-infected luminal fluid (7.5 $\times$ dilution) were made in PBS. One aliquot of each solution was heated at 95°C for 10 min. All solutions were clarified by microfuge centrifugation. To the supernatant of each sample was added 0.6 μl of Bio-PK (4 μM in DMSO). After 35-min incubation, 14 μl of each of the above solutions was added to 5 μl of 4 $\times$ sample buffer (250 mM Tris, pH 6.8/8% SDS/40% glycerol/0.08% bromophenyl blue) and 1 μl of DTT (1 M). Samples were heated (5 min, 80°C), resolved by SDS/PAGE, and transferred to poly(vinylidene difluoride) (Hybond-P, Amersham Pharmacia) membranes. Membranes were blocked with 2% casein and 0.1% Tween-20 in PBS (PBST), washed in PBST, treated with VectaStain (Vector Laboratories) in PBST, washed, treated with ECL reagents (Amersham Pharmacia), and exposed to film.

Western blotting detection of trypsin and granzyme A. Samples were separated on SDS/12% PAGE gels and transferred to poly(vinylidene difluoride) membranes. Membranes were washed (20 mM Tris/137 mM NaCl, pH 7.5/0.1% Tween-20), blocked with 5% skim milk, and incubated with goat polyclonal granzyme A antiserum (A-15) at a 1:100 dilution in 5% skim milk at 4°C overnight or rabbit polyclonal anti-human pancreatic trypsin antibody (Athens Research and Technology, Athens, GA) at a 1:2,000 dilution in 5% skim milk solution for 2 h at room temperature. The membranes were washed, treated with secondary antibody (anti-goat or anti-rabbit IgG-HRP) at a 1:15,000 dilution in 5% skim milk solution for 1 h, washed, treated with ECL reagents, and exposed to film.

Protein Sequence and Identification. Trypsin digestion, mass fingerprinting, sequencing of tryptic peptides, database searching, and protein identification were all performed at Wemb Biochem (Toronto) according to published procedures (23).

Cleavage of a 20-mer Peptide (P20) Corresponding to the Cleavage/Activation Sequence of wt-rPAR₂ Monitored by HPLC. The procedure used was similar to that described in ref. 13. Trypsin (25 ng), granzyme A (450 ng), and *C. rodentium*- or sham-infected luminal fluid (3 μl) was incubated with P20 peptide (200 μM), which corresponds to the cleavage/activation site of the wt-rPAR₂ N terminus [³⁰GPNSKGR ↓ SLIGRLDT⁴⁵P-YGGC (↓, trypsin cleavage site, -YGGC was added for affinity column coupling for another project)] in 50 mM Hepes/150 mM NaCl, pH 7.5 (75 μl). At various time points, the reactions were stopped with 0.1% trifluoroacetic acid (300 μl). The hydrolysis products were resolved by HPLC (Waters, 5 μm , C18 column) using a gradient of 0.1% trifluoroacetic acid in acetonitrile (0–70%), visualized at 214 nm, collected, and analyzed by mass spectrometry (MALDI).

Data Analysis. Comparisons among groups were made by using two-tailed Student's *t* test with Bonferroni correction. Data are expressed as mean $\pm$ SEM, and a *P* value < 0.05 was considered significant.

Results

Failure to Detect Proteolytic Activity Released by *C. rodentium*. Because bacteria are known to contain serine proteinases (24, 25), we tested various preparations of *C. rodentium* for the release of trypsin-like activity using Boc-Gln-Ala-Arg-AMC as a substrate (26, 27). Incubation of this substrate in the presence of either intact or outer membrane preparations of *C. rodentium* did not demonstrate trypsin-like activity. Bacteria grown under

Fig. 1. *C. rodentium*-infected luminal fluid exhibits trypsin-like proteinase activity. The rates of cleavage of the substrate Boc-Gln-Ala-Arg-AMC (75 μ M) by luminal fluids collected at different time points (3, 7, and 10 days) from sham- and *C. rodentium*-infected, wild-type (PAR₂^{+/+}) mice, treated or not with STI, compared to the rate of cleavage by trypsin (4 nM). *, Significantly different from sham; Ψ , significantly different from *C. rodentium*-infected PAR₂^{+/+}, $P < 0.05$, $n = 8$ in each group.

anaerobic conditions also failed to cleave this substrate (data not shown).

Proteolytic Activity Specifically Released *in Vivo* After *C. rodentium* Infection. Next, we tested the hypothesis that “trypsin-like” proteinases could be released *in vivo* from *C. rodentium*-infected host animals. The luminal fluid washes collected from *C. rodentium*-infected and sham-treated mice were tested for proteinase activity by using the same Boc-Gln-Ala-Arg-AMC substrate. Luminal fluid collected 3, 7, and 10 days after *C. rodentium* infection in mice exhibited considerable proteolytic activity compared to sham-infected mice (Fig. 1). The trypsin-like activity of the *C. rodentium*-infected luminal fluid was completely inhibited by adding STI (2 μ g/ml) to the samples (data not shown) and was significantly reduced in mice treated with STI (40 mg per mouse per day) (Fig. 1).

Proteinase(s) Released *In Vivo* on *C. rodentium* Infection Activate PAR₂. Luminal fluid, collected 10 days after *C. rodentium* infection, with trypsin-like activity was evaluated for activating PAR₂ in a PAR₂-transfected KNRK cell calcium mobilization assay (13, 14). Luminal fluids from 17 of 26 (65%) *C. rodentium*-infected mice induced calcium mobilization in PAR₂-transfected KNRK cells (Fig. 2), but not in the nontransfected cells (not shown). Using calcium ionophore A23187 (2 μ M) as a standard, *C. rodentium*-infected luminal fluid (100 μ l) provided an average response of $29 \pm 6\%$ ($n = 26$) (Fig. 2A). In contrast, there was no calcium mobilization in response to either sham-infected luminal fluid (100 μ l) ($n = 12$) or STI-treated (2 μ g/ml) *C. rodentium*-infected luminal fluid (100 μ l) ($n = 3$).

Colonic homogenates from *C. rodentium*-infected mice also induced a calcium mobilization response (Fig. 2A). The response to the colonic homogenates (200 μ l) was $12 \pm 7\%$ of the calcium ionophore (2 μ M)-induced signal ($n = 4$). Half of the samples elicited a response, whereas half were inactive. None of the colonic homogenates from sham-infected mice induced calcium mobilization.

To confirm that *C. rodentium*-infected luminal fluid-induced calcium mobilization in KNRK-PAR₂ cells was caused by activation of PAR₂, a cross-desensitization assay was done (19). The PAR₂-selective agonist SLIGRL-NH₂ (50 μ M) caused robust calcium mobilization (Fig. 2*B*). Ensuring that all PAR₂ receptors had been desensitized, the second dose of SLIGRL-NH₂ (50 μ M), added 5 min after the initial treatment, did not mobilize

Fig. 2. Proteinase(s) released upon *C. rodentium* infection activate PAR₂. (A) Activation of calcium signaling in PAR₂-transfected KNRK cells by sham-infected (*n* = 12) and *C. rodentium*-infected (*n* = 26) luminal fluid, *C. rodentium*-infected luminal fluid plus STI (*n* = 3), and sham- and *C. rodentium*-infected colonic homogenates (*n* = 4) at 10 days after infection. (B) Desensitization of the *C. rodentium*-infected luminal fluid calcium signal by prior desensitization of PAR₂. The left side of the tracing shows a continuous recording of calcium-mediated fluorescence (*E*₅₃₀) of a suspension of rPAR₂-transfected KNRK cells exposed to receptor-desensitizing concentrations of the PAR₂-activating peptide SLIGRL-NH₂ before treatment with *C. rodentium*-infected luminal fluid (*n* = 4). The calcium signal caused by a sample of *C. rodentium*-infected luminal fluid in a cell suspension that had not been previously exposed to SLIGRL-NH₂ is shown on the right side of the tracing (*n* = 4). (C) There exists a direct correlation between the trypsin-like activity and the calcium signaling in PAR₂-transfected KNRK cells by *C. rodentium*-infected luminal fluid.

calcium. The subsequent addition of *C. rodentium*-infected luminal fluid (100 μ l) to the PAR₂ desensitized cells did not elicit a calcium mobilization response ($n = 4$), whereas the addition of the same luminal samples to nondesensitized cells did (Fig. 2B). The drop in calcium level is due to a decrease in the concentration of cells. These results demonstrated pharmacologically

Fig. 3. Detection of trypsin-like activity and the presence of trypsin and granzyme A in the luminal fluid of *C. rodentium*-infected mice. (A and B) Fluorescent in-gel assays using the substrate Boc-Gln-Ala-Arg-AMC copolymerized in the SDS/PAGE gel. (C) Labeling using the activity-based probe, Biotin-Pro-Lys-diphenylphosphonate. (D) Western blot analysis of trypsin expression. (E) Western blot analysis of granzyme A expression. All pictures are representative of three to four samples.

that the calcium response to *C. rodentium*-infected luminal fluids was indeed caused by the activation of PAR₂. There was a direct correlation between the trypsin-like activity in samples of *C. rodentium*-infected luminal fluid and the degree of calcium signaling in PAR₂-transfected KNRK cells (Fig. 2C).

Identification of Proteinases Released On *C. rodentium* Infection. Because the trypsin-like activity in the *C. rodentium*-infected luminal fluid activated PAR₂, the next step was to identify the activating proteinase(s). Fluorescent in-gel assays were performed to identify simultaneously the molecular masses and substrate cleavage by proteinases with trypsin-like activity (20). Luminal fluid collected 10 days after *C. rodentium* infection contained highly active proteinases (Fig. 3A) with molecular masses of ≈ 60 and 24 kDa (lane 2). The sham-infected luminal fluid was devoid of proteinase activity (lane 3). Tryptic digestion followed by mass fingerprinting and sequencing of the tryptic peptides of the fluorescent gel pieces identified trypsinogen 16 and granzyme A in both the upper and lower bands.

To show that the proteinase activity from the *C. rodentium*-infected luminal fluid was trypsin-like, the samples were treated with STI (1 μ g) for 10 min before treatment with sample loading buffer and SDS/PAGE (Fig. 3B). The activity of trypsin (Fig. 3B, lane 2) and *C. rodentium*-infected luminal fluid (Fig. 3B, lane 4) was inhibited by STI treatment, compared to controls (Fig. 3B, lanes 1 and 3) ($n = 4$). Because the proteinase-activity was inhibited by STI, *C. rodentium*-infected luminal fluid was purified by affinity chromatography using an STI-agarose column. Mass spectral analysis of the eluent fractions with trypsin-like activity identified granzyme A, kallikrein B, and trypsinogen 16 from the *Mus musculus* genome in the sample.

Tryptic digestion followed by mass fingerprinting and sequencing of the tryptic peptides was also done on unpurified

samples of *C. rodentium*- and sham-infected luminal fluid collected 10 days after infection. The top 100 “hits” of both samples were compared. The proteinases trypsin 3, trypsinogen 9, trypsinogen 11, tryptase 4, and trypsinogen 16 (listed from highest score to lowest score) from the *M. musculus* genome were identified in the crude *C. rodentium*-infected luminal fluid sample. No other proteinases were found from the database, including prokaryotes. Trypsinogen 16 was the only proteinase found both in the *C. rodentium*-infected and in the sham-infected luminal fluid samples.

Serine proteinases can also be identified by using activity-based probes (ABPs). The serine proteinase specific ABP, Biotin-Pro-Lys-diphenylphosphonate (Bio-PK) was incubated with luminal fluid followed by separation on SDS/PAGE and visualization using standard Western blotting techniques. The ABP labeled a 28-kDa active serine proteinase in *C. rodentium*-infected luminal fluid (Fig. 3C, lane 2), but not in the sham-infected luminal fluid (Fig. 3C, lane 3) ($n = 4$). Its molecular weight distinguished this active proteinase from that of porcine pancreatic trypsin (Fig. 3C, lane 1). Upon heating the samples, ABP labeling was diminished for trypsin (Fig. 3C, lane 4) and abrogated for the *C. rodentium*-infected luminal fluid (Fig. 3C, lane 5).

Western blot analyses using trypsin and granzyme A antisera confirmed the presence of trypsin (Fig. 3D) ($n = 4$) and granzyme A (Fig. 3E) ($n = 3$) in luminal fluids collected 10 days after infection in *C. rodentium*-infected mice, but not sham-infected mice.

Granzyme A in *C. rodentium*-Infected Luminal Fluid May Activate PAR₂

It is well known that trypsin activates PAR₂, but it has not yet been shown that granzyme A activates PAR₂. Although calcium mobilization experiments did not show activation of PAR₂ by granzyme A (data not shown), treatment of a peptide containing residues 30–45 of the rat PAR₂ sequence (P20 contains the PAR₂ cleavage-activation sequence) with granzyme A (194 nM) for 20 h yielded $22 \pm 2\%$ ($n = 3$) conversion to the PAR₂-activating peptide SLIGRL... , as confirmed by mass spectral analysis. In addition, treatment of P20 for 2 h with luminal fluid collected 10 days after *C. rodentium* infection provided $94 \pm 4\%$ ($n = 4$) of the receptor-activating peptide SLIGRL... ($n = 4$), whereas the sham-infected luminal fluid samples did not ($n = 4$). As a positive control, P20 treated with trypsin (14 nM) for 20 min gave $94 \pm 2\%$ ($n = 3$) of the PAR₂-activating peptide SLIGRL... .

PAR₂ Activation and “Trypsin-Like” Proteolytic Activity Are Implicated in *C. rodentium* Infectious Colitis Pathogenicity.

Thus far, our results showed that proteolytic activity was released specifically upon *C. rodentium* infection in the colonic lumen, and that we could identify the PAR₂-cleaving enzymes trypsin and granzyme A as the major proteinases released upon infection. Moreover, *C. rodentium* infection-induced proteolytic activity was able to cleave and activate PAR₂. Next, we evaluated whether proteolytic activity and PAR₂ activation are important for the development of murine infectious colitis induced by *C. rodentium*. In preliminary experiments, we observed that, compared with sham-infected mice, colon samples from *C. rodentium*-infected mice exhibited a number of inflammatory indices such as increased macroscopic damage score (Fig. 4A), increased wall thickness (characteristic of hyperplasia, Fig. 4B), granulocyte infiltration (Fig. 4C), and bacterial translocation to peritoneal organs. Bacterial translocation was maximum at 10 days (Fig. 4D). The appearance of these inflammatory signs (Fig. 4) correlates with the presence of proteolytic activity in luminal washes (Fig. 1). In wild-type (PAR₂^{+/+}) mice, all of these inflammatory indices were significantly higher in *C. rodentium*-infected mice compared with sham-treated mice at 3, 7, and 10

other proteinases were identified from the database, including prokaryotes. After STI-agarose affinity purification, mass spectral analysis identified sequences consistent with granzyme A, kallikrein B, and a proteinase similar to airway trypsin. Granzyme A and trypsin immunoreactivity were also detected in the *C. rodentium*-infected luminal fluid by Western blot analysis. Thus, rather than secreting a bacterial proteinase, *C. rodentium* infection *in vivo* induces host tissues to release PAR₂-activating serine proteinases. Granzyme A and kallikrein B are both known to be inhibited by STI (36, 37), and it is possible that the inhibition by STI of these proteinases could account for its antiinflammatory effects *in vivo*. It also needs to be recognized that human mesotrypsin (38) and trypsin IV (39) are not inhibited by STI, and, in fact, mesotrypsin degrades STI. This finding could explain why the proteolytic activity was not completely abolished in the in-gel assays shown in Fig. 3B. However, it is not known which murine trypsins correspond to these human trypsins.

Here, we have described bacterial-induced proteinase release from host tissues. Results obtained with the PAR₂ null animals

and with the oral administration of STI indicate that this bacterial-induced release of proteolytic activity by the host tissue, in concert with the activation of PAR₂, plays a major role in the host inflammatory response. Given the striking PAR₂-dependent inflammatory effect of bacterially released proteinases in the murine model, we suggest that a comparable mechanism could be operational in humans. The identification of the precise target (e.g., enteric nerves) of bacterially mediated PAR₂ activation should provide insights for the design of novel therapeutic modalities for the treatment of infectious intestinal inflammatory diseases.

This work was supported by Group and operating grants from the Canadian Institutes of Health Research (CIHR) (to M.D.H., P.M.S., J.L.W., and N.V.). K.K.H. is an Alberta Heritage Foundation for Medical Research (AHFMR) Fellow. P.M.S. was the recipient of an AHFMR Visiting Scholar Award and holds a Canada Research Chair in Gastrointestinal Disease. J.L.W. holds a Canada Research Chair in Inflammation Research and an AHFMR Scientist award. N.V. is an AHFMR Scholar and a CIHR Investigator.

- Hollenberg, M. D. & Compton, S. J. (2002) *Pharmacol. Rev.* **54**, 203–217.
- Déry, O., Corvera, C. U., Steinhoff, M. & Bunnett, N. W. (1998) *Am. J. Physiol.* **274**, C1429–C1452.
- Vergnolle, N., Wallace, J. L., Bunnett, N. W. & Hollenberg, M. D. (2001) *Trends Pharmacol. Sci.* **22**, 146–152.
- Cenac, N., Garcia-Villar, R., Ferrier, L., Larauche, M., Vergnolle, N., Bunnett, N. W., Coelho, A.-M., Fioramonti, J. & Bueno, L. (2003) *J. Immunol.* **170**, 4296–4300.
- Cenac, N., Coelho, A.-M., Nguyen, C., Compton, S., Andrade-Gordon, P., MacNaughton, W. K., Wallace, J. L., Hollenberg, M. D., Bunnett, N. W., Garcia-Villar, R., *et al.* (2002) *Am. J. Pathol.* **161**, 1903–1915.
- Fiorucci, S., Mencarelli, A., Palazzetti, B., Distrutti, E., Vergnolle, N., Hollenberg, M. D., Wallace, J. L., Morelli, A. & Cirino, G. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 13936–13941.
- Kaper, J. B., Nataro, J. P. & Mobley, H. L. T. (2004) *Nat. Rev. Microbiol.* **2**, 123–140.
- Luperchio, S. A. & Schauer, D. B. (2001) *Microbes Infect.* **3**, 333–340.
- Vallance, B. A., Deng, W., Jacobsen, K. & Finlay, B. B. (2003) *Infect. Immun.* **71**, 3443–3453.
- Stephen, J. (2001) *Can. J. Gastroenterol.* **15**, 669–683.
- Vallance, B. A., Chan, C., Robertson, M. L. & Finlay, B. B. (2002) *Can. J. Gastroenterol.* **16**, 771–778.
- Law, D. (2000) *J. Appl. Microbiol.* **88**, 729–745.
- Compton, S. J., Renaux, B., Wijesuriya, S. J. & Hollenberg, M. D. (2001) *Br. J. Pharmacol.* **134**, 705–718.
- Saifeddine, M., Al-Ani, B., Cheng, C. H., Wang, L. & Hollenberg, M. D. (1996) *Br. J. Pharmacol.* **118**, 521–530.
- Al-Ani, B., Saifeddine, M., Kawabata, A., Renaux, B., Mokashi, S. & Hollenberg, M. D. (1999) *J. Pharmacol. Exp. Ther.* **290**, 753–760.
- Al-Ani, B., Saifeddine, M., Wijesuriya, S. J. & Hollenberg, M. D. (2002) *J. Pharmacol. Exp. Ther.* **300**, 702–708.
- Vergnolle, N., MacNaughton, W. K., Al-Ani, B., Saifeddine, M., Wallace, J. L. & Hollenberg, M. D. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 7766–7771.
- Hansen, K. K., Saifeddine, M. & Hollenberg, M. D. (2004) *Immunology* **112**, 183–190.
- Kawabata, A., Saifeddine, M., Al-Ani, B., Leblond, L. & Hollenberg, M. D. (1999) *J. Pharmacol. Exp. Ther.* **288**, 358–370.
- Yasothornsrikul, S. & Hook, V. Y. H. (2000) *BioTechniques* **28**, 1166–1173.
- Zhao, Z. & Russell, P. J. (2003) *Electrophoresis* **24**, 3284–3288.
- Zhao, Z., Raftery, M. J., Niu, X. M., Daja, M. M. & Russell, P. J. (2004) *Electrophoresis* **25**, 1142–1148.
- Zhang, R., Barker, L., Pinchev, D., Marshall, J., Rasamoeliso, M., Smith, C., Kupchak, P., Kireeva, I., Ingratta, L. & Jackowski, G. (2004) *Proteomics* **4**, 244–256.
- Paetzel, M., Dalbey, R. E. & Strynadka, N. C. J. (2000) *Pharmacol. Ther.* **87**, 27–49.
- Rawlings, N. D. & Barrett, A. J. (1994) *Methods Enzymol.* **244**, 19–61.
- Zimmerman, M., Yurewicz, E. & Patel, G. (1976) *Anal. Biochem.* **70**, 258–262.
- Kanaoka, Y., Takahashi, T., Nakayama, H., Takada, K., Kimura, T. & Sakakibara, S. (1977) *Chem. Pharm. Bull.* **25**, 3126–3128.
- Vergnolle, N. (2004) *Br. J. Pharmacol.* **141**, 1264–1274.
- Vergnolle, N., Ferazzini, M., D'Andrea, M. R., Buddenkotte, J. & Steinhoff, M. (2003) *Trends Neurosci.* **26**, 496–500.
- Nguyen, C., Coelho, A. M., Grady, E., Compton, S. J., Wallace, J. L., Hollenberg, M. D., Cenac, N., Garcia-Villar, R., Bueno, L., Steinhoff, M., *et al.* (2003) *Can. J. Physiol. Pharmacol.* **81**, 920–927.
- Mazelin, L., Theodorou, V., Fioramonti, J. & Bueno, L. (1999) *Peptides* **20**, 1367–1374.
- McCafferty, D. M., Wallace, J. L. & Sharkey, K. A. (1997) *Am. J. Physiol.* **272**, G272–G280.
- Spiller, R. C. (2002) *Gut* **51**, 759–762.
- Brunder, W., Schmidt, H., Frosch, M. & Karch, H. (1999) *Microbiology* **145**, 1005–1014.
- Lourbakos, A., Potempa, J., Travis, J., D'Andrea, M. R., Andrade-Gordon, P., Santulli, R., Mackie, E. J. & Pike, R. N. (2001) *Infect. Immun.* **69**, 5121–5130.
- Tsuzuki, S., Kokado, Y., Satomi, S., Yamasaki, Y., Hirayasu, H., Iwanaga, T. & Fushiki, T. (2003) *Biochem. J.* **372**, 227–233.
- Fujiyoshi, T., Hayashi, I., Ohishi, S., Kuwashima, M., Iida, H., Dozen, M., Taniguchi, N., Ikeda, K. & Ohnishi, H. (1989) *Agents Actions* **3–4**, 332–334.
- Szmola, R., Kukor, Z. & Sahin-Toth, M. (2003) *J. Biol. Chem.* **278**, 48580–48589.
- Cottrell, G. S., Amadesi, S., Grady, E. F. & Bunnett, N. W. (2004) *J. Biol. Chem.* **279**, 13532–13539.